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Conjugated Polymer-Based Real-Time Fluorescence Caspase Assays

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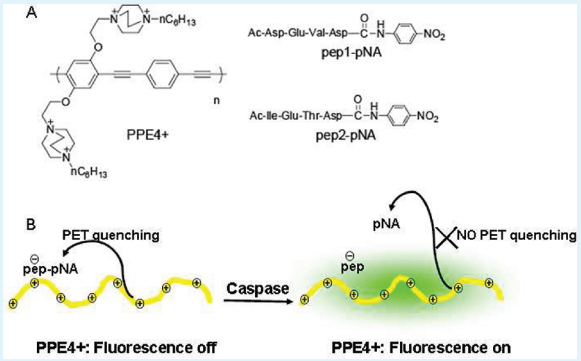
ABSTRACT: We have developed conjugated polyelectrolyte-based fluorescence turn-on assays for caspase 3 and 8. These assays are composed of a cationic polyphenylene ethynylene polymer PPE4+ and p-nitroaniline modified caspase peptide substrate. The fluorescence of the assay is initially turned-off because of the efficient quenching of the polymer by *p*-nitroaniline moiety on anionic peptide substrates. A turn-on effect is observed due to the cleavage of the peptide by the enzyme and formation of the neutral p-nitroaniline unit which has no quenching on the polymer. We validated this assay design and obtained kinetic parameters of caspase 3 and caspase 8. These assays demonstrated good sensitivity as in pmol/L (0.1 units/mL) for caspase 3 and nmol/L (0.2 units/mL) for caspase 8. This method also showed high specificity by using caspase 3 assay as a model system and the results demonstrated that other proteases including caspase 8, papain, pepsin, and trypsin did not show observable fluorescence turn-on effect. The dose−response curve of a caspase inhibitor Z-VAD-FMK was evaluated by caspase 3 assay, by which the IC_{50} value was determined to be 0.73 μM and was in a good agreement with the literature reported value at 0.62 μM. This design could be applied into the in vitro screening of small molecular inhibitors for drug discovery.



KEYWORDS: *conjugated polymer, polyphenylene ethynylene, p-nitroanilide, caspase 3, caspase 8, fluorescence assay*

INTRODUCTION

Apoptosis, also known as programmed cell death, is a physiological phenomenon to keep the balance of cells in vivo. Dysregulation of apoptosis can cause a number of diseases, including tumor and Alzheimer's disease.[1] Cascade activation of caspase (cysteine aspartic acid specific proteases) family proteins plays important roles in cell apoptosis. Specifically speaking, caspase 8 is one of the apoptosis initiators activated by Fas and various apoptotic stimuli, whereas caspase 3 is the executioner which leads directly to cell apoptosis. Small molecular inhibitors and inducers have been developed to regulate the apoptosis pathway by targeting caspases. For example, a phase II drug Idun, developed by Pfizer, was hoped to reduce cell death from ischarmia-reperfusion injury.[2] Therefore, the development of sensitive and selective caspase assays has attracted significant attention because of their applications in drug discovery, disease diagnosis, and signal transduction pathway study.[3]

Colorimetric and fluorescence assays are two types of commonly used caspase assays. These assays are based on the detection of the cleaved chromophore like *p*-nitroaniline (pNA)[4] or fluorophores including rhodamin 110,[5] 7-amido-4-methylcoumarin,[6,7] 7-amido-4-trifluoromethylcoumarin,[4] etc., by UV−visible absorption or fluorescence emission spectroscopy, respectively. However, sensitivities of such assays are limited either due to the detection limit of common absorption spectrometers, or background fluorescence interference and spectral overlap of the fluorophore-labeled peptide substrates and their fluorescent products.[5,8−10] Bioluminescent peptide substrates for caspase assays were reported with improved sensitivity and applicability for high-throughput-screening (HTS), however, making such assays is usually laborious and time-consuming and thus make the commercial product more expensive than other types of assays.[11] Label-free mass spectrometry based strategy was developed,[12] but it needs complicated postprocessing and the real-time activity monitoring is not supported.

Over the past decades, conjugated polyelectrolytes (CPEs) have emerged as a novel biosensing platform because of their special optical properties, including strong light absorption, high fluorescence quantum yields, and most important of all, the amplified quenching property.[13] CPEs have been used in building highly sensitive biosensors for proteins,[14] enzymes,[15−17] DNAs,[18−20] and carbohydrates.[21] CPE fluorescence quenching based protease assays were reported by Schanze and Whitten et al., in which a quencher-modified peptide substrate was used and the fluorescence of the polyphenylene ethynylene (PPE) was either quenched or recovered depending on the electrostatic attraction of the quencher and the polymer before and after proteolysis.[17,22] Along this strategy, several PPE fluorescence quenching based assays have been developed to target different proteases, including peptidase, thrombin, papain, enterokinase, caspase 3 and 7, and β-secretase,[22] which used the strong and specific

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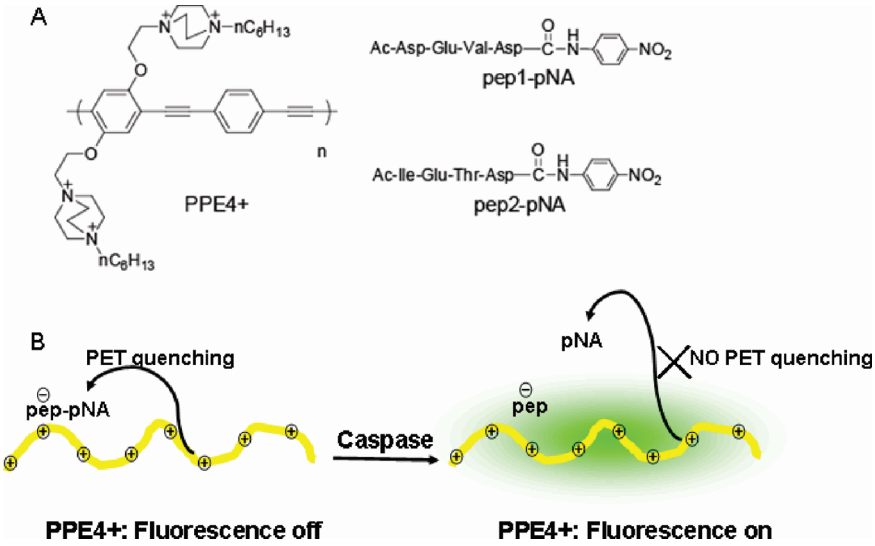

Figure 1. (A) Structures of PPE4+ and pNA-modified peptide substrates pep1-pNA and pep2-pNA. (B) Principle of the CPE-based fluorescence turn-on assay for caspases.

binding between the biotin modification on the substrate and avidin modification on CPE microspheres. Other approaches include the CPE conformational change-based assays,[15,23,24] and the bioconjugated CPE quenching assay.[25]

On the basis of our previous study, we report herein a new and simple real-time fluorescence turn-on assay for caspase 3 and 8, which consists of a cationic PPE and a pNA-modified peptide substrate. This assay demonstrated good sensitivity and high specificity with potential wide applications in protein activity determination and drug screening.

EXPERIMENTAL SECTION

Materials. The synthesis and characterization of PPE4+ was previously described.[26] The polymer was purified by dialysis against deionized water, and it was stored as an aqueous stock solution in the dark under an argon atmosphere. The polymer stock solution was 1.38 mM corresponding to the concentration of polymer repeat unit (PRU). The stock solution was diluted as needed to prepare solutions used for spectroscopic experiments and assays. Final concentrations of the diluted PPE4+ solutions were determined based on the polymer's molar extinction coefficient as 6.44×10^4 M^{-1} cm^{-1}. *p*-Nitroaniline (pNA)-modified peptide substrates pep1-pNA and pep2-pNA were purchased from GL Biochem Ltd. (Shanghai, China) and used as received. The pep1-pNA and pep2-pNA stock solutions were prepared in HPLC grade DMSO (TCI, Japan). Caspase 3 and 8 were purchased from Enzo Life Sciences Inc. (USA) and used as guided. Papain, pepsin and trypsin were obtained from Aladdin Reagent Inc. (Shanghai, China). The broad-spectrum caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) was purchased from Beyotime Institute of Biotechnology (Wuxi, China). The water used in all experiments was prepared on a SG water purification system and displayed a resistivity of ≥18.2 MΩ cm^{-1}.

Instrumentation. Absorption spectra were obtained on a Beckmann DU 800 UV−visible absorption spectrometer. Steady state fluorescence quenching experiments were carried out on a SPEX FL3 spectrometer with 380 nm as the excitation wavelength and 400−650 nm as the emission scan range. Fluorescence turn-on assays were measured on a Thermo-Fisher Varioskan Flash Multimode Reader with 380 nm as the excitation wavelength and 500 nm as the emission wavelength.

Steady-State Fluorescence Quenching. Fluorescence quenching experiments of PPE4+ by pep1-pNA, pep2-pNA and pNA were carried out by microtitration in a fluorescence cuvette. In a typical titration quenching experiment, 2.0 mL of a 10 μM PPE4+ solution was placed in a 1 cm quartz fluorescence cell and the fluorescence of the polymer before and after addition of μL aliquots of a concentrated quencher solution were recorded. The mixing time for PPE4+ with quenchers were 1 min before each measurement.

Fluorescence Turn-on Assays for Caspase 3 and 8. The fluorescence turn-on assays for caspase 3 and caspase 8 were carried out in optimized buffer condition as 10 mM Hepes buffer with 10 mM KCl, 0.1% (m/m) CHAPS, 10% (m/m) sucrose, 0.5 mM EGTA, and 20 mM β-mercaptheanol, at 37 °C on a Corning 96-well black plate. In a typical assay measurement, the enzyme was incubated in the assay buffer at ambient temperature for 15 min first. pNA-modified peptide substrate at the desired concentration was then added followed by prompt addition of PPE4+. The fluorescence intensities were measured right after the addition of PPE4+ and monitored vs time up to 240 min for caspase 3 and 75 min for caspase 8.

Inhibitor Screening Assay. In the inhibitor screening assays, the inhibitor Z-VAD-FMK was preincubated with caspase 3 for 30 min before the assay measurements. The fluorescent intensities were recorded as in the fluorescence turn-on assays.

RESULTS AND DISCUSSION

Assay Mechanism. Structures of the polymer (PPE4+) and the pNA-modified peptide substrates (pep1-pNA and pep2-pNA) are illustrated in Figure 1A. PPE4+ is a cationic poly(phenylene ethynylene) (PPE) that features cationic diazabicyclooctane (DABCO)-based bis-alkylammonium side groups. The synthesis and the basic photophysical characterization of PPE4+ was reported previously, as well as its amplified quenching by a negatively charged quencher.[26]

Both pep1-pNA and pep2-pNA are tetra-peptide substrates containing an aspartic acid residue, which can be specifically recognized and cleaved at the carbonyl end to release the pNA moiety by caspase 3 and caspase 8, respectively.[27] PNA modified peptide substrates have been widely used in colorimetric assays for caspases, in which a rise of the absorbance at 405 nm upon the enzymatic reaction was observed because of the accumulation of pNA. Although it is a convenient and economical method, the sensitivity is limited to micromolar to sub-micromolar range, because of the detection limit of UV−visible absorption spectrometers.

The pNA moiety was reported as a strong quencher and was therefore used with PPE-SO3, an anionic PPE polymer, in a turn-on assay for peptidase and thrombin.[17] On the basis of the

reported strategy, we made advantage of the cationic PPE4+ and anionic caspase substrates which have glutamic acid (Glu) and aspartic acid (Asp) residues, and thus designed the fluorescence turn-on assays for both caspase 3 and 8. As shown in Figure 1B, the negatively charged pep-pNA is in the close proximity to PPE4+ due to the electrostatic attraction and it quenches the PPE4+ fluorescence by means of photoinduced electron transfer (PET) mechanism before the enzymatic reaction; whereas after the proteolysis, the product pNA is neutral and has minimum quenching effect on the polymer and therefore the fluorescence turn-on effect is predicted to take place.

Steady-State Fluorescence Quenching of PPE4+ by pNA-Modified Peptide Substrates. Since the sensitivity of this assay design depends mostly on the quenching efficiency of the polymer, fluorescence titration experiments of PPE4+ with the pep1-pNA, pep2-pNA, and pNA were first carried out in Hepes buffer for proof of principle. Using pep1-pNA as an example, a continuous fluorescence emission decreasing was observed in the quenching experiment as shown in Figure 2.

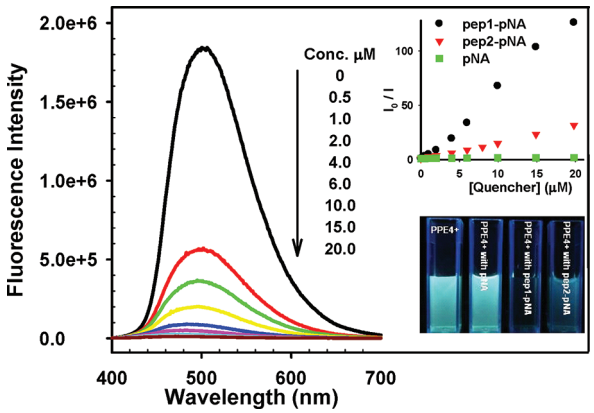

Figure 2. Fluorescence quenching spectra of 10 μM PPE4+ with pep1-pNA (0 to 20 μM) in 10 mM Hepes buffer. Inset: (top) SV plots of PPE4+ quenching by pep1-pNA (black circles), pep2-pNA (red triangles), and pNA (green squares), respectively. (bottom) Pictures of 10 μM PPE4+ alone and 10 μM PPE4+ with pNA, pep1-pNA, and pep2-pNA at 10 μM.

The fluorescence quenching data were analyzed using the Stern–Volmer (SV) equation as in eq 1.

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \tag{1}$$

where I_0 is the initial fluorescence intensity of PPE4+ solution, I is the fluorescence intensity at any quencher concentration [Q], and K_{SV} is the SV constant. As shown in the SV plots in the inset of Figure 2, both pep1-pNA and pep2-pNA demonstrate efficient quenching, whereas as a distinctive comparison, no obvious quenching takes place in the presence of pNA at the similar concentration range up to 20 μM. For a quantitative comparison, the K_{SV} values for pep1-pNA, pep2-pNA, and pNA are 6.7×10^6 M^{-1}, 1.5×10^6 M^{-1}, and 4.2×10^4 M^{-1}, respectively. The less efficient quenching by pep2-pNA than pep1-pNA is believed to be due to the lower anionic charge and thus weaker electrostatic attraction with PPE4+.

It was reported that solvent played an important role in CPEs' quenching properties, such as buffer concentration, ionic strength, and surfactant.[28,29] To optimize the quenching sensitivity as well as maintain necessary enzymatic conditions, we compared different buffers with varying factors (data not shown). Hepes was chosen as the appropriate buffer in our assay based on the literature report.[30] The assay buffer was determined as 10 mM Hepes, 10 mM KCl, 0.5 mM EGTA, 20 mM β-mercaptoethanol, 0.1% (m/m) CHAPS, and 10% sucrose.

Real-Time Fluorescence Turn-on Assay. In order to demonstrate the suitability of the real-time fluorescence turn-on assays, fluorescence intensity measurements were carried out using 10 μM PPE4+ with pep-pNA at varying enzyme concentrations in the optimized assay buffer. The fluorescence intensity data of PPE4+ vs time were recorded as shown in Figure 3A (caspase 3) and 3D (caspase 8). The substrate is usually about 200 μM in commercially available colorimetric caspase assays which can typically leads to the observable absorbance change. However, only 10–20 μM substrate was used in our method to achieve obvious fluorescence intensity change. Specifically speaking, in each assay at the beginning of the enzymatic reaction, the fluorescence of PPE4+ was nearly 93% quenched by 10 μM pep1-pNA and 75% quenched by 20 μM pep2-pNA, respectively. The fluorescence intensity remained quenched when no enzyme existed in the assay; whereas all the other curves corresponding to assays with various amounts of enzymes demonstrated obvious fluorescence turn-on effect to different extents. The most significant change is that the emission enhancement is more than 10 folds in the caspase 3 assay when the enzyme is 75 pM within 2 h.

To quantitatively analyze the enzymes' kinetics, we derived fconcentrations of unhydrolyzed pep-pNAs at different times rom fluorescence intensity data based on eq 2.[17]

$$[S]_t = [S]_0 \frac{I_0/I_t - 1}{I_0/I_q - 1} \tag{2}$$

where $[S]_t$ is the substrate concentration at time t; $[S]_0$ is the initial substrate concentration; I_0 is the fluorescence intensity of the polymer with the enzyme in a set of control experiments without the substrate; I_q is the fluorescence intensity quenched by the substrate before the reaction of enzyme; and I_t is the fluorescence intensity at time t after reaction. Panels B and E in Figure 3 illustrate the changes of concentrations of pep1-pNA and pep2-pNA, respectively, as the function of time. It is obvious that the concentrations of substrates decrease more rapidly with more enzyme for both caspase 3 and caspase 8 assays. There is an observable substrate hydrolysis when the enzyme concentration is as low as 7.5 pM (0.1 unit/mL) for caspase 3 and 0.7 nM (0.2 unit/mL) for caspase 8 within 1 h. As plotted in Figure 3C, F, the initial rates V (calculated from the slopes of the plots in Figure 3B, E using the beginning 10 points) as a function of enzyme concentrations appeared to be linear in the range of about 0–80 pM for caspase 3 and 0–15 nM for caspase 8. This proved the principle of this assay and suggested that such assays are practicable in determining caspases' activitities in vitro.

Determination of Kinetic Parameters of Caspase 3 and 8. Kinetic parameters were further determined with assays at different initial substrate concentrations in the range from 1 to 10 μM for caspase 3 (Figure 4A) and 5 to 50 μM for caspase 8 (Figure 4B). Enzyme concentrations were set to be 75 pM for caspase 3 assay and 7.2 nM for caspase 8 assay for optimized performance. Lineweaver–Burk plots were obtained (insets of Figure 4) using double reciprocal data of initial rates vs

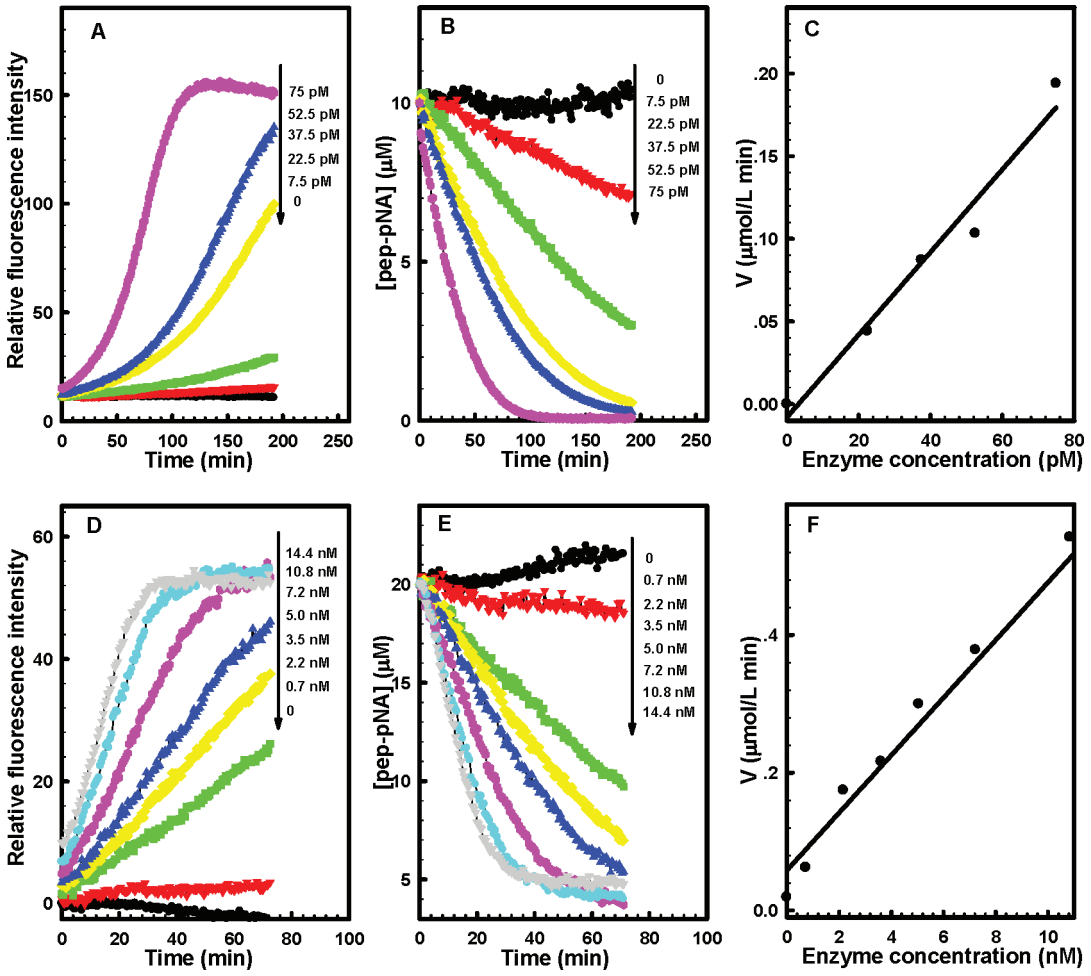

Figure 3. Fluorescence turn-on assays for caspase 3 and caspase 8. Caspase 3 assays were carried out with 10 μM PPE4+, 10 μM pep1-pNA in assay buffer with different concentrations of caspase 3 (0, 7.5, 22.5, 37.5, 52.5, and 75.0 pM). (A) Real-time fluorescence turn-on effect of caspase 3 assays. (B) Derived pep1-pNA concentrations as the function of time for caspase 3 assays. (C) Initial rate vs enzyme concentration for caspase 3 assays. Caspase 8 assays were carried out with 10 μM PPE4+, 20 μM pep2-pNA in assay buffer with different concentrations of caspase 8 (0, 0.7, 2.2, 3.6, 5.0, 7.2, 10.8, and 14.4 nM). (D) Real-time fluorescence turn-on effect of caspase 8 assays. (E) Derived pep2-pNA concentrations as the function of time for caspase 8 assays. (F) Initial rate vs enzyme concentration for caspase 8 assays.

substrate concentrations as in eq 3

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (3)$$

where V is the initial rate; [S] is the initial substrate concentration; $[E_0]$ is the initial enzyme concentration; K_m is the Michaelis constant; V_{max} is the maximal velocity. Among the parameters, the Michaelis constant K_m is a measure of the substrate's affinity to the enzyme and it is commonly used to evaluate the affect of the modification on substrate. The K_m values are determined to be 9.4 and 313.7 μM for caspase 3 and caspase 8, respectively, among which the K_m for caspase 3 is in a good agreement with the literature reported data as 11.0 μM for the same substrate used in both colorimetric and fluorescent assays,[7,31] however, the K_m for our caspase 8 assay is about 5 times greater than the literature reported value as 66 ± 5 μM, indicating less affinity of the this substrate to the enzyme.

Specificity of the Caspase 3 Turn-on Assay. Proteins have been reported to cause substantial perturbations on the fluorescence of CPEs and therefore affected the specificities of CPE based biosensing.[32] Our caspase assays composed of PPE4+ and peptide substrate with enzyme-recognition-sequence demonstrated good specificities at low enzyme concentrations in the range of pM to sub-nM. As an example, assay with pep1-pNA was selected to demonstrate its high specificity to caspase 3. Figure 5 shows the fluorescence recovery comparison of 5 different enzymes, including caspase 3, caspase 8, papain, pepsin, and trypsin. Among these 5 enzymes, caspase 3 is the only one that can recognize the sequence of pep1-pNA and cleave the pNA moiety. The literature reported K_m value for the substrate pep1-pNA with caspase 8 is about 167 ± 12 μM,[33] indicating less affinity of this substrate with caspase 8 than caspase 3. Papain, trypsin, and pepsin are common proteases, among which papain is a cysteine hydrolase, trypsin has specificity to substrate with positively charged lysine and arginine side chains, and pepsin is able to preferentially cleave aromatic amino acids at the carboxyl side. As a result, the fluorescence intensity is about 9-fold enhancement for 75 pM caspase 3, whereas it remains almost unchanged within 4 h for all other proteins even at 10–20 nM concentration. This indicates that by using high specific enzyme substrate, this strategy can be applicable to other enzyme assays with high specificity.

Caspase Inhibitor Screening Assay. The feasibility of using this assay as a screening tool for caspase inhibitors was

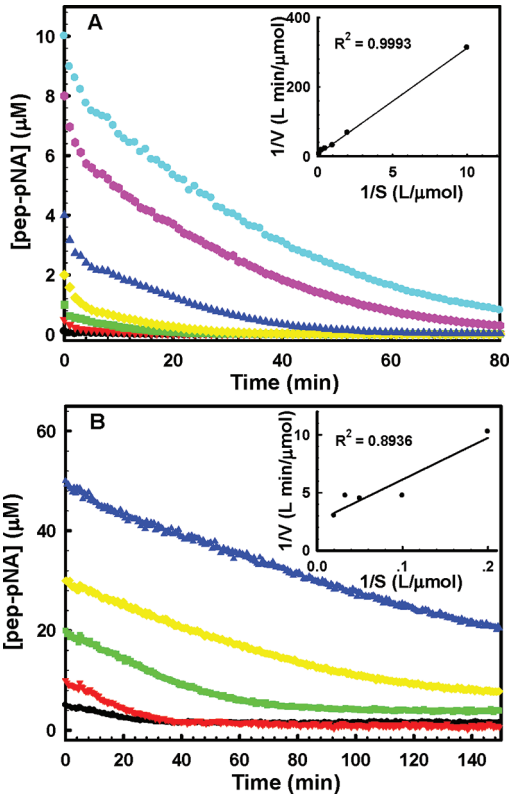

Figure 4. Enzymatic kinetics of caspase 3 and caspase 8 assays at different substrate concentrations. (A) pep1-pNA concentration as a function of time at various substrate concentrations (0.1, 0.5, 1, 2, 4, 8, and 10 μM). (B) pep2-pNA concentration as a function of time at various substrate concentrations (5, 10, 20, 30, and 50 μM). Insets of (A) and (B): Lineweaver–Burk plots using double reciprocal data of initial rates vs substrate concentrations for caspase 3 and caspase 8, respectively.

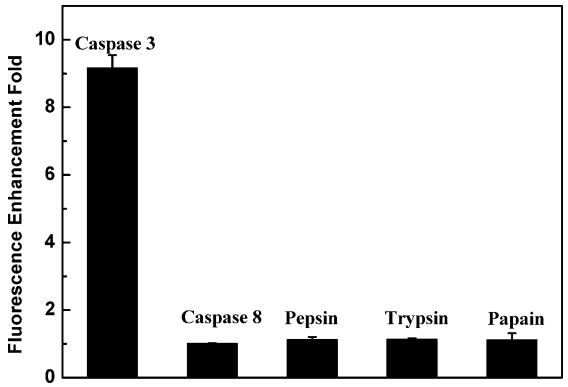

Figure 5. Specificity of caspase 3 assay. Fluorescene intensity change at 500 nm after 4 h of incubation with 75 pM caspase 3, 14.4 nM caspase 8, 20 nM papain, 20 nM pepsin, and 20 nM trypsin.

further evaluated using a known broad-spectrum inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK).[34,35] For inhibitor screening assays, caspase 3 was preincubated with the varying concentrations of inhibitor (up to 1 μM) for 30 min and then the fluorescence intensities were continuously measured as same as in the enzyme kinetic study. As shown in Figure 6, the remaining unhydrolyzed pep1-pNA concentrations after the reaction reached saturation were plotted vs the inhibitor concentrations. The inhibition effect

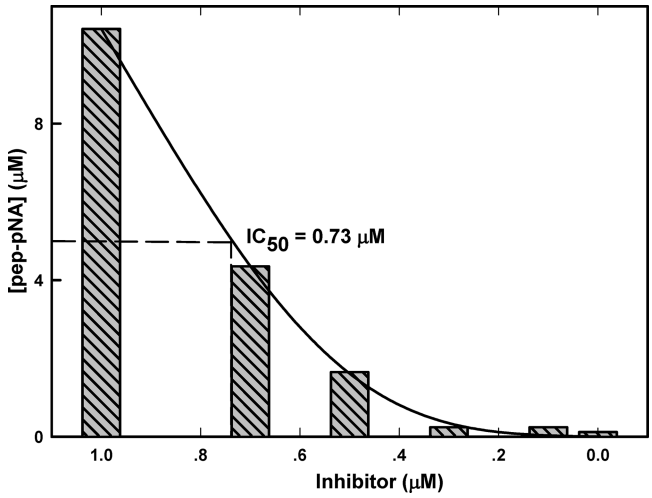

Figure 6. Fluorescence inhibition assay for caspase 3. Assays composed of 10 μM PPE4+, 10 μM pep1-pNA, and 75.0 pM caspase 3 in buffer were preincubated with varying concentrations of the inhibitor Z-VAD-FMK (0, 0.1, 0.3, 0.5, 0.7, and 1.0 μM) for 30 min before fluorescence measurements.

increased as the inhibitor concentration increased. The IC_{50} value of 0.73 μM was derived as the inhibitor concentration at which the initial rate was 50% as that without the inhibitor. This value is in a good agreement with the literature reported cellular activity at 0.63–0.65 μM.[36]

CONCLUSION

In summary, we have demonstrated the feasibility of a novel cationic CPE fluorescence quenching based caspase real-time assay for in vitro inhibitor screening as well as caspase activity measurement with good sensitivity and high specificity, by taking advantage of the amplified fluorescence quenching of CPEs as well as the high specificity of peptide substrates for enzymes. This method is applicable to be easily adapted to other protease assays by changing the substrate peptide sequence.

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