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Hairpin Peptide Beacon: Dual-Labeled PNA-Peptide-Hybrids for Protein Detection

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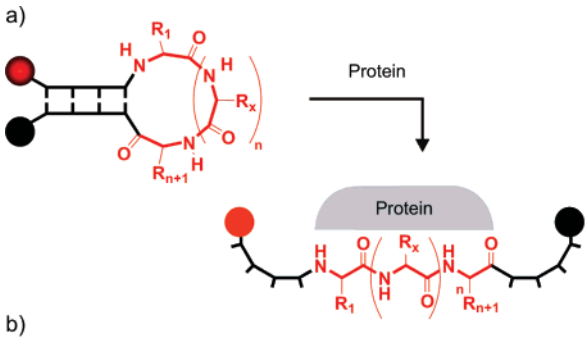
Fluorescent probe molecules are key to the analysis and detection of biomolecules in complex environments. For example, nucleic acid-based probes have been developed to signal the presence of a specific DNA or RNA target by changes of fluorescence.[1,2] Usually, nucleic acid probes are designed to recognize target molecules by means of Watson−Crick base pairing. By comparison, the design of peptide-based probes that report on binding to (and dissociation from) specific protein targets by changes of fluorescence intensity is more challenging, owing to the lack of a universal peptide−protein recognition code.[3]

Previously, peptides have been modified at or near the terminal ends with interacting fluorescence dyes.[4,5] Binding of antibodies or a SH2 protein has been shown to induce changes of probe fluorescence due to relief of contact quenching or dye dimer formation. The probe design drew upon the flexibility of the unbound peptide, which enabled weak hydrophobic interactions between selected chromophores such as rhodamine or pyrene residues, and protein induced folding of the peptide probe. We sought a new, rational probe concept, in which protein binding is signalled by a mechanism that is independent of (a) the binding interactions between two fluorophores and (b) the flexibility of the recognized peptide segment. We herein introduce hairpin peptide beacons (HPB), which are designed to maximize fluorescence quenching in the absence of a protein target by forming stable hairpins. The particular advantage of the hairpin peptide beacons is the increased fluorescence responsiveness and the generic design which should be applicable to any peptide that interacts with a protein while allowing almost unrestricted access of interesting fluorophores.

HPBs consist of a central, protein specific peptide sequence flanked by two DNA-analogous self-complementary peptide nucleic acid (PNA) arm segments (Figure 1a).[6,7] These chimeric molecules are designed in analogy to DNA molecular beacons. They form a hairpinlike structure that is maintained by a double helical PNA stem region. The loop region contains a peptide motif that is recognized by the protein target. The hairpinlike structure enforces interactions between terminally attached chromophores. Thus, decay channels may be opened due to FRET, static, or collisional quenching or excimer formation. The binding of a protein to the peptide sequence induces a structural reorganization and results in the opening of the closed structure, provided that the conformation of the protein-bound peptide is different from the loop conformation in the closed state. The chromophores are separated, the decay channels are closed, and, hence, the fluorescence is increased.

In a paradigm study, we chose to develop HPBs for the detection of Src kinase.[8] The Src protein offers three target sites; the SH2, the SH3, and the tyrosin kinase domain.[9] Crystal structure analysis revealed that pTyr-containing peptides containing the recognition motif pTyr-Xxx-Xxx-Ile bind Src-SH2 in an extended conformation.[10] A known Src-SH2 binder, the phosphotyrosyl peptide Glu-Pro-Gln-pTyr-Glu−Glu-Ile-Pro was equipped with self-complementary PNA arms. HPB **1** (Figure 1b) was prepared by Fmoc-based solid-phase synthesis (Supporting Information (SI)). The ϵ-amino groups of terminal lysine residues were labeled with pyrenebutanoic acid. Thermal denaturation measurements of **1** showed sigmoidal melting curves, which indicates cooperative base pairing (Supporting Information Figure S2a). The $T_M = 60$ °C was independent of the concentration of HPB **1** suggesting intramolecular hybridization of the PNA arms (Figure S2b). It was concluded that HPB **1** is likely to form a hairpin structure at room temperature.



Hairpin Peptide Beacons:

Ac-Lys-t t g g-Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-c c a a-Lys-Gly^{CONH_2} **1**
Pyb Pyb

Ac-Lys-t t g g-Pro-His-Pro-Phe-His-Stat-Val-Ile-c c a a-Lys-Gly^{CONH_2} **2**
NIR Dabcyl

(PNA-) Peptide Beacons:

Ac-Lys-g t c a-Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-c c a a-Lys-Gly^{CONH_2} **3**
Pyb Pyb

Ac-Lys-Glu-Pro-Gln-pTyr-Glu-Glu-Ile-Pro-Lys-Gly^{CONH_2} **4**
Pyb Pyb

Ac-Lys-Pro-His-Pro-Phe-His-Stat-Val-Ile-Lys-Gly^{CONH_2} **5**
NIR Dabcyl

Figure 1. (a) Hairpin peptide beacons are composed of a double helical peptide nucleic acid (PNA) stem and a recognition peptide. Binding of target protein results in opening of the hairpin and enhancement of fluorescence. (b) Sequences of studied hairpin peptide beacons (HPB) and (PNA-)peptide beacons. Lower case letters indicate PNA nucleotides; amino acids are denoted in three letter code with pTyr = phosphotyrosine and Stat = statine; Pyb = pyrene butanoic acid, NIR = *NearInfraRed-667*, Dabcyl = 4-(4-dimethylaminophenylazo)benzoic acid.

Hairpin-formation is expected to align the terminally appended pyrene residues of **1** in close proximity and thus pyrene excimer emission should prevail. Indeed, the fluorescence spectrum of HPB **1** at 25 °C was dominated by a broad excimer band centered at 490 nm (Figure 2a). Addition of the Src-SH2 protein resulted in a more than 10-fold increase of pyrene monomer emission at 380 nm, presumably by stabilizing the open state (Figure 2b).[11]

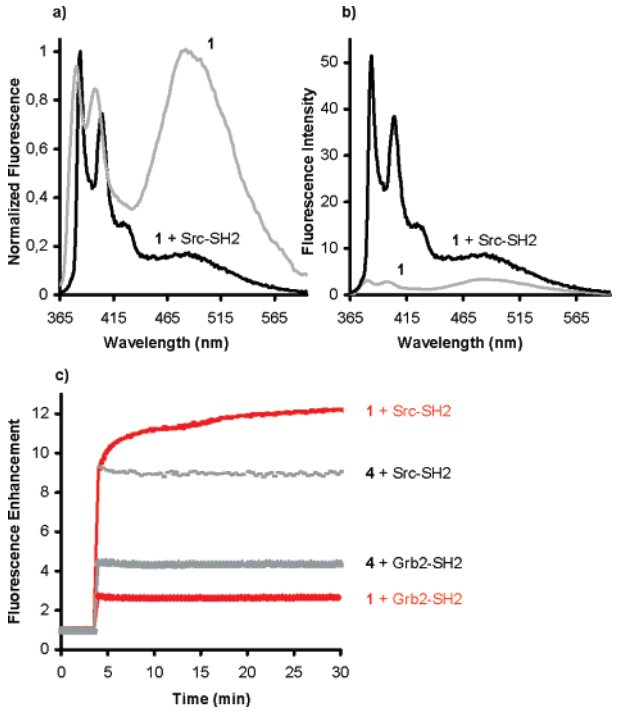


Figure 2. Fluorescence of HPB **1** and PB **4**: (a) normalized fluorescence spectra of **1** before (gray) and after addition of Src-SH2 (black); (b) fluorescence spectra of **1** before (gray) and after addition of Src-SH2 (black line); (c) time course of relative fluorescence intensity at 380 nm of **1** (red) or **4** (black) after addition (5 min) of Src-SH2 and Grb2-SH2. Conditions: $\lambda_{ex} = 340$ nm, 1 μM of **1** in buffer (20 mM NaH_2PO_4, 100 mM NaCl, 2 mM DTT, pH 7.4, 25 °C) and 16 μM SH2 protein (required for saturation of signaling) when added.

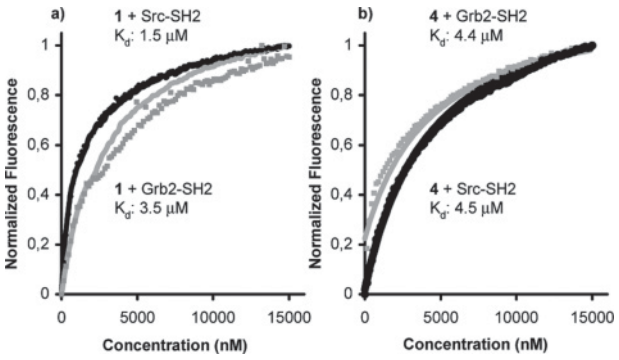


Figure 3. Determination of dissociation constants of HPB **1** and PB **4** vs Src-SH2 and Grb2-SH2; binding curve and nonlinear regression analysis of (a) HPB **1** and (b) PB **4** titrated with Src-SH2 (black) and Grb2-SH2 (gray). Conditions: $\lambda_{ex} = 340$ nm; $\lambda_{em} = 380$ nm; 20 nM of **1** or **4** in buffer (20 mM NaH_2PO_4, 100 mM NaCl, 2 mM DTT, pH 7.4, 25 °C) and 16 μM SH2 protein (final concentration). Data are shown as the average of three independent measurements.

The fluorescence signaling of protein binding was a fast process that occurred within seconds after adding Src-SH2 (Figure 2c). Control experiments with the hydrophobic protein bovine serum albumin (Figure S3) and a SH2 domain from another protein, the adaptor Grb2 (Figure 2c), confirmed that the large fluorescence enhancements are due to binding of the HPB **1** to the Src-SH2 target.

To assess the influence of the hairpin structure and the PNA-arms we compared HPB **1** with pyrene-labeled peptide beacons **3** and **4**. Fluorescence titration revealed a dissociation constant $K_d =$ 1,5 μM of the HPB **1**·Src-SH2 complex (Figure 3a, see also Figure S4). The PNA-peptide conjugate **3** contained noncomplementary PNA-arms and exhibited a significantly lower $K_d = 0.11$ μM. A

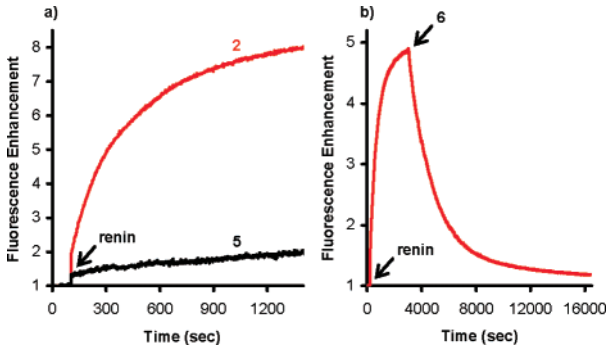


Figure 4. (a) Time courses of relative fluorescence emission of **2** (100 nM) or **5** (100nM), respectively, in buffer after addition of 120 nM renin (for **2**) and 600 nM renin (for **5**). (b) Time course of relative fluorescence emission of **2** (100 nM). After 25 min, 0.3 mM Ac-Pro-His-Pro-Phe-His-Statine-Val-Ile-Gly-NH2 **6** was added to the protease-HPB complex. Conditions: $\lambda_{ex} = 667$ nm; $\lambda_{em} = 690$ nm;. Buffer: 0.05 M TRIS, 0.1 M NaCl, 1 mM EDTA, 0.1% BSA, pH 7.4, 37 °C.

comparison with the $K_d = 0.2$ μM measured for a nonmodified peptide[12] and the $K_d = 4.5$ μM for excimer peptide beacon **4** suggested that PNA *per se* provided no hindrance to protein-peptide binding. This property has also been observed in DNA-responsive PNA-peptide switches.[7] PNA may rather serve as a spacer that separated the bulky hydrophobic pyrene residues from the binding site. The decreased affinity of HPB **1** for Src-SH2 compared to PNA-PB **3** suggested that the formation of the hairpin caused an energetic penalty to protein-HPB binding, as expected. Notably, structured HPB **1** was found to bind the Src-SH2 target with higher specificity than excimer peptide beacon **4**. The affinity of PB **4** for Grb2-SH2 ($K_d = 4.4$ μM) was as high as the affinity for Src-SH2 ($K_d = 4.5$ μM, Figure 3b). As a result, fluorescence signaling of PB **4** was less efficient in discriminating against Grb2-SH2 (Figure 2c) than HPB **1** which had a significantly reduced affinity for Grb-SH2 ($K_d = 3.5$ μM). This interesting behavior, improvement of target specificity, has previously been observed for DNA molecular beacons.[13]

To explore the versatility of the HPB design, we explored another protein target. The aspartic acid protease renin is involved in blood pressure regulation and electrolyte homeostasis through the renin−angiotensin system. It was the aim to develop a probe that, in contrast to most other protease reporters,[14] would not be subject to cleavage. We envision that noncleavable probes could facilitate measurements of both increases and decreases of protease concentration. The fluorescent group of such a probe will not diffuse away from the target as observed with cleavable probes. Rather, fluorescence should remain associated to the target, which may be of use when attempting the imaging of proteases in live tissues. The recognition motif used for the design of a renin probe was based on the known renin peptide-inhibitor sequence Pro-His-Pro-Phe-His-Statine-Val-Ile wherein the amino acid analogon statine replaced the natural cleavage site Val-Leu or Leu-Leu of renin substrates.[15] The peptide was flanked by self-complementary PNA arms and equipped with the near-infrared emitter NIR-664 and the Dabcyl dye as fluorescence quencher. The HPB **2** was found to signal the presence of 120 nM renin by up to 8-fold increases of NIR emission (Figure 4a, see also Figure S6). By contrast, only 2-fold fluorescence enhancement was observed for the nonstructured peptide probe **5** despite exposure to an increased renin concentration (600 nM). HPLC analysis was used to confirm the proteolytic stability of HPB **2** (Figure S7). Thus, it can be assumed that the binding of HPB **2** to the active site of renin is sufficient to induce fluorescence increases. Further evidence was provided by competition experiments which involved the nonmodified renin inhibitor

Ac-Pro-His-Pro-Phe-His-Statine-Val-Ile-Gly-NH_2, **6**. The addition of an excess of unlabeled competitor **6** to HPB **2** bound by renin resulted in a continious decrease in fluorescence (Figure 4b). It can be concluded that the inhibitor **6** quantitatively displaced HPB **2**, which prefers to adopt the closed state in the unbound form. This experiment also proved the reversibility of fluorescence signaling and suggests that continuous monitoring of protease expression may be feasible. Furthermore, the investigation highlighted the advantage of the hairpin peptide beacon design. The renin peptide-inhibitor contains two proline residues and the β-branched amino acids valine and isoleucine. These amino acids are known to reduce the flexibility of peptides.[16] As result, end-to-end contacts are impeded, dye−dye collisions in peptide beacon **5** are less likely to occur, and the fluorescence responsiveness is low. However, the self-complementary PNA-arms in hairpin peptide beacon **2** apparantly provided sufficient binding energy to enforce dye−dye interactions, presumably by inducing a hairpinlike structure.

Most current techniques of homogeneous protein detection rely on conversion of probes such as the fluorogenic and dual-labeled peptides used for the detection of proteases.[14] These methods can only be used for the detection of proteins that have enzymelike activity. However, numerous proteins, which lack enzyme activity, are involved in important protein−protein interaction networks. These proteins are currently visualized by immunostaining or as fluorescent fusion proteins.[17,18]

Labeled DNA aptamer beacons have been shown to change fluorescence upon noncovalent interaction with target proteins.[19] In resemblance to antibody probes, aptamer probes are generated through an evolution process. A rational design of protein recognition probes has been demonstrated for end-labeled peptide beacons.[4,5] The binding of antibodies or the C-p85 SH2 protein domain was reported to induce changes of TAMRA or pyrene fluorescence. However, the results obtained with renin-specific probes (Figure 4) point to limitations of the peptide beacon design which may arise when less flexible recognition peptides are used.

The hairpin peptide beacons developed by us signal the presence of the target protein by 8−11-fold increases of fluorescence. The results obtained with two different protein targets and two different combinations of labels suggest a broad applicability. These new probes are, to the best of our knowledge, the first peptide-based protein reporters designed to feature a stable hairpin. The stability of the hairpin structure can be tuned by adjusting the length of the double helical PNA stem. It may thus prove feasible to incorporate almost any recognition peptide into a HPB structure. It is conceivable that the observed enhancements of target specificity are a characteristics of structured HPBs as previously shown for duplex and triplex based DNA molecular beacons.[13,20] Future studies will be aimed at studying the generality and specificity of HPB signaling.

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Supporting Information Available: Complete ref 15a; synthesis, experimental details, and fluorescence measurement data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) The presence of an excimer signal upon the addition of Src-SH2 to HPB **1** could, in principle, suggest the continued existence of a hybridized PNA stem. It is difficult to provide rigorous proof for hairpin opening other than by crystal structure analysis. The fluorescence measurements have been performed under conditions for saturation of binding. Thus, all HPB **1** is bound in the complex. Most of the data is in agreement with loss of hybridization upon protein binding, in particular the Src affinity differences for peptides **1** ($K_d = 1.5\ \mu$M) and **3** ($K_d = 0.11\ \mu$M) where a 14-fold loss in binding affinity is observed for the complementary versus mismatched PNA sequences in the stem regions. This loss of binding affinity is most readily explained by assuming an energetic penalty when the hairpin is opened. We speculate that excimer-like signals may also occur in an opened, protein-bound complex. It is conceivable that the length and flexibility of the two PNA 4-mer arms may be sufficient to allow contacts between terminally appended dyes in a small population of protein-bound HPB **1**. Pyrene excimers formed in the unhybridized, protein-bound state may have higher fluorescence quantum yield than the excimers in the hybridized state, due to less efficient charge transfer-mediated quenching by G through nonstacked nucleobases. Alternatively, pyrene may have the ability to form excimer-like structures with unhybridized nucleobases.

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