Fluorescence Probes

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# Protease Probes that Enable Excimer Signaling upon Scission**

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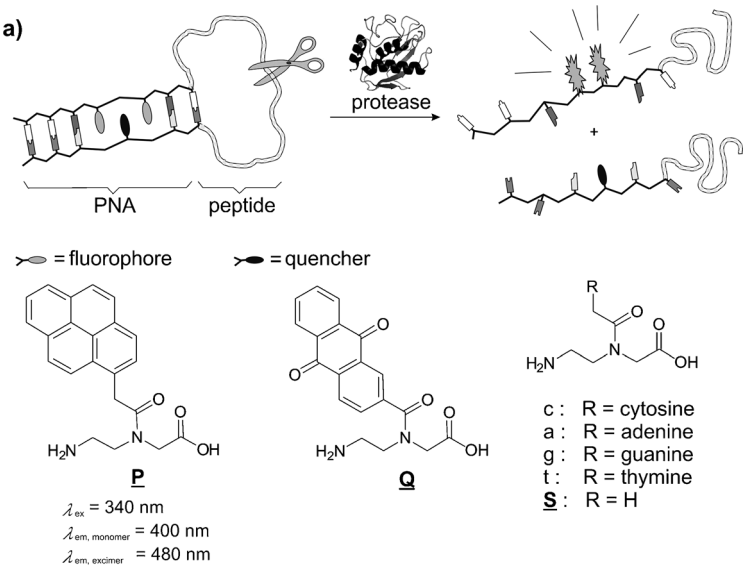
***Abstract:*** *Peptide-based probes that fluoresce upon proteolytic cleavage are invaluable tools for monitoring protease activity. The read-out of protease activity through pyrene excimer signaling would be a valuable asset because the large Stokes shift and the long lifetime of the excimer emission facilitate measurements in autofluorescent media such as blood serum. However, proteolytic cleavage abolishes rather than installs the proximity relationships required for excimer signaling. Herein, we introduce a new probe architecture to enable the switching on of pyrene excimer emission upon proteolytic scission. The method relies on hairpin-structured peptide nucleic acid (PNA)/peptide hybrids with pyrene units and anthraquinone-based quencher residues positioned in a zipper-like arrangement within the PNA stem. The excimer hairpin peptide beacons afforded up to a 50-fold enhancement of the pyrene excimer emission. Time-resolved measurements allowed the detection of matrix metalloprotease 7 in human blood serum.*

Stimuli-responsive fluorescent probes enable the detection of biomolecules in complex native environments.[1] The design of such probes usually relies on two distinct approaches: 1) the use of environmentally sensitive fluorescent dyes or 2) the perturbation of energy/electron transfer upon changes in the distance between two or more interacting dyes.[2] Pyrene has frequently been applied in both approaches.[3] The distance-dependent formation of excimers is particularly useful. In a commonly applied format, the targeted biomolecular interactions are used to bring two pyrene units into the necessary proximity required for the formation of the excited-state dimers (= excimer). Pyrene excimer emission provides large Stokes shifts (ca. 140 nm) and long fluorescence lifetimes (40–60 ns), which facilitate measurements when the background resulting from the autofluorescence of biological species is high. The advantages of pyrene excimer signaling have been exploited for the detection/imaging of nucleic acid targets[3c,4] and in membrane biophysics/bioimaging.[5]

Applications of pyrene excimer signaling in the field of protein detection/imaging are rare.[3b,6] The majority of fluorescent probes applied in the protein sciences are directed against proteases, which are amongst the most frequently addressed drug targets.[7] Proteases catalyze the cleavage of peptide bonds and, therefore, abrogate rather than install proximity relationships. By contrast, excimer signaling requires proximity, which probably explains why this mechanism of signaling has not yet been considered in the design of protease reporters.




b)

| | |
|---|---|
| eHPB1 | H-cca-P-P-ac-***RPLA LWRS***-gt-Q-tgg-G-$NH_2$ |
| PB1 | H-K(Pyr)K(Pyr)-***RPLA LWRS***-K(C1Anthr)-$NH_2$ |
| eHPB2 | H-cca-P-P-ac-Ahex-***RPLA LWRS***-Ahex-gt-Q-tgg-G-$NH_2$ |
| eHPB3 | H-DDD-cca-P-P-ac-Ahex-***RPLA LWRS***-Ahex-gt-Q-tgg-G-$NH_2$ |
| eHPB4 | H-DDD-cca-P-S-P-ac-Ahex-***RPLA LWRS***-Ahex-gt-S-Q-S-tgg-G-$NH_2$ |

***Figure 1.*** a) Cleavage of the excimer-signaling hairpin peptide beacon (eHPB) by the protein of interest = MMP-7 (PDB: 1MMQ) and structures of PNA monomers. b) Sequences used in this study; small letter = PNA monomer; capital letter = amino acid; P, Q, S = modified PNA monomers, Ahex = aminohexanoic acid.

Herein, we introduce a conceptually new and seemingly counterintuitive approach which enables the pyrene excimer emission to be switched on upon protease-catalyzed scission of a peptide substrate (Figure 1). The method relies on hairpin-shaped peptide nucleic acid (PNA)/peptide hybrids.[8] A peptide segment that serves as the protease substrate is equipped with self-complementary PNA arms. The formed PNA duplex leads to a hairpin-type arrangement, in which suitably appended labels are forced into proximity (Figure 1a). We took inspiration from studies on DNA-based

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hybridization probes and incorporated two pyrene units into the helical base stack of the N-terminal PNA arm.[9] We assumed that an anthraquinone unit at an appropriate position in the C-terminal arm would quench the pyrene emission and disrupt excitonic interactions.[10] Proteolytic cleavage within the peptide segment should remove the entropic advantage provided by intramolecular hybridization and, thereby, induce dissociation of the PNA-PNA duplex stem. The accompanying withdrawal of the anthraquinone disruptor should enable the formation of pyrene excimers, which would report peptide cleavage through a characteristic emission signal.

The pyrene and anthraquinone units were introduced as base surrogates (Figure 1a).[10,11] We expected that this would facilitate coaxial stacking interactions, which on the one hand should assist hairpin formation and on the other hand foster the contact quenching of pyrene emission. As proof-of-concept, we chose to develop a probe that signals the presence of the extracellular matrix metalloproteinase 7 (MMP-7).[12] MMPs are zinc-dependent endopeptidases and important biomarkers for inflammation and tumor progression.[13] Elevated levels of MMP-7 have been found, amongst others, in colorectal cancers and adenomas as well as in rectal and hepatocellular carcinomas and in various gliomas. Our probes contain the peptide RPLALWRS, which is known to be a substrate for MMP-7 (Figure 1b).[14] Solid-phase synthesis was used to equip this peptide with PNA arms. Each PNA arm contained five nucleobases for base pairing. The pyrene and anthraquinone units were introduced by means of aminoethylglycine-based PNA submonomers, followed by coupling of the chromophores on a solid support.

We examined whether the pyrene and anthraquinone units introduced at internal positions of probe eHPB1 support PNA-PNA interactions. Fluorometrically detected melt experiments at different concentrations showed a sigmoidal profile ($T_M = 44\,°C$), indicative of intramolecular PNA-PNA hybridization (see Figure S9a in the Supporting Information). We next measured the emission spectra of eHPB1 at 25 °C (Figure 2a). In the closed state, the monomer and excimer emission was low. Incubation with MMP-7 resulted in a 48-fold enhancement of the excimer emission at $\lambda = 480$ nm, which suggests that the enzyme was able to open the hairpin-type structure. Indeed, HPLC analysis of the probe before and after incubation with MMP-7 confirmed the quantitative cleavage of the Ala-Leu peptide bond (Figure 2b, see also Figure S11 in the Supporting Information). Melting experiments with the cleavage products confirmed that intermolecular hybridization after the cleavage is inefficient (see Figure S12 in the Supporting Information). Kinetic measurements showed the time-dependent increase in the excimer signaling, which was absent when MMP-7 was omitted (Figure 2c). For comparison, the peptide PB1 was studied. This probe also contained two pyrene units and an anthraquinone quencher, but lacked the C- and N-terminal PNA arms required for hairpin formation. In this case, the emission intensity at $\lambda = 480$ nm remained virtually unchanged, thus highlighting the importance of the hairpin design (Figure 2a,c).

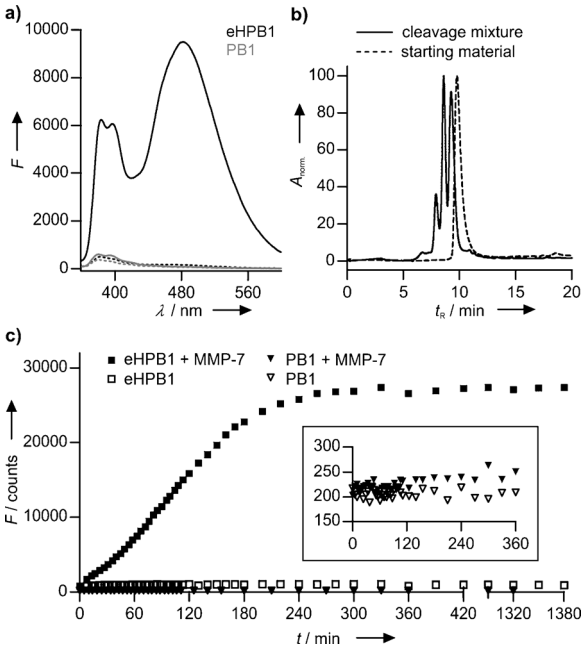


***Figure 2.*** a) Emission spectra of eHPB1 and PB1 at 25 °C with (——) and without (-----) MMP-7. b) HPLC traces of eHPB1 at $\lambda = 260$ nm. c) Time courses of the fluorescence signal at $\lambda = 480$ nm of eHPB1 and PB1 at 37 °C with and without MMP-7. Insert: amplified view. Conditions: 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.05 w/v% CHAPS, pH 7.4; 1 μM probe, 25 nM MMP-7, $\lambda_{ex} = 340$ nm. HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Kinetic measurements demonstrated that cleavage of eHPB1 occurred at a useful rate. However, faster reactions would be desirable. An aminohexanoic acid (Ahex) linker was inserted between the peptide and PNA segments in eHPB2. This modification was intended to increase the flexibility of the peptide part, whereby access of MMP-7 should be improved. Indeed, the cleavage rate was improved ($t_{1/2} = 61$ min; Table 1 and Figure 3a), however, at the cost of fluorescence enhancement ($F/F_0$(eHPB2) = 18 versus $F/F_0$-(eHPB1) = 48). Concentration-dependent melting experiments suggested that eHPB2 does not adopt a hairpin

***Table 1:*** Properties of excimer-signaling hairpin peptide beacons used in this study.

| Probe | $T_M$[a] | $F_0$[b] | $t_{1/2}$[c] | $F/F_0$[d] |
|---|---|---|---|---|
| eHPB1 | 44 | 408 | 103 | 48 |
| eHPB2 | /[e] | 1477 | 61 | 18 |
| eHPB3 | 50 | 328 | 32 | 29 |
| eHPB4 | 51 | 252 | 24 | 49 |

[a] Melting temperature of intramolecular hybridization in °C. [b] Background signal (relative fluorescence intensity) of the probe at $\lambda = 480$ nm and 25 °C. [c] Time of half maximal cleavage in minutes. [d] Ratio of the relative fluorescence intensities at λ480 nm measured after ($F$) and before ($F_0$) probe cleavage as a measure for the cleavage-induced fluorescence enhancement, 25 °C. [e] This probe shows ambiguous melting curves (see Figure S9b in the Supporting Information). Conditions: 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.05 w/v% CHAPS, pH 7.4, 1 μM probe, 25 nM MMP-7, $\lambda_{ex} = 340$ nm.


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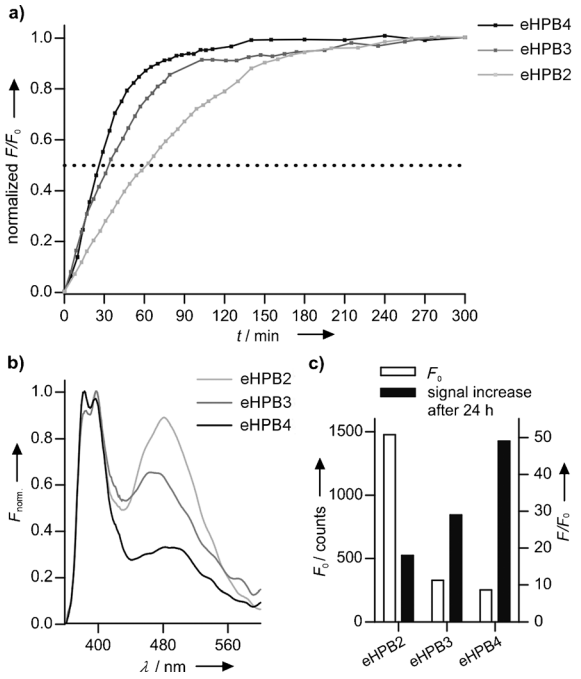


***Figure 3.*** a) Time courses of the fluorescence signal at $\lambda = 480$ nm of eHPB2-4 incubated at 37 °C with MMP-7. b) Fluorescence spectra of eHPB2–4 normalized to maximal monomer emission at $\lambda = 380$ or 400 nm. c) Improvement of the eHPBs. Conditions: 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.05 w/v% CHAPS, pH 7.4; 1 μM probe, 25 nM MMP-7, 25 °C, $\lambda_{ex} = 340$ nm.

structure (see Figure S9b in the Supporting Information). The three aspartate residues in eHPB3 were introduced with the aim to improve the solubility. Astonishingly, the half-maximal cleavage time was reduced to 32 min. In addition, we found, perhaps surprisingly, that the three aspartate residues also stabilized the hairpin ($T_M = 50$ °C). The underlying cause for this effect is currently unclear. Regardless of the mechanism, the increased stability of the PNA stem apparently allowed a better disruption of the excimer emission in the absence of MMP-7 ($F_0$(eHPB3) = 328 versus $F_0$(eHPB1) = 408). We reasoned that a further improvement in the quenching in the closed state and, therefore in the signal-to-background ratio ($F/F_0$), should be feasible by fostering the pyrene-anthraquinone contact within the hairpin. In eHPB4, the pyrene and anthraquinone chromophores were paired against abasic site PNA monomers (S). We presumed that the zipper-like arrangement could eliminate options for an extrahelical arrangement of anthraquinone. The efficiency of excimer disruption can be assessed by means of the monomer/excimer emission ratio. This ratio increased from 1.1 in eHPB2 to 2.8 in eHPB4 (Figure 3b). In fact, eHPB4 exhibited the lowest background signals (Figure 3c). However, incubation with MMP-7 led to a rapid increase in the excimer emission, which amounted to $F/F_0 = 49$. The half-maximal cleavage of the probe by MMP-7 was obtained after 24 min (Figure 3, Table 1).

We next assessed whether excimer-signaling hairpin peptide beacons facilitate measurements in biological media, where background is high due to autofluorescence. Incubation of eHPB4 with MMP-7 in buffer provided a 49-fold increase in the steady-state excimer emission. The cell medium (Dulbecco's cell growth medium with 10% fetal calf serum and 1% antibiotics; DMEM*) showed strong autofluorescence between 360 and 500 nm, which increased the background at the wavelength (480 nm) used for measuring the excimer emission (Figure 4a). As a result, the enhancement in the signal upon cleavage by MMP-7 was decreased from 49-fold in HEPES buffer to 5-fold in DMEM*. The set of experiments was repeated, but this time the cleavage of eHPB4 was monitored by means of time-resolved fluorometry (Figure 4b,c). The fluorescence signal of the HEPES buffer and the cell medium rapidly decayed within ≤6 ns. After this time, uncleaved eHPB4 still provided notable fluorescence signals. Importantly, the fluorescence decay was significantly slower when the probe eHPB4 was incubated with MMP-7 (Figure 4d). Integration of the fluorescence decay curve in the 50–250 ns time interval demonstrated that eHPB4 signaled proteolysis in cell medium with a signal-to-

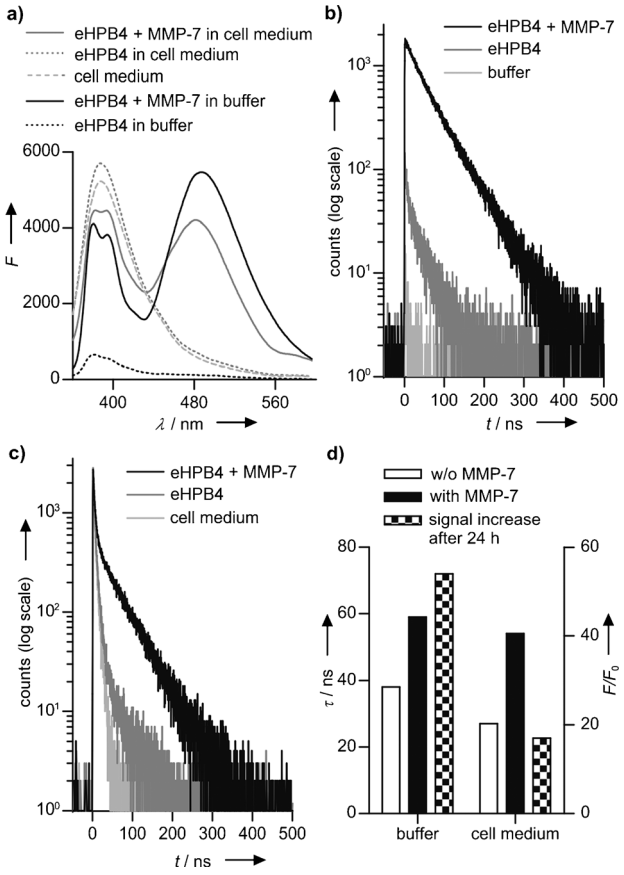


***Figure 4.*** a) Steady-state measurement of eHPB4 in buffer and cell medium with excitation at $\lambda = 340$ nm. Emission decay traces of eHPB4 at $\lambda = 480$ nm in b) buffer and c) cell medium with excitation at $\lambda = 280$ nm. d) Lifetime of eHPB4 and signal increase over 50–250 ns in buffer and cell medium. Conditions: 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.05 w/v% CHAPS, pH 7.4; 1 μM probe, 25 nM MMP-7, cell medium = DMEM*, Dulbecco's cell growth medium with 10% fetal calf serum and 1% streptomycin/penicillin.

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background of 17 (Figure 4d). In comparison, only a 5-fold signal increase was obtained by steady-state fluorometry (Figure 4a).

We evaluated the potential of eHPB4 to detect MMP-7 directly in blood serum (Figure 5). A concentration of 1 nM MMP-7 is regarded as a critical threshold, indicative of metastatic colorectal cancer.[15] Hence, human serum was spiked with 1 nM MMP-7 and incubated with 1 μM eHPB4 for 24 h at 37 °C. To hamper cleavage by nontargets, the protease inhibitor Pefabloc SC was added. The read-out from time-resolved fluorometry revealed that the presence of MMP-7 induced a 4-fold increase in the integral emission intensity (50–200 ns). This finding suggests that protease probes based on excimer-signaling hairpin peptide beacons enable a direct read-out of protease activity in optically dense matrices such as blood serum.

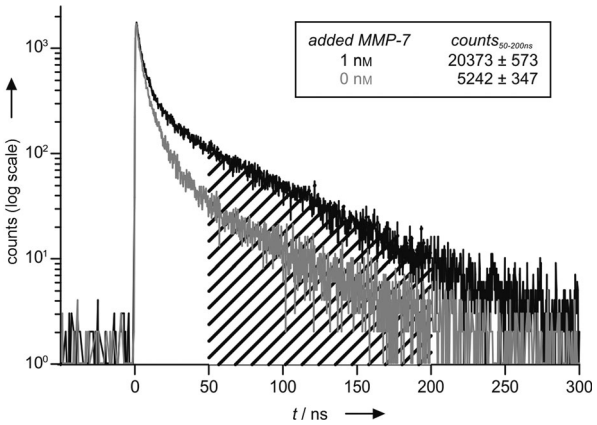


***Figure 5.*** Emission decay traces of eHPB4 in human serum at $\lambda = 480$ nm with (black) and without (gray) 1 nM MMP-7; inset: means of integrated counts of three measurements with standard deviation. Conditions: addition of 10 mM Pefabloc SC, 1:10 diluted with 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, 0.05 w/v% CHAPS, pH 7.4; 1 μM probe, $\lambda_{ex} = 280$ nm.

In summary, we presented a novel design for protease probes that enables long-lived excimer emission after scission has occurred. The precise positioning of an anthraquinone chromophore within excimer-signaling hairpin peptide beacons (eHPBs) is the prerequisite for efficient perturbation of the excitonic interaction between two adjacently aligned pyrene chromophores. Target-induced cleavage led to an approximately 50-fold enhancement in the pyrene excimer emission as a result of disruption of the hairpin structure. The long lifetime of the excimer emission is noteworthy, which enabled the detection of the biomarker matrix metalloproteinase 7 (MMP-7) within an optically dense, autofluorescent matrix (blood serum) at a physiologically relevant concentration, despite a short excitation wavelength. The probe design can be adapted for other protein targets simply by altering the peptide sequence within the loop segment of the eHPBs. Furthermore, the eHPBs should not be restricted to the use of pyrene dyes, but rather may be expanded to other intercalating dyes. Based on this and the opportunities provided by wash-free assays in biological environments, we expect that excimer-signaling hairpin peptide beacons will prove useful as fluorogenic tools in protease research.

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