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An Optical Approach for Drug Screening Based on Light-Harvesting Conjugated Polyelectrolytes**

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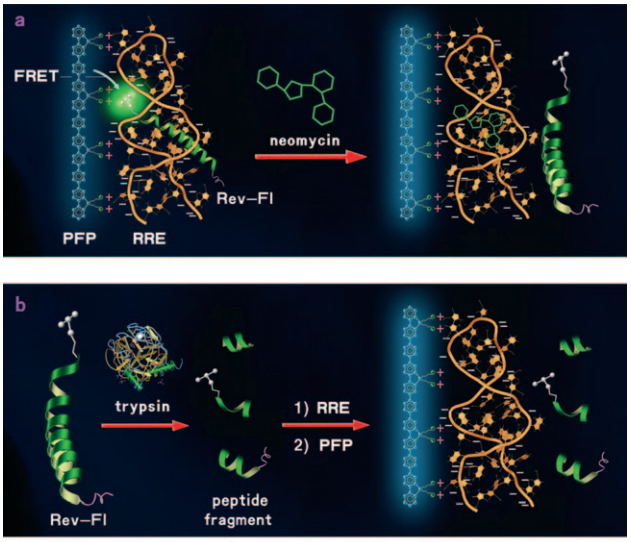
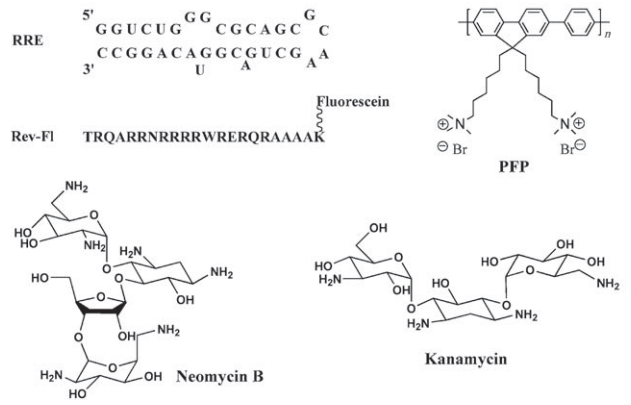
Conjugated polyelectrolytes (CPEs) are water-soluble polymers that contain a π-delocalized backbone bearing pendant ionic functionalities. The polarizable electronic structure allows facile intra- or interchain migration of optical excitations; thus, it is possible to coordinate the action of a large number of absorbing units and obtain amplification of fluorescence signals relative to those of small-molecule dyes.[1,2] Furthermore, the charged groups allow electrostatic complexation and, thereby, can trigger of fluorescence resonance energy transfer (FRET) upon molecular-recognition events that change the net charge of the macromolecule bearing the reporter (acceptor) dye.[3,4] These features have allowed CPEs to function as the optical platform in highly sensitive chemical and biological sensors.[5–22]

In comparison to previous applications tailored for the detection of specific targets, we report herein on a different function: the use of CPEs for the real-time screening of potential drugs. Our strategy builds on specific RNA–protein interactions that mediate the replication cycles of pathogenic viruses, such as the human immunodeficiency virus type 1 (HIV-1) and picornaviruses.[23–25] Small organic molecules that target the viral RNA sites and thereby prevent RNA–protein complexation are sought as initial candidates for drug discovery.[26–31] Furthermore, CPEs can also be used as a key component for monitoring proteases that disturb recognition interactions by virtue of peptide-cleavage reactions.[10,21] These methods depart from traditional screening methods, such as gel shift mobility, NMR spectroscopy, or fluorescence assays,[26–29,32–34] which are time consuming and material intensive and which often require the labeling of multiple components.

To demonstrate the concept, the regulatory peptide (Rev) binding to the Rev responsive element (RRE) sequence in the RNA of HIV-1 was chosen as the RNA/peptide binding pair (see molecular structures in Scheme 1).[32] The stem–loop IIB of RRE RNA, which contains a purine-rich internal bulge, and a fluorescein (Fl) labeled Rev peptide with 17 amino acids (Rev-Fl) were used as the model for in vitro studies.[28,32] Poly{[(9,9-bis(6′-*N*,*N*,*N*-trimethylammonium)hexyl)-fluorenylene-phenylene] dibromide} (PFP) was used as the cationic CPE in the energy-transfer experiments. The absorption spectrum of the fluorescein (acceptor) overlaps with the emission of PFP (donor) and thus satisfies the conditions for efficient FRET. Furthermore, irradiation at 380 nm selec-





Scheme 1. Chemical structures of RRE, Rev-Fl, PFP, neomycin B, and kanamycin, together with schematic representations of a) the mechanism for detection of neomycin action and b) how digestion of Rev-Fl by trypsin leads to a reduction in FRET efficiency.

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tively excites PFP. These features allow for simple evaluation of the FRET efficiencies between PFP and fluorescein.[6,8]

Scheme 1 diagrammatically shows the design and anticipated function of the PFP/Rev-Fl/RRE system. The arginine-rich Rev peptide is positively charged at neutral pH values. Upon addition of negatively charged RRE to a solution of Rev-Fl, the Rev-Fl/RRE complex forms and the *net* charge in this complex is negative. Addition of the cationic PFP results in formation of PFP/Rev-Fl/RRE electrostatic complexes, in which PFP resides in close proximity to fluorescein; the distance requirement for FRET is thus met. Neomycin B is a small-molecule antibiotic commonly used as a competitive inhibitor towards the RRE/Rev complex.[28,33] It is positively charged at neutral pH values and can bind to the major groove of duplex RNA through electrostatic interactions mediated by the ammonium groups. When neomycin is titrated into a PFP/RRE/Rev-Fl solution, the three-way binding equilibrium shifts toward the release of free Rev-Fl. This process results in the progressive removal of Rev-Fl from the vicinity of PFP and a concomitant reduction in FRET efficiency (Scheme 1 a). Thus, PFP provides an optical and structural basis for modeling drug screening in a real-time fashion. Scheme 1 b shows the anticipated function of trypsin, a serine protease that can cleave the Rev-Fl peptide on the C-terminal side of any lysine and arginine amino acid residues.[35] Breakdown of Rev-Fl generates short peptide fragments that do not associate with RRE. The reaction products also do not associate with PFP. Thus, digestion of Rev-Fl should also lead to an increase in the distance between the optical partners and a reduction in FRET efficiency.

Figure 1 compares the emission observed upon addition of PFP to a solution of Rev-Fl with RRE RNA. Also shown are the results when RRE RNA was replaced with a randomly chosen dsDNA to test the possibility of nonspecific binding. In these experiments, the Rev-Fl probe was premixed with the RRE RNA or dsDNA in buffer at 4 °C; the concentrations of the reagents are given in the figure legend.[36] After addition of PFP, a comparison of the fluorescence intensities from the fluorescein upon excitation of PFP at 380 nm shows a larger FRET ratio with Rev-Fl/RRE than with the nonspecific Rev-Fl/dsDNA pair. Also shown are the results with PFP/Rev-Fl and PFP/RRE. These control experiments show that fluorescein is more efficiently sensitized by FRET from PFP when it is present in the Rev-Fl/RRE complex, that is, under conditions where the acceptor is found in a macromolecule with net negative charge.

Two aminoglycoside antibiotics, neomycin B and kanamycin, which are competitive inhibitors towards RRE–Rev complexation, albeit with different binding efficiencies, were selected to study how the PFP/Rev-Fl/RRE system can be used to screen small molecules that inhibit Rev–RRE interactions. In these experiments, solutions containing Rev-Fl and RRE were incubated at 4 °C for 15 minutes in buffer; this was followed by the addition of PFP and then different concentrations of neomycin B. After incubation of the mixture at 4 °C for 2 minutes, the fluorescence spectra were measured upon excitation of PFP at 380 nm. As shown in Figure 2 a, addition of neomycin B in the concentration range from 0 up to 2.2 mM leads to a progressive increase in the emission intensity of PFP at 425 nm (I_{425nm}) and a decrease in fluorescein emission at 526 nm (I_{526nm}). From Figure 2 b, we can see that, when [neomycin B] = 2.2 mM, the ratio of the intensity at 425 nm versus that at 526 nm increases approximately seven-fold. These experiments show that the addition of neomycin B into the PFP/RRE/Rev-Fl system causes

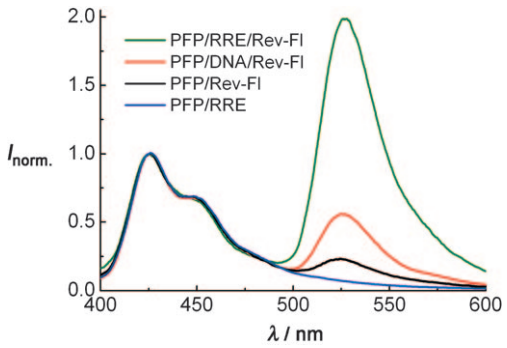

Figure 1. Emission spectra from solutions containing PFP with RRE/Rev-Fl (green line), double-stranded (ds) DNA/Rev-Fl (red line), RRE (blue line), and Rev-Fl (black line). Conditions: [PFP] = 4.0×10^{-7} M in repeat units (RUs), [RRE] = [Rev-Fl] = 1.0×10^{-8} M, [dsDNA] = 6.8×10^{-9} M (in strands). Measurements were performed at 4 °C in a buffer solution (pH 7.4) containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 140 mM NaCl. The excitation wavelength was 380 nm. Spectra are normalized with respect to PFP emission for ease of comparison.

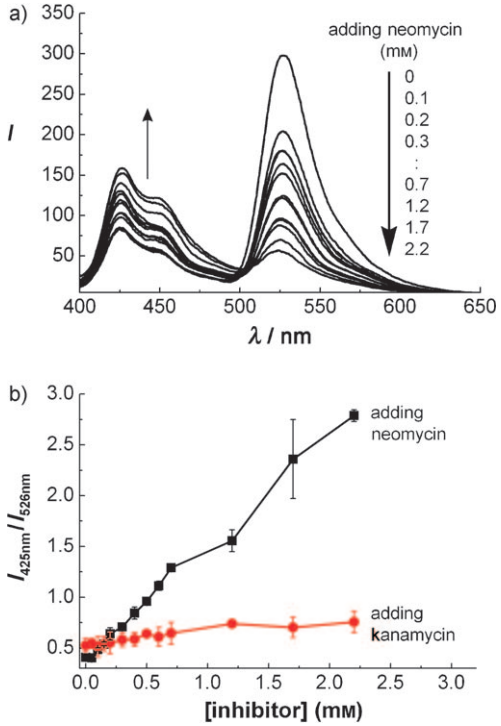

Figure 2. a) Emission spectra of PFP/RRE/Rev-Fl as a function of neomycin B concentration. [Neomycin] = 0.0–2.2 mM. b) I_{425nm}/I_{526nm} ratio of PFP/RRE/Rev-Fl as a function of the antibiotic concentration. [Neomycin] = [Kanamycin] = 0.0–2.2 mM, [PFP] = 4.0×10^{-7} M in RUs, [RRE] = [Rev-Fl] = 1.0×10^{-8} M. Measurements were performed at 4 °C in a buffer solution (pH 7.4) containing 20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 140 mM NaCl. The excitation wavelength was 380 nm. The error bars represent the standard deviation of triplicate measurements.

displacement of Rev-Fl from the complex and disturbs the energy-transfer process. It is also significant that kanamycin only leads to a negligible change of the I_{425nm}/I_{526nm} ratio under similar experimental conditions to those used with neomycin B (Figure 2b). The much larger change in the I_{425nm}/I_{526nm} ratio with neomycin B, relative to that with kanamycin, is related to its more efficient inhibition of Rev–RRE complexation (dissociation constant $K_d = 2.2 \times 10^{-7}$ M for neomycin B; $K_d = 2.5 \times 10^{-5}$ M for kanamycin).[33b] Thus, the PFP/RRE/Rev-Fl system provides a sensitive, rapid, and convenient protocol to screen promising small-molecule leads for anti-HIV drugs.

We now examine how digestion of the Rev fragment by trypsin influences the collective optical response. In these experiments, a solution containing Rev-Fl and trypsin is first incubated at 37 °C for a specific period of time. RRE is then added, and the resulting solution is allowed to incubate at 4 °C for 4 minutes. The final step involves addition of PFP and measurement of the fluorescence spectra upon excitation at 380 nm. As shown in Figure 3a, the emission intensity of PFP at 425 nm increases and that of fluorescein at 526 nm decreases as the trypsin-incubation time increases from 0 to 21 minutes. Hydrolysis of Rev-Fl catalyzed by trypsin thus leads to easily distinguishable optical signatures from the PFP/Rev-Fl/RRE system and is consistent with the overall mechanism proposed in Scheme 1b. Furthermore, the I_{425nm}/I_{526nm} ratio can be related to the population of cleaved Rev fragments. This feature can be used to evaluate the trypsin activity, as illustrated in Figure 3b, which shows the I_{425nm}/I_{526nm} values as a function of [trypsin] and digestion time. That larger I_{425nm}/I_{526nm} ratios are observed with higher trypsin concentrations provides evidence of the faster cleavage reaction rates and demonstrates the viability of this method for evaluating enzyme activity. Control experiments were also done with the nonspecific proteins bovine serum albumin (BSA) and butyrylcholinesterase (BchE). As shown in Figure 3c, there is virtually no change in the I_{425nm}/I_{526nm} value with BSA and BchE, which is consistent with the specific cleavage of Rev-Fl by trypsin. It is also interesting to note that nonspecific PFP–protein interactions that can lead to perturbations in fluorescence are not significant within this set of reactants.[37] The collected evidence is consistent with specific cleavage of Rev-Fl by trypsin controlling the change in the I_{425nm}/I_{526nm} value; these results highlight the potential of the PFP/Rev-Fl/RRE platform for screening potential enzyme agents that target recognition peptides.

We have thus demonstrated how to integrate CPE materials into assays for screening agents that disturb critical recognition events in viral replication cycles. Hits in these assays constitute a preliminary, but vital, step forward in the search for new small molecules or proteins that have therapeutic potential. The method in Scheme 1 incorporates the inhibition of specific RNA–protein (RRE–Rev) interactions, in combination with the light-harvesting properties of CPEs. We recognize that the nature of the CPE/RNA/protein complexes most likely involves higher order aggregates, rather than well-defined ternary systems as depicted in Scheme 1.[38] Regardless of these uncertainties, the CPE-based detection protocol offers several significant practical

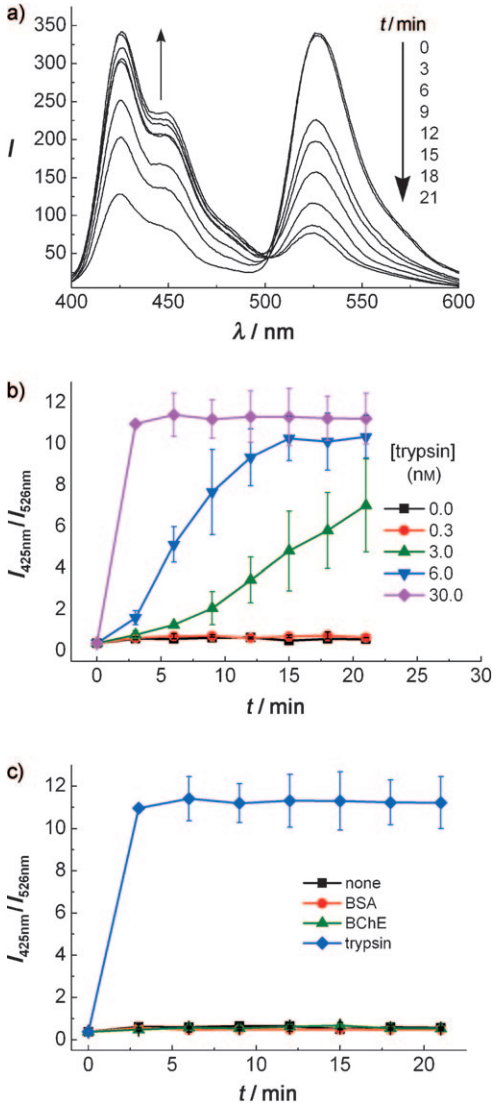

Figure 3. a) Emission spectra of PFP/RRE/Rev-Fl as a function of trypsin digestion time. The final concentration of trypsin is 3.0 nM. b) Plot of I_{425nm}/I_{526nm} versus digestion time with various trypsin concentrations. The final concentration of trypsin is 0.0–30 nM. c) Plot of I_{425nm}/I_{526nm} versus digestion time in the presence of trypsin and nonspecific proteins. Final concentrations: [protein] = 30 nM, [PFP] = 4.0×10^{-7} M in RUs, [RRE] = [Rev-Fl] = 1.0×10^{-8} M. Measurements were performed at 4 °C in buffer solution (pH 7.4) containing 20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 140 mM NaCl. The excitation wavelength was 380 nm. Error bars represent the standard deviation of triplicate measurements.

features. First, it offers a convenient "mix-and-detect" approach for the rapid screening of small molecules. Second, the CPE offers significant amplification of the detection signal, which imparts assays with high sensitivity. Third, in contrast to reported FRET-based methods, our strategy does not require labeling of the RNA probes, which should reduce costs. Furthermore, such fluorescence screening systems could be expanded to high-throughput protocols. Additionally, the successful evaluation of the action of trypsin promises to expand the function of CPE materials into the monitoring of protease activity. The composite set of properties is general and should be straightforward to adapt not only

to screening anti-HIV chemical and biological molecules with abilities to disrupt and alter specific RRE–Rev interactions but also to the targeting of other important pathogens in viral and bacterial diseases.[39]

Experimental Section

Materials and measurements: The RRE RNA and oligonucleotide DNA were custom made by TaKaRa Biotechnology Co. Ltd. (Dalian), and their concentrations were determined by measuring the absorbance at 260 nm in 200 μL quartz cuvettes. The RNA was renatured by incubation in a buffer solution (pH 7.4) containing 20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 14 0 mM NaCl for 2 min at 90 °C, followed by slow cooling to room temperature. The polypeptide modified by fluorescein on the N terminus (Rev-Fl) was made by GL Biochem Ltd. (Shanghai), and its concentration was determined by measuring the absorbance at 280 nm in 200 μL quartz cuvettes. Neomycin B and kanamycin were purchased from Amresco and used without further purification. PFP was prepared according to literature procedure.[6] UV/Vis absorption spectra were taken on a JASCO V-550 spectrophotometer. Fluorescence measurements were recorded on a Hitachi F-4500 spectrophotometer equipped with a xenon-lamp excitation source. All fluorescence spectra were measured at an excitation wavelength of 380 nm. Water was purified by using a Millipore filtration system.

Trypsin cleavage assays: Rev-Fl (2.5 μM) was added into 5 buffer solutions (20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 140 mM NaCl, pH 7.4) with a total volume of 30 μL each, and then trypsin was added to each in various amounts (0, 0.3, 3.0, 6.0, or 30.0 nM). The samples were incubated at 37 °C, and after a specific incubation period, aliquots (4.0 μL) of the solutions were drawn out and diluted to 1000 μL with buffer containing RRE (1.0×10^{-8} M). After incubation for 4 min at 4 °C, PFP ([PFP] = 4.0×10^{-7} M in RUs) was added, and the fluorescence spectra were recorded at 4 °C. Plots of the fluorescence-intensity ratio (I_{425nm}/I_{526nm}) versus the incubation time were then obtained.

Inhibitor screening: A 3 mL plastic cuvette containing RRE (1.0×10^{-8} M) and Rev-Fl (1.0×10^{-8} M) in buffer solution (20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 140 mM NaCl, pH 7.4) was incubated for 15 min at 4 °C. After incubation, PFP was added to the cuvette ([PFP] = 4.0×10^{-7} M in RUs). Neomycin B was then added successively (0.0–2.2 mM) at 4 °C, and the fluorescence spectra were measured. Plots of the fluorescence-intensity ratio (I_{425nm}/I_{526nm}) versus the incubation time were then obtained. The assay procedure for kanamycin was same as that for neomycin B except that kanamycin was used in place of neomycin B.

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