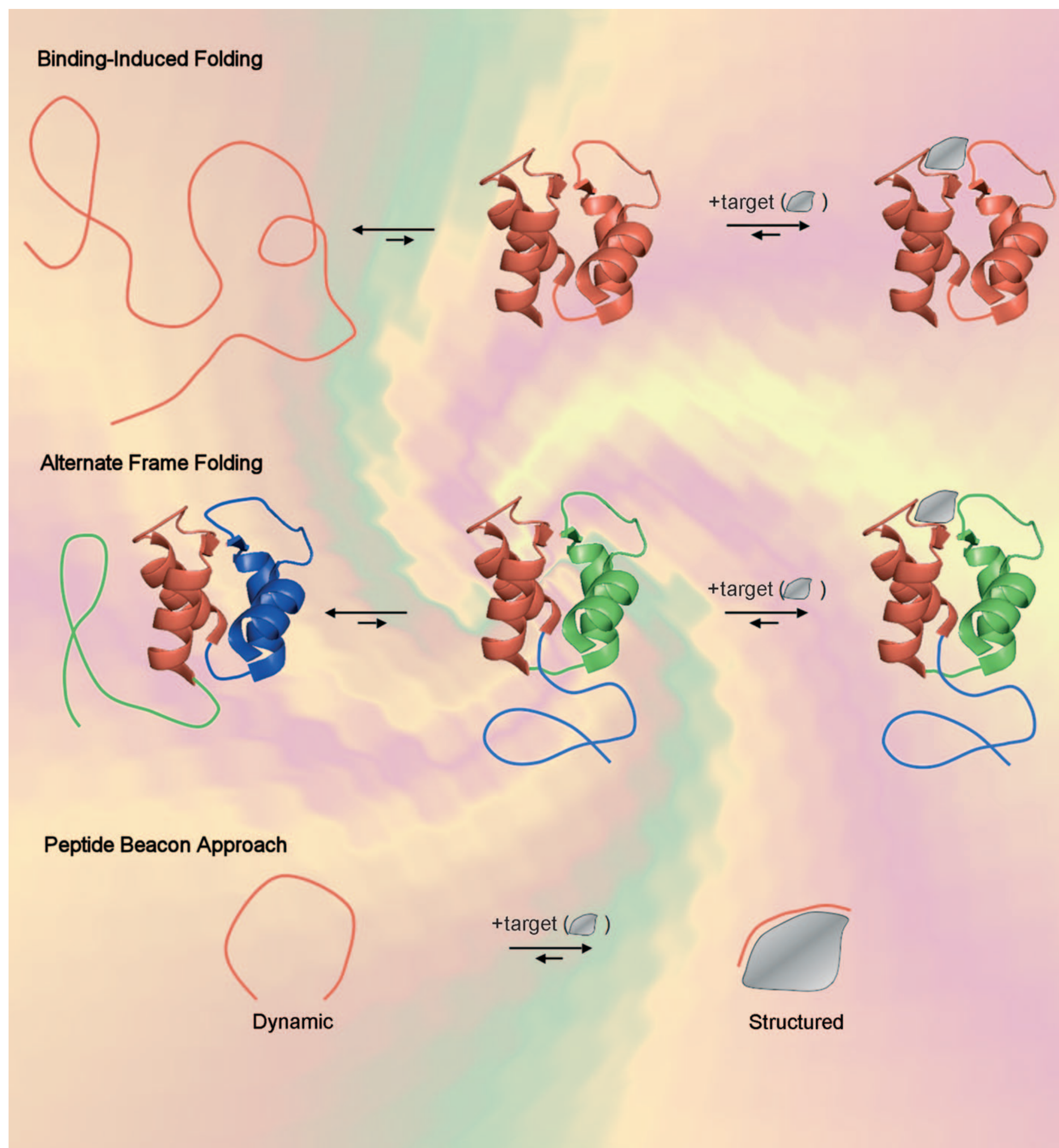
CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200701748

Beyond Molecular Beacons: Optical Sensors Based on the Binding-Induced Folding of Proteins and Polypeptides

Kenneth J. Oh,[b] Kevin J. Cash,[c] and Kevin W. Plaxco*[a]



WILEY InterScience DISCOVER SOMETHING GREAT

© 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Abstract: Many polypeptides and small proteins can be readily engineered such that they only fold upon binding a specific target ligand. This approach couples target recognition with a considerable change in polymer structure and dynamics. Recent years have seen the development of a number of biosensors that couple these large changes to readily measurable optical (fluorescent) outputs. These sensors afford the detection of a wide variety of macromolecular targets including proteins, polypeptides, and nucleic acids. Here we describe the design of such biosensors, from the first iterations as protein engineering experiments, to the development of biosensors targeting a range of protein and nucleic acid targets.

Keywords: binding-induced folding · biosensors · molecular beacons · proteins · rational design

Introduction

Motivated by the exceptional affinity and specificity of biomolecular interactions, enormous effort has gone into the development of generic sensing technologies based on biological recognition elements such as proteins,[1] DNA[2] and even whole cells.[3] Consistent with the promising attributes of biomolecular recognition, biosensors often achieve impressive sensitivity and selectivity relative to many other sensing approaches.[4] Moreover, novel proteins can be produced in the laboratory (through immunological or in vitro selection approaches) that target a wide range of water-soluble analytes with high affinity and high specificity.[5] This suggests that a generic mechanism for detecting protein-target interactions could translate into a valuable, highly general sensing platform. Despite these potential advantages, however, the number of commercially viable, electronic or optical biosensors remains quite limited.[4a]

The drawback that has prevented widespread adaptation of biosensors outside of the laboratory originates, ultimately, from the fact that most proteins do not produce an easily measurable output signal upon target binding. As a result, most protein-based biosensing architectures reported to date rely on sandwich assays (in which a labeled secondary reagent specifically signals the adsorption of the target molecule to the sensor surface), competition assays (the displacement of previously placed, labeled target molecules), or the indirect detection of binding through changes in mass, charge, or index of refraction that occur when a target analyte binds to a biomolecule-coated surface. While sandwich and competition assays are well established (the western blot, enzyme-linked immunosorbent assay (ELISA), and fluorescence polarization assays that dominate clinical pathology laboratories fall into these categories), they require time- and labor-intensive batch processing and thus are ill suited for applications such as continuous monitoring or detection at the point-of-care. In contrast, sensors that function by monitoring changes in the adsorbed mass, charge, or index of refraction, which include microcantilevers,[6] quartz-crystal microbalances,[4b] field-effect transistors[7] and surface-plasmon-resonance-based sensors,[8] achieve admirable operational convenience. As such, these devices have become mainstay research instruments in research settings. These adsorption-based sensors have not, however, reached a similar level of success in clinical settings, in which the non-specific adsorption of even a small fraction of the large number of proteins "contaminating" a typical clinical sample produces overwhelming background signals.[9]

In contrast to adsorption-based sensors, an optical platform termed "molecular beacons"[10] (MBs) has been successfully employed for the label-free detection of nucleic acids in a wide range of realistically complex sample matrices.[11] MBs are composed of a stem-loop DNA modified with terminally attached quencher and fluorophore moieties. In the absence of target, the stem holds the fluorophore/quencher pair in proximity, minimizing fluorescence emission. Hybridization of a target oligonucleotide to the MB loop separates the reporter groups, resulting in a large increase in fluorescence emission. Critically, because MB signaling is linked to a binding-specific conformational change (and not simply adsorption of charge, mass etc. to a surface), this approach has proven of utility in applications ranging from real-time polymerase chain reaction (PCR) analysis[12] to in-situ hybridization.[13]

The success of molecular beacons illustrates a key and presumably general issue regarding biosensors: sensing mechanisms based on specific, binding-induced changes in the physical properties of the biomolecule provide a means of circumventing the false positives that plague adsorption-based approaches to label-free biosensing. This suggests that biosensors relying on specific signal transduction mechanisms—sensors that monitor a binding-specific change in the biomolecule (or the substrate) rather than adsorption—may prove suitable for deployment even in complex, contaminant-ridden sample matrices. This argument has motivated

[a] Prof. K. W. Plaxco
Department of Chemistry and Biochemistry
Department of Biomolecular Science and Engineering
University of California, Santa Barbara
Santa Barbara, California 93106 (USA)
Fax: (+1) 805-893-4120
E-mail: kwp@chem.ucsb.edu

[b] K. J. Oh
Department of Chemistry and Biochemistry
University of California, Santa Barbara
Santa Barbara, California 93106 (USA)

[c] K. J. Cash
Department of Chemical Engineering
University of California, Santa Barbara
Santa Barbara, California 93106 (USA)

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

the development of protein-engineering methods aimed at coupling the binding of any arbitrary target to a large change in the physics of a specific recognition protein. In this paper we review recent polypeptide- and protein-based biosensors that, like DNA-based molecular beacons, couple binding-induced changes in polymer structure and dynamics with robust optical outputs.

Signal Transduction

While the rationale for polypeptide analogues of molecular beacons is clear, the means of fabricating such sensors is less so. The problem is that, unlike stem-loop DNAs, the structure and dynamics of most proteins do not change significantly upon target binding. To date, however, two complementary solutions to this dilemma have been reported. The first is to identify the few proteins that, in contrast to most, do exhibit a large, binding-induced conformational change and then re-engineer their binding specificity such that they recognize the desired target. The second is to select a protein with the appropriate binding characteristics and then modify it such that it undergoes a large-scale conformational change upon target binding.

An illustrative example of the first approach is that of Hellinga and co-workers, who have demonstrated a family of sensors based on a large, binding-induced conformational change that occurs when members of the maltose-binding protein (MBP) family bind their target ligands.[14] MBP adopts two conformations: a ligand-free "open" form and a ligand-bound "closed" conformation that interconvert through a combined bending–twisting motion around a hinge region.[15] The interconversion of these conformations is linked to the opening and closing of a deep cleft at a site opposite the ligand binding site. Hellinga and co-workers have used this conformational change to transduce the binding event into an optical signal by placing a fluorescent reporter group in the cleft that is sensitive to the change in solvation that occurs when target binding opens it. The first-generation of these sensors employed near native sequences of the maltose binding protein and its close homologues (the family of periplasmic binding proteins), producing sensors to the proteins' natural targets.[16] Hellinga and co-workers have also employed computational protein engineering methods to convert maltose binding protein into a zinc sensor by replacing key residues in the ligand binding site with zinc binding amino acids.[14] The introduction of multiple and cooperative zinc-binding sites and optimization of the geometry of the zinc-binding amino acids lead to improvements in zinc affinity with a dissociation constant of 350 nM.

While the family of biosensors based on the maltose binding protein framework described above has shown the merits of re-engineering the binding sites of proteins that naturally support a large, binding-induced conformational change, the approach remains a difficult, cutting edge technology with few successful examples reported to date. A second limitation appears to be that the number of protein "scaffolds" that exhibit a suitable binding-induced conformational change may be limited: to date this approach has only been demonstrated for maltose binding protein[14] and calmodulin.[17] Given these potential pitfalls, we argue that the approach of identifying a protein that recognizes the target of interest and re-engineering it to undergo a large scale change in physics upon binding may prove more straightforward.

How does one couple target binding with a large change in the conformation or dynamics of an arbitrary polypeptide? Two solutions to this question have been reported to date. The first solution, first reported by our laboratory, employs mutations to destabilize the native fold, thus creating a protein that shifts between its native, target-binding configuration and the inactive, unfolded state (Figure 1a).[18] The second, pioneered by Loh and co-workers, stabilizes an alternative, non-native, non-binding state that, nevertheless, adopts a native-like configuration upon binding to target ligand (Figure 1b).[19]

The folding of small, single-domain proteins is highly cooperative[20] and, thus, mutations that destabilize the native state sufficiently, including terminal deletions,[21] internal de-

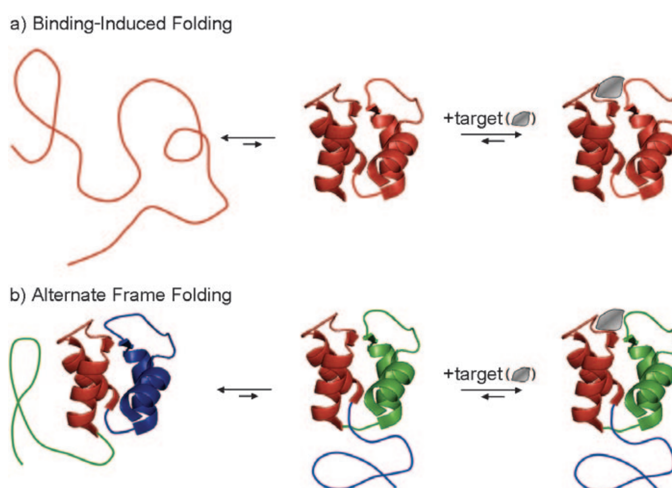


Figure 1. Binding-induced folding occurs when the formation of a protein–target complex overcomes an unfavorable folding free energy and drives the polypeptide into the properly folded state. a) The folding equilibrium constants of small proteins are generally rather marginal and only favor the native state by three to four orders of magnitude. When coupled with the cooperative folding of small proteins, this renders it relatively straightforward to mutate a protein altering the equilibrium constant to favor the unfolded state. Even the mutant protein, however, continues to sample the native state. Molecules transiently sampling the native state are competent to bind the protein's target thus "trapping" the native configuration through the favorable free energy gained through formation of the complex. b) Loh and co-workers have demonstrated an alternate method by which proteins can be rationally re-engineered to populate two conformations.[19] Their approach, termed alternate frame folding (AFF), involves the duplication of a portion of a protein's sequence, which introduces an alternate fold containing the duplicate sequence element, as well as the introduction of a mutation to inhibit target binding through the native fold. The presence of target will push the equilibrium towards the alternate fold, thus coupling recognition with a large-scale conformational change suitable for signal generation.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

letions ,[22] and internal mutations,[23] produce fully unfolded proteins. In contrast to the unfolded state, however, only a protein's native conformation supports target binding. Because of this, proteins that have been "engineered" to unfold by mutation will refold upon binding their target ligand if the binding free energy is sufficient to overcome the unfavorable free energy of folding[20,21a,b,22,23a,c,24] (Figure 1 a). For example, Shortle has demonstrated that a truncated, largely unfolded variant of staphylococcal nuclease binds to and hydrolyzes its substrate and refolds upon titration with high affinity nuclease inhibitors.[24] Similarly, Matthews and co-workers have created T4 lysozyme variants containing methionine substitutions in almost all core positions, thereby "simplifying" the hydrophobic core of the protein, but observing a complete loss of structure. This unfolded variant, however, still retains 20% of the activity of the wild type protein,[25] indicating that it is likewise capable of folding into the native structure and binding to (even catalyzing the hydrolysis of) the protein's natural substrate. Motivated by this observation, and the serendipitous examples of the effect described above, we set out to demonstrate that it is possible to systematically engineer binding-induced folding into an arbitrary small protein.[18] Specifically, we performed a series of carboxy-terminal truncations on a small protein, the Fyn SH3 domain. The truncations resulted in a change in the folding equilibrium, favoring the unfolded state. The target ligand, in this case a short polypeptide (p85α-2), only binds the native conformation and thus the presence of the target rapidly shifts the equilibrium towards the folded state.

In contrast to our approach, Loh and co-workers created a "bistable" protein by stabilizing an alternative, native-like fold for their polypeptide chain.[19] Their approach, termed alternate frame folding (AFF), involves the duplication of a portion of a protein's sequence, which introduces an alternate fold containing the duplicate sequence element. As the alternate fold does not bind the target, the presence of target will again push the equilibrium back towards the "native" fold, thus coupling recognition with a large-scale conformational change suitable for signal generation (Figure 1 b).

Signal Output

The above examples of binding-induced folding couple target recognition with an enormous change in the dimensions and, often, physical properties of the polypeptide chain, suggesting that they might prove ideal signal transduction mechanisms for protein-based biosensors. Binding-induced folding, however, does not lead directly to a viable biosensor. To do this, we need to transduce the binding-induced folding event to a reporter (e.g., optical or electrochemical) that reports on folding and , in turn, indicates the presence of the target. For DNA-based molecular beacons this is commonly performed by using a pair of fluorophores that undergo Förster resonance energy transfer (FRET).[26] The large separation (typically several nanometers) of the reporter groups that occurs when a target binds to the stem-loop coupled with the inverse sixth-power distance dependence of FRET results in efficient fluorescence emission from the target-beacon complex. This mechanism, however, has proven of only limited utility for sensors based on binding-induced folding. The problem is that the distance changes arising upon the binding-induced folding of polypeptides and single-domain proteins are short (1–2 nm) relative to the 4–6 nm Förster radii of typical, visible-light FRET pairs (such as cyan and yellow fluorescent proteins), leading to efficient transfer in both the bound and unbound states and only small signal changes upon target binding (reviewed in reference [27]). Fortunately, quenching mechanisms with much stronger distance dependencies than that of FRET are available.

The aromatic amino acid tryptophan is an efficient contact quencher of a number of fluorophores through photo-induced electron transfer (PET).[28] Using such a fluorophore we have converted our truncated FynSH3 domain into an optical biosensor (Figure 2). In the unfolded, target-free state the FynSH3 domain's two tryptophan residues are solvent exposed, allowing them to quench a dipyrromethene boron difluoride (Bodipy) fluorophore conjugated to a nearby cysteine. Binding-induced folding of the protein sequesters these residues, producing a large increase in Bodipy emission. This allows us to detect the target at concentrations as low as a few hundred micromolar despite the approximately 100-fold[21d] diminished affinity of the modified FynSH3 domain for this target.

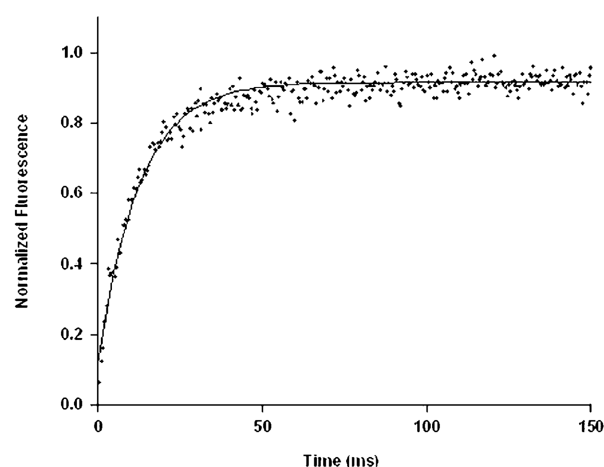


Figure 2. Binding-induced folding supports rapid sensor response times. Shown is the response (fluorescence emission) of a biosensor based on the binding-induced folding of the FynSH3 domain when it is rapidly mixed with its target ligand.[18] A single-exponential increase in fluorescence is observed with a time constant within error of that reported for the folding of the full-length protein,[21c] suggesting that, rather than serving as a template around which the protein folds, the target traps transiently folded molecules by binding to them.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Our laboratory's use of tryptophan quenching of an exogenous fluorophore was inspired by the work of Sauer and co-workers, who have shown that tryptophan is a contact quencher of a number of organic dyes, including oxazine MR 121, ATTO 655, and Rhodamine 6G (see reference [28] for a review). Using this effect, Neuweiler and co-workers[29] have themselves fabricated a sensor for the detection of antibodies against the nuclear tumor suppressor protein p53. They achieved this by modifying two short (15 and 20 residues), tryptophan-containing sequences excised from p53 with the tryptophan-quenched oxazine dye MR121. This epitope (antigenic sequence), like the vast majority of short polypeptides,[30] is flexible, and thus supports dye-quencher contact which, in turn, reduces MR121 fluorescence. Upon complex formation the epitope adopts its native conformation (since the antibody was raised against the native antigen, the unstructured polypeptide likely adopts its native conformation when bound to the antibody[31]) which, in this case, sequesters the tryptophan residue from the MR121 dye. This, in turn, leads to a two-fold increase in fluorescence. By using this approach, antibodies are easily detected to concentrations as low as a few nanomolar using standard fluorometric approaches.

Sauer and co-workers have further advanced their approach by employing confocal microscopy to push the detection limit of their sensors an order of magnitude below the dissociation constant of the sensing polypeptide.[29] The small sample volumes interrogated in confocal microscopy minimize background fluorescence and scattering to such an extent that single molecules can be observed as they diffuse through the detection volume, at which they produce bursts of fluorescence with high signal-to-background ratios.[32] Using this approach, the authors report the detection of p53 antibodies directly in human serum.[29] By using fluorescence intensities to distinguish between bound and free p53 probes, Neuweiler and co-workers quantified the ratio of free to target bound sensor at target concentrations an order of magnitude below the dissociation constant of the complex, thus achieving the detection of anti-p53 antibodies at concentrations as low as 10 pM in buffer, and with results comparable to ELISA in serum samples from tumor patients.

As illustrated by the two examples described above, tryptophan-quenched dyes provide a convenient means of monitoring the binding-induced folding of polypeptides that sequester a tryptophan residue upon binding-induced folding. Tryptophan, however, is the least common of the encoded amino acids, perhaps limiting the approach's utility. Fortunately, however, a new platform has been developed, which we have named peptide beacons (PBs), which invokes other fluorophore-quencher pairs to overcome this limitation (Figure 3).

The first class of fully exogenous reporters adapted to the PB architecture are dyes that form stable dimers and, in doing so, modulate their emission intensity. For example, Herron and co-workers,[33] labeled an antigenic, 13-residue polypeptide from human chorionic gonadotrophin (hCG) with fluorescein and tetramethylrhodamine (TMR) at the amino- and carboxy-termini, respectively. When the polypeptide conjugate is free in solution, fluorescein and TMR form an intramolecular dimer in which dipole–dipole interactions result in fluorescence quenching.[34] When complexed with the target antibody, the dimer is disrupted, increasing the fluorescence intensity up to 7.8-fold. Similarly, Hoth and co-workers[35] have described a PB based on the formation of a TMR homodimer and a polypeptide antigen from type II collagen. The binding of a target antibody to this construct disrupts the dye–dye dimer and increases the construct's emission approximately fourfold.

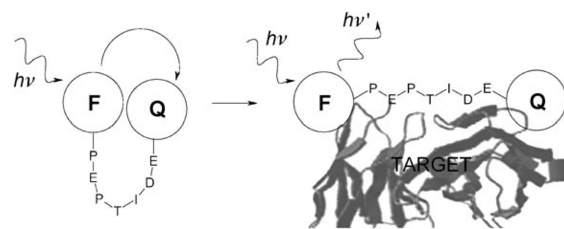


Figure 3. Shown is the mechanism of a generic peptide beacon. Left: In the absence of target, the polypeptide is flexible, allowing an attached fluorophore to contact a second element that modulates its emission (e.g., a quencher, as shown, or an excimer partner). Right: Upon target binding, the fluorophore is segregated from its partner, resulting in increased or decreased fluorescence intensity. Of note, because of the limited distance changes that can be generated by the folding of polypeptides and small proteins, Förster resonance energy transfer (FRET)-based quenching (which varies inversely with distance to the sixth power) generally does not support high-gain signaling. Instead PB sensors generally require higher-order distance-dependent phenomena, such as electron transfer-based quenching (which is exponentially distance dependent) or excimer pair formation.

Pyrene forms an excimer, a complex between a molecule in its excited state with a ground state counterpart that emits at a wavelength distinct from that of the monomeric dye,[36] providing another reporter group for peptide beacon applications.[37] Of note, excimer formation is highly dependent on the precise geometry (distance and angle) of the dye–dye complex,[38] and thus even binding-induced changes in the contact angle of the pyrene pair are sufficient to alter excimer emission. To illustrate the importance of this point we have recently demonstrated[37] a pyrene-based PB that can detect its target—the TAR RNA hairpin of HIV-1—even though target binding results in the polypeptide folding into a hairpin[39] rather than the extended conformation required of other PB architectures (Figure 4). Loh and co-workers[19] similarly employed pyrene reporters with a version of the protein calbindin re-engineered to undergo alternate frame folding (AFF) to generate a series of fluorescent calcium sensors with detection limits in the low micromolar regime. Finally, Tcherkassaya and co-workers[40] have used pyrene-labeled prion peptides to monitor the presence of prions, although it remains unclear whether this represents a sensor based on binding-induced (aggregation-induced) folding or instead based on interpeptide excimer formation in the aggregated state.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

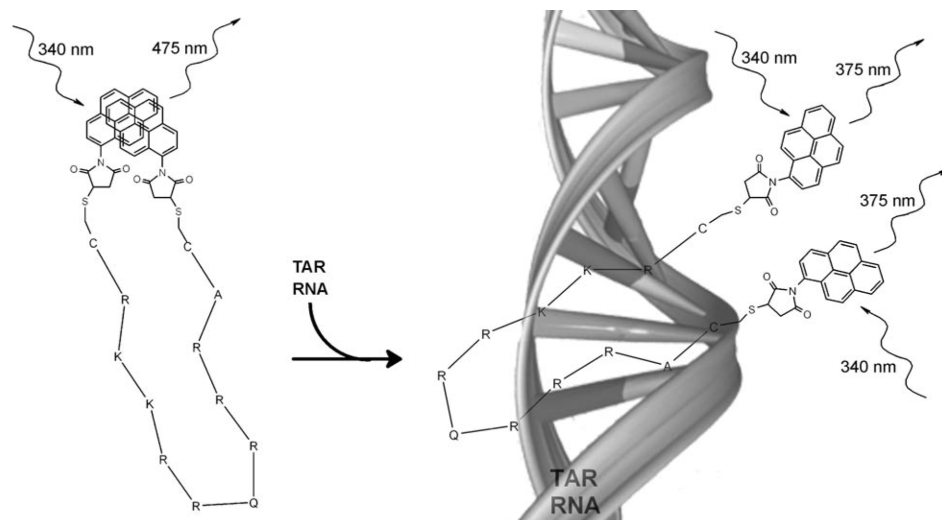


Figure 4. Excimer pair formation, such as shown here between two pyrenes, is strongly dependent on the geometry (distance and angle) of the excited state duplex. Consistent with this, an excimer-based PB architecture composed of two *N*-(1-*p*yrene)maleimide functional groups conjugated to a recognition peptide can be used to detect targets that bring the PB into a hairpin structure,[39] as long as binding introduces geometric constraints that inhibit excimer formation.

The above-described peptide beacons employ exogenous dyes that hold the termini of the unfolded polypeptide together in a manner analogous to the stem structure in a DNA molecular beacon. Taking this to what is perhaps its logical conclusion, two groups have fabricated "chimeric" molecules containing a polypeptide recognition element flanked by peptide nucleic acid (PNA) sequences that hybridize to form a double-stranded stem. PNA is a DNA analogue composed of the four naturally occurring nucleobases, but containing a modified polypeptide backbone rather than a sugar–phosphate backbone. As such, PNA can readily be incorporated into polypeptides comprised of α-amino acids during chemical synthesis. Using this approach we have demonstrated a "chimeric" PNA–polypeptide–PNA peptide beacon that forms a stable stem-loop structure in the absence of target, thus quenching the fluorescence of a terminal Bodipy through collisions with a tryptophan residue on the opposite terminus.[41] Target binding disrupts the PNA stem, leading to a threefold increase in Bodipy emission and allowing detection of the target antibody at subnanomolar concentrations. Seitz and co-workers have, similarly, used this approach to develop sensors that they term hairpin peptide beacons" (HPBs) for the detection of the SH2 domain of Src kinase and the protease renin.[42] Using NIR-664 as their fluorophore and DABCYL as their quencher, they achieve an eightfold increase in fluorescence upon recognition of renin and a sub-120 nM detection limit. This represents a significant increase relative to the twofold gain produced by the analogous sensor lacking the PNA stem. They report that the inclusion of the PNA hairpin also increases the specificity of the sensor relative to the stemless variant, although at the cost of decreased affinity.

All of the above-described peptide beacons are directly analogous to DNA molecular beacons in that target binding disrupts the formation of a stable loop structure (held in place either by interactions between the dye molecules or by formation of a PNA stem). An alternate PB signaling mechanism, based instead on a binding-induced change in the dynamics of the polypeptide, has also been demonstrated. That is, whereas folded proteins are relatively rigid, the two ends of an unfolded, ten-residue polypeptide collide with a time constant of a few nanoseconds[43]—a change that can be coupled to large changes in fluorescence emission. Based on this observation we have developed a stemless PB using the electron-accepting quencher viologen (effectively a contact quencher) and a long-lived, ruthenium-based fluorophore with an excited state lifetime that is an order of magnitude longer than the intermolecular collision rates typical of unfolded polypeptides[44] (Figure 5). In the absence of the target antibody, a relatively rapid rate of end-to-end collisions ensures that the viologen efficiently suppresses the emission of the ruthenium fluorophore. Upon addition of the target, the peptide is bound in an extended conformation and the fluorescence intensity increases by a factor of six.

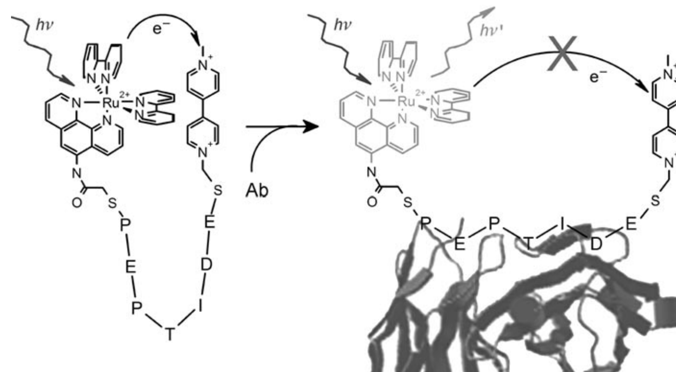


Figure 5. The PB architecture does not require the formation of a stable complex between the dye and the modulator. For example, we have developed a "dynamics" based peptide beacon architecture that is comprised of a long-lived fluorophore and an electron-accepting quencher conjugated to a recognition peptide.[44] Rapid intramolecular collisions that occur in the absence of target (left) allow for collision-induced fluorescence quenching. Upon target binding (right), the fluorophore is separated from the quencher, preventing electron transfer and significantly enhancing fluorescence.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

PB signaling is linked to a very specific, binding-induced conformational change and not, as is the case in many biosensor architectures (such as those based on SPR, FETs and QCMs), on the measurement of mass, charge, or index of refraction of analyte adsorbed to the surface of the sensor. As such, PBs should be less susceptible to the false positives associated with nonspecific binding that typically plague adsorption-based biosensing approaches.[45] Being optical approaches, however, their sensitivity can be degraded significantly by the presence of fluorescent contaminants, a problem that is particularly severe at shorter excitation wavelengths. For example, our excimer-based PB architecture fails when challenged in blood serum due to the significant autofluorescence of serum at the UV excitation wavelength of pyrene. In contrast, however, our ruthenium-based PB excites in the visible and is thus capable of detecting target antibody in complex media such as human saliva and blood serum,[44] albeit even still with somewhat reduced sensitivity due to background fluorescence. Nevertheless, the sensor's gain is unchanged between serum and buffer, suggesting that none of the hundreds of proteins in human serum activate the PB.

Conclusions

Binding-induced folding appears to be a fairly general signal-transduction mechanism for polypeptide-based biosensors. The criteria for choosing a suitable target/ligand system requires only that target binding can be used to segregate an optical reporter pair by at least a few angstroms, and that the reporting group pair does not disrupt target binding. Also of benefit, many PB architectures can be fabricated with readily available dye conjugates, often during solid-phase synthesis of the polypeptide recognition element. Finally, invoking large libraries of engineered protein or polypeptide recognition elements by phage and bacterial display[46] techniques increases the number of possible molecular targets. The combination of these attributes suggests a wide range of applications are within reach of the PB sensing approach.

[1] B. Leca-Bouvier, L. J. Blum, *Anal. Lett.* **2005**, *38*, 1491–1517.
[2] G. A. Rivas, M. L. Pedano, N. F. Ferreyra, *Anal. Lett.* **2005**, *38*, 2653–2703.
[3] B. S. Edwards, T. Oprea, E. R. Prossnitz, L. A. Sklar, *Curr. Opin. Cell Biol.* **2004**, *16*, 392–398.
[4] a) J. Castillo, S. Gaspar, S. Leth, M. Niculescu, A. Mortari, I. Bontidean, V. Soukharev, S. A. Dorneanu, A. D. Ryabov, E. Csoregi, *Sens. Actuators B* **2004**, *102*, 179–194; b) M. A. Cooper, *Anal. Bioanal. Chem.* **2003**, *377*, 834–842.
[5] a) T. J. Vaughan, J. K. Osbourn, P. R. Tempest, *Nat. Biotechnol.* **1998**, *16*, 535–539; b) D. R. Wilson, B. B. Finlay, *Can. J. Microbiol.* **1998**, *44*, 313–329; c) P. Mathonet, J. Fastrez, *Curr. Opin. Struct. Biol.* **2004**, *14*, 505–511.
[6] a) M. B. Viani, L. I. Pietrasanta, J. B. Thompson, A. Chand, I. C. Gebeshuber, J. H. Kindt, M. Richter, H. G. Hansma, P. K. Hansma, *Nat. Struct. Mol. Biol.* **2000**, *7*, 644–647; b) C. Ziegler, *Anal. Bioanal. Chem.* **2004**, *379*, 946–959.
[7] E. Katz, I. Willner, *Electroanalysis* **2003**, *15*, 913–947.
[8] J. Homola, *Anal. Bioanal. Chem.* **2003**, *377*, 528–539.
[9] A. N. Asanov, W. W. Wilson, P. B. Oldham, *Anal. Chem.* **1998**, *70*, 1156–1163.
[10] S Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303–308.
[11] S. S. M. Sum, D. K. H. Wong, M. F. Yuen, H. J. Yuan, J. Yu, C. L. Lai, D. Ho, L. Zhang, *J. Clin. Microbiol.* **2004**, *42*, 3438–3440.
[12] L. Wang, J. R. Blasic, Jr., M. J. Holden, R. Pires, *Anal. Biochem.* **2005**, *344*, 257–265.
[13] S. Ganguly, S. Ghosh, D. Chattopadhyay, P. Das, *RNA Biol.* **2004**, *1*, 48–53.
[14] J. S. Marvin, H. W. Hellinga, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 4955–4960.
[15] F. A. Quiocho, *Philos. Trans. R. Soc. London Ser. B* **1990**, *326*, 341–351.
[16] a) J. S. Marvin, E. E. Corcoran, N. A. Hattangadi, J. V. Zhang, S. A. Gere, H. W. Hellinga, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4366–4371; b) R. M. de Lorimier, J. J. Smith, M. A. Dwyer, L. L. Looger, K. M. Sali, C. D. Paavola, S. S. Rizk, S. Sadigov, D. W. Conrad, L. Loew, H. W. Hellinga, *Protein Sci.* **2002**, *11*, 2655–2675; c) M. A. Dwyer, H. W. Hellinga, *Curr. Opin. Struct. Biol.* **2004**, *14*, 495–504; d) N. C. Vercillo, K. J. Herald, J. M. Fox, B. S. Der, J. D. Dattelbaum, *Protein Sci.* **2007**, *16*, 362–368.
[17] a) A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura, R. Y. Tsien, *Nature* **1997**, *388*, 882–887; b) A. Persechini, J. A. Lynch, V. A. Romoser, *Cell Calcium* **1997**, *22*, 209–216.
[18] J. E. Kohn, K. W. Plaxco, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10841–10845.
[19] M. M. Stratton, D. M. Mitrea, S. N. Loh, *ACS Chem. Biol.* **2008**, *3*, 723–732.
[20] H. S. Chan, S. Shimizu, H. Kaya, *Methods Enzymol.* **2004**, *380*, 350–379.
[21] a) J. P. Sluka, S. J. Horvath, A. C. Glasgow, M. I. Simon, P. B. Dervan, *Biochemistry* **1990**, *29*, 6551–6561; b) C. W. Adams, O. Nanassy, R. C. Johnson, K. T. Hughes, *Mol. Microbiol.* **1997**, *24*, 1235–1247; c) A. T. Alexandrescu, C. Abeygunawardana, D. Shortle, *Biochemistry* **1994**, *33*, 1063–1072; d) A. R. Viguera, J. L. R. Arrondo, A. Musacchio, M. Saraste, L. Serrano, *Biochemistry* **1994**, *33*, 10925–10933; e) G. de Prat Gay, J. Ruiz-Sanz, J. L. Neira, F. J. Corrales, D. E. Otzen, A. G. Ladurner, A. R. Fersht, *J. Mol. Biol.* **1995**, *254*, 968–979; f) J. L. Neira, A. R. Fersht, *J. Mol. Biol.* **1999**, *285*, 1309–1333; g) J. L. Neira, A. R. Fersht, *J. Mol. Biol.* **1999**, *287*, 421–432.
[22] N. Kouvatsos, J. K. Meldrum, M. S. Searle, N. R. Thomas, *Chem. Commun.* **2006**, 4623–4625.
[23] a) O. Zhang, J. D. Forman-Kay, *Biochemistry* **1997**, *36*, 3959–3970; b) E. S. Cobos, V. V. Filimonov, M. C. Vega, P. L. Mateo, L. Serrano, J. C. Martinez, *J. Mol. Biol.* **2003**, *328*, 221–233; c) M. S. Yousef, W. A. Baase, B. W. Matthews, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11583–11586.
[24] D. Shortle, A. K. Meeker, *Biochemistry* **1989**, *28*, 936–944.
[25] N. C. Gassner, W. A. Baase, B. W. Matthews, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12155–12158.
[26] S. Tyagi, D. P. Bratu, F. R. Kramer, *Nat. Biotechnol.* **1998**, *16*, 49–53.
[27] S. B. VanEngelenburg, A. E. Palmer, *Curr. Opin. Biotechnol.* **2008**, *19*, 60–65.
[28] S. Doose, H. Neuweiler, M. Sauer, *ChemPhysChem* **2005**, *6*, 2277–2285.
[29] H. Neuweiler, A. Schultz, A. C. Vaiana, J. C. Smith, S. Kaul, J. Wolfrum, M. Sauer, *Angew. Chem.* **2002**, *114*, 4964–4968; *Angew. Chem. Int. Ed.* **2002**, *41*, 4769–4773.
[30] M. R. Pincus, *Biopolymers* **1992**, *32*, 347–351.
[31] P. Rezacova, J. Lescar, J. Brynda, M. Fabry, M. Horejsi, J. Sedlacek, G. A. Bentley, *Structure* **2001**, *9*, 887–895.
[32] S. Nie, D. T. Chiu, R. N. Zare, *Science* **1994**, *266*, 1018–1021.
[33] A. P. Wei, D. K. Blumenthal, J. N. Herron, *Anal. Chem.* **1994**, *66*, 1500–1506.
[34] a) I. L. Arbeloa, *J. Chem. Soc. Faraday Trans. 2* **1981**, *77*, 1735–1742; b) I. L. Arbeloa, *J. Chem. Soc. Faraday Trans. 2* **1981**, *77*,

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

1725–1733; c) I. López Arbeloa, P. R. Ojeda, *Chem. Phys. Lett.* **1982**, *87*, 556–560.
[35] K. F. Geoghegan, P. J. Rosner, L. R. Hoth, *Bioconjugate Chem.* **2000**, *11*, 71–77.
[36] a) F. M. Winnik, *Chem. Rev.* **1993**, *93*, 587–614; b) S. S. Lehrer, *Methods Enzymol.* **1997**, *278*, 286–295.
[37] K. J. Oh, K. J. Cash, K. W. Plaxco, *J. Am. Chem. Soc.* **2006**, *128*, 14018–14019.
[38] M. Masuko, H. Ohtani, K. Ebata, A. Shimadzu, *Nucleic Acids Res.* **1998**, *26*, 5409–5416.
[39] J. D. Puglisi, L. Chen, S. Blanchard, A. D. Frankel, *Science* **1995**, *270*, 1200–1203.
[40] O. Tcherkasskaya, E. A. Davidson, M. J. Schmerr, C. S. Orser, *Biotechnol. Lett.* **2005**, *27*, 671–675.
[41] K. J. Oh, K. J. Cash, A. A. Lubin, K. W. Plaxco, *Chem. Commun.* **2007**, 4869–4871.
[42] S. Thurley, L. Röglin, O. Seitz, *J. Am. Chem. Soc.* **2007**, *129*, 12693–12695.
[43] L. J. Lapidus, W. A. Eaton, J. Hofrichter, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7220–7225.
[44] K. J. Oh, K. J. Cash, V. Hugenberg, K. W. Plaxco, *Bioconjugate Chem.* **2007**, *18*, 607–609.
[45] C. Kosslinger, E. Uttenthaler, S. Drost, F. Aberl, H. Wolf, G Brink, A. Stanglmaier, E. Sackmann, *Sens. Actuators B* **1995**, *24*, 107–112.
[46] P. H. Bessette, J. J. Rice, P. S. Daugherty, *Protein Eng. Des. Sel.* **2004**, *17*, 731–739.

Published online: February 3, 2009

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim