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A Folding-Based Approach for the Luminescent Detection of a Short RNA Hairpin

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Supporting Information

ABSTRACT: We report the rational design of a 20-mer basic peptide, derived from the transcriptional antitermination protein N of bacteriophage P22, equipped with a luminescent DOTA[Tb^{3+}] macrocyclic complex and a sensitizing tryptophan antenna. Folding of this peptide into an α helical conformation, which occurs upon binding to its target *boxB* RNA hairpin, results in a large increase in luminescence emission. Therefore, the peptide construct works as a highly sensitive and selective probe for this RNA hairpin.

In addition to its well-established functions as intermediary in protein expression, as proposed by Francis Crick in the central dogma, RNA has progressively been recognized as a cornerstone in cell biochemistry, playing critical roles ranging from biocatalysis to post-transcriptional regulation of gene expression.[1] Consequently, RNA represents an attractive therapeutic target and will undoubtedly become a major biomarker for diagnosis purposes.[2] Currently, most of the methods for detecting specific RNA sequences rely on the hybridization of denatured RNAs with synthetic complementary probes.[3] However, the direct recognition and sensing of native secondary RNA structures, which might lead to new research and diagnostic tools, remain a largely unexplored challenge,[4] and while a handful of methods for labeling of RNA aptamers using large biosynthetic protein reporters have been described,[5] to the best of our knowledge, synthetic probes capable of specifically signaling naturally occurring RNA folded structures are still unknown.

Inspired by the recognition of natively folded RNA hairpins by short basic peptides that undergo large conformational changes upon binding,[6] we considered that this folding process could be exploited for the modulation of the sensitization of a lanthanide ion and thus for the selective optical signaling of the target RNA.[7] Lanthanide luminescence is particularly useful for biological purposes owing to their narrow, long wavelength-emission bands and the possibility of avoiding background autofluorescence using time-resolved experiments.[8] As a model system to test this sensing approach, we selected the interaction between an arginine-rich motif of the transcriptional antitermination protein N of phage P22 (P22-N) and a short RNA hairpin (*boxB*). This interaction is a key component of a ribonucleoprotein antitermination complex that allows the RNA polymerase to read through terminator elements and transcribe downstream genes required for the progress of the lytic infection.[9] The P22-N peptide is largely unstructured in solution and only folds into an α helical conformation upon binding to the major groove of its target *boxB* RNA hairpin.[10] Therefore, the P22-N/*boxB* system provides an excellent platform to explore whether the geometrical changes associated with the folding/recognition process can be exploited for modulating the sensitization of a Tb^{3+} ion by a nearby antenna.[11]

The RNA binding domain of P22-N comprises the N-terminal region of the protein, from residues Asn14 to Ile33 (NAKTRRHERRRKLAIERDTI, Figure 1). Extensive mutational studies have shown that only a handful of the many contacts between the *boxB* hairpin and the P22-N RNA binding domain are base specific, and in addition to residues Ala15, Arg18, Arg22 and Arg23, which are conserved across P22, λ and φ21 N proteins, only Arg19 seems absolutely required for high-affinity binding of P22-N to the *boxB* hairpin.[12] Based on this functional information as well as on the NMR structure of the RNA binding domain of the P22-N protein to a *boxB* hairpin RNA,[13] we selected two solvent-exposed and mutation-tolerant positions for introducing the required sensing components. Lys25 was selected for attaching the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelate, and the

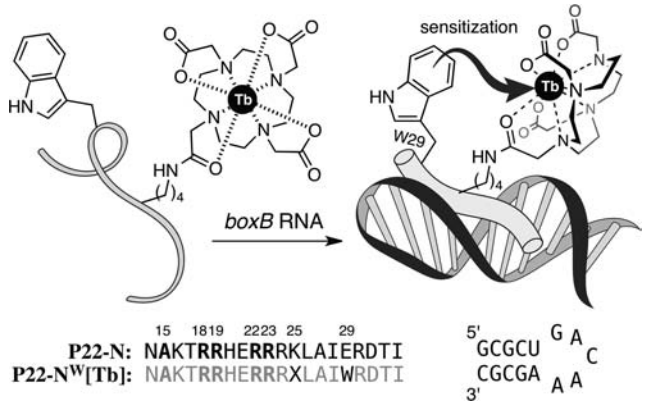

Figure 1. Sensing mechanism: The conformational changes associated with RNA binding bring together the sensitizing antenna and the lanthanide chelate. Sequences of the natural P22-N RNA binding domain, the modified luminescent **P22-N^{W}[Tb]**, X = Lys(DOTA-[Tb^{3+}]), and of the *boxB* RNA hairpin used in our studies.

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sensitizing Trp was introduced four residues away in place of the natural Glu29 (Figure 1 and Supporting Information, SI). Thus, the DOTA[Tb^{3+}] complex and the antenna would be positioned adjacent to each other upon folding of the peptide chain in the major groove of the *boxB* RNA hairpin, thus maximizing the sensitization of the lanthanide ion upon binding.

Detailed procedures are given in the SI. In short, the sensor was synthesized following standard Fmoc solid-phase synthesis procedures.[14] The lysine residue was introduced with its side chain orthogonally protected as alloc carbamate and selectively deprotected by Pd catalysis once the peptide sequence was fully assembled in the solid phase.[15] The DOTA chelating macrocycle was then coupled to the ε-amino of the Lys side chain as its tris-(*t*-Bu)-ester derivative,[16] while the peptide was still attached to the solid support. Finally, the N-terminal Fmoc was removed, and the peptide was subjected to a standard cleavage/deprotection step.[17] The resulting DOTA–peptide product was purified by reverse-phase HPLC and incubated with $TbCl_3$ in HEPES buffer to give the desired peptide chelate **P22-N^W[Tb]**, which was subsequently repurified to remove any trace of unbound Tb^{3+}.

In order to avoid the RNA inner filter effect observed in preliminary experiments when irradiating the peptide–RNA mixtures at 280 nm, we decided to perform our studies by exciting the Trp at 300 nm. At this wavelength the RNA is virtually transparent, while the tryptophan indole still has a relatively noticeable absorption (ε = 507 M^{-1} cm^{-1}).[18] Thus, irradiation of a series of mixtures containing the **P22-N^W[Tb]** peptide (100 nM) and increasing concentrations of the *boxB* RNA hairpin resulted in a set of spectra with progressively increased emission intensity of the terbium ion bands at 488, 544, and 585 nm (Figure 2). The increase of the luminescence emission band at 544 nm could be fitted to a binding model corresponding to the formation of a specific peptide/RNA complex plus a contribution from nonspecific binding, probably arising from electrostatic interactions between the highly charged **P22-N^W[Tb]** peptide and the polyanionic RNA. This

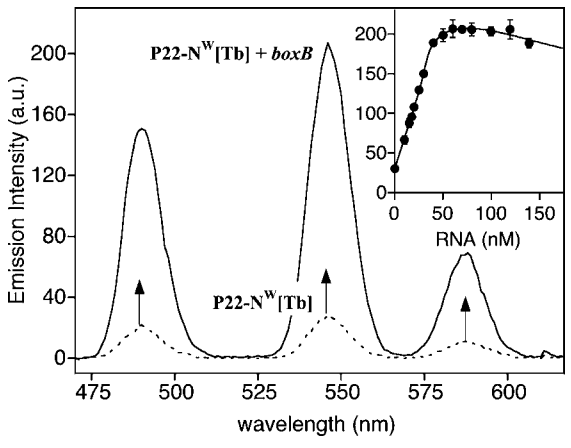

Figure 2. Time-resolved luminescence emission (0.2 ms delay time) of a 100 nM solution of the peptide **P22-N^W[Tb]** sensor in 10 mM HEPES buffer, 100 mM NaCl, pH 7.6 (dashed line), and the same peptide in the presence of 1 equiv of *boxB* RNA (solid line). Inset: Emission intensity at 544 nm represented as a function of the concentration of *boxB* RNA and best fit to a 1:1 binding model including nonspecific contributions. Each point in the binding curve is the mean of three independent titrations.

resulted in a K_D for the 1:1 complex of ≈12 nM (Figure 2, inset),[19] in agreement with the low nM dissociation constant reported for the natural **P22-N** peptide.[20] The detection limit under those conditions was found to be ≈1 nM.[21]

Control experiments demonstrated that no significant variation in the terbium luminescence was induced either by single- or double-stranded DNA oligonucleotides or by the closely related bovine immunodeficiency virus (BIV) TAR hairpin, specifically recognized by the BIV Tat protein (see the SI).[6d,22] As a further demonstration of the specificity, incubation of an equimolar mixture of **P22-N^W[Tb]** and the *boxB* RNA with increasing concentrations of a peptide containing the RNA binding domain of the P-22N protein resulted in a progressive decrease in the terbium luminescence (see the SI). This reduction in the emission intensity was consistent with the competitive displacement of the **P22-N^W[Tb]** peptide probe by the natural P-22N peptide binding to the same recognition site in the *boxB* RNA hairpin.

Importantly, we also observed a significant increase in the terbium luminescence in response to the *boxB* RNA, even in a complex biological medium (cell extracts with total protein concentration of 300 ng/mL), and although the dynamic range of the sensor was reduced in comparison to that observed with a pure RNA sample, this experiment demonstrated the high specificity and viability of the sensing strategy under demanding biologically relevant conditions (Figure 3, left). This experi-

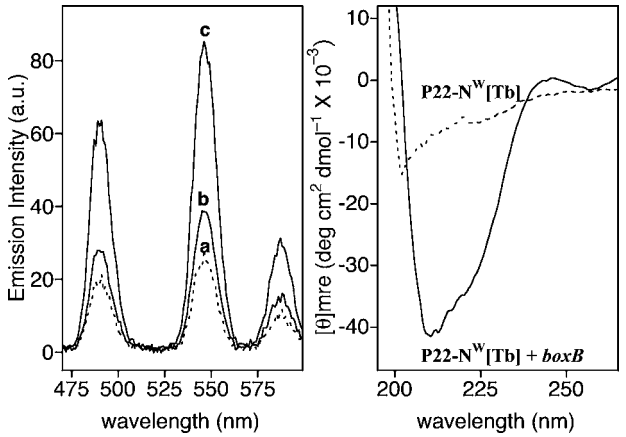

Figure 3. Left: (a) Time-resolved luminescence emission (0.2 ms delay time) of a 100 nM solution of **P22-N^W[Tb]** in 10 mM HEPES buffer, 100 mM NaCl, pH 7.6; (b) in the presence of 300 ng/mL total protein cell lysate; (c) after addition of 1 equiv of *boxB* RNA. Right: CD of a 5 μM solution of the peptide **P22-N^W[Tb]** sensor in 10 mM phosphate buffer, 100 mM NaCl, pH 7.5 (dashed line), and in the presence of 1 equiv of *boxB* RNA (solid line).

ment also demonstrates the advantages of the long luminescence lifetime of the lanthanide ions, as it allows the use of time-gated conditions to isolate the terbium luminescence emission from the short-lived fluorescence of the biological background.

Finally, in order to confirm that the large emission increase in the presence of the *boxB* RNA was caused by the folding of the disordered peptide chain into an alpha helical structure upon specific binding, we measured the circular dichroism (CD) spectra of a 5 μM solution of the **P22-N^W[Tb]** peptide before and after the addition of 1 equiv of the target *boxB* RNA (Figure 3, right). As expected, the **P22-N^W[Tb]** sensor shows a CD spectrum consistent with a random coil peptide lacking

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secondary structure; addition of 1 equiv of the target *boxB* RNA results in a large increase of the negative intensity of the mean residue ellipticity at 208/222 nm, indicating a significant folding of the peptide chain into an α helical conformation[23] and therefore supporting the proposed sensing mechanism.

In summary, we have demonstrated that the conformational changes experienced by a peptide chain upon recognition of a biologically important RNA target can be exploited for the effective modulation of lanthanide sensitization processes and hence for the development of a RNA-specific luminescent sensor. Our probe shows good dynamic range and excellent sensitivity and selectivity; it is compatible with complex biological media and benefits from the unique luminescence properties of the lanthanide ion. Therefore, this approach should be suitable for the development of sensors directed toward other structured RNA targets and in general for studying other biomolecular events in which recognition is coupled to the folding of disordered proteins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Hobert, O. *Science* **2008**, *319*, 1785−1786. (b) Mandal, M.; Breaker, R. R. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 451−463. (c) Lönnberg, T.; Lönnberg, H. *Curr. Opin. Chem. Biol.* **2005**, *9*, 665−673. (d) Okamura, K.; Lai, E. C. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 673−678.

(2) (a) Armitage, B. A. *Curr. Opin. Chem. Biol.* **2011**, *15*, 806−812. (b) Sivaraman, D.; Biswas, P.; Cella, L. N.; Yates, M. V.; Chen, W. *Trends Biotechnol.* **2011**, *29*, 307−313.

(3) (a) Guo, J.; Ju, J.; Turro, N. J. *Anal. Bioanal. Chem.* **2011**, *402*, 3115−3125. (b) Kummer, S.; Knoll, A.; Socher, E.; Bethge, L.; Herrmann, A.; Seitz, O. *Angew. Chem., Int. Ed.* **2011**, *50*, 1931−1934.

(4) (a) Zengeya, T.; Gupta, P.; Rozners, E. *Angew. Chem., Int. Ed.* **2012**, *51*, 12593−12596. (b) Qi, X.; Xia, T.; Roberts, R. W. *Biochemistry* **2010**, *49*, 5782−5789. (c) Hagihara, M.; Fukuda, M.; Hasegawa, T.; Morii, T. *J. Am. Chem. Soc.* **2006**, *128*, 12932−12940.

(5) (a) Schifferer, M.; Griesbeck, O. *J. Am. Chem. Soc.* **2012**, *134*, 15185−15188. (b) Valencia-Burton, M.; McCullough, R. M.; Cantor, C. R.; Broude, N. E. *Nat. Methods* **2007**, *4*, 421−427.

(6) (a) Gosser, Y.; Hermann, T.; Majumdar, A.; Hu, W.; Frederick, R.; Jiang, F.; Xu, W.; Patel, D. J. *Nat. Struct. Biol.* **2001**, *8*, 146−150. (b) Schärpf, M.; Sticht, H.; Schweimer, K.; Boehm, M.; Hoffmann, S.; Rösch, P. *FEBS J.* **2000**, *267*, 2397−2408. (c) Ye, X.; Gorin, A.; Frederick, R.; Hu, W.; Majumdar, A.; Xu, W.; McLendon, G.; Ellington, A.; Patel, D. J. *Chem. Biol.* **1999**, *6*, 657−669. (d) Puglisi, J. D.; Chen, L.; Blanchard, S.; Frankel, A. D. *Science* **1995**, *270*, 1200−1203. (e) Puglisi, J. D.; Tan, R.; Calnan, B. J.; Frankel, A. D.; Williamson, J. R. *Science* **1992**, *257*, 76−80.

(7) Work of our group relying on lanthanide sensitization, see (a) Pazos, E.; Jiménez, A.; Mascareñas, J. L.; Vazquez, M. E. *Chem. Sci.* **2011**, *2*, 1984−1987. (b) Pazos, E.; Goličnik, M.; Mascareñas, J. L.; Eugenio Vázquez, M. *Chem. Commun.* **2012**, *48*, 9534−9536. (c) Vazquez, O.; Sánchez, M. I.; Mascareñas, J. L.; Vazquez, M. E. *Chem. Commun.* **2010**, *46*, 5518−5520. (d) Pazos, E.; Torrecilla, D.; Vázquez López, M.; Castedo, L.; Mascareñas, J. L.; Vidal, A.; Vazquez, M. E. *J. Am. Chem. Soc.* **2008**, *130*, 9652−9653. (e) Pazos, E.; Vazquez, O.; Mascareñas, J. L.; Vazquez, M. E. *Chem. Soc. Rev.* **2009**, *38*, 3348−3359.

(8) (a) Gunnlaugsson, T.; Leonard, J. P. *Chem. Commun.* **2005**, 3114−3131. (b) Pandya, S.; Yu, J.; Parker, D. *Dalton Trans.* **2006**, 2757−2766. (c) Gunnlaugsson, T.; Harte, A. J.; Leonard, J. P.; Nieuwenhuyzen, M. *Chem. Commun.* **2002**, 2134−2135. (d) Eliseeva, S. V.; Bunzli, J.-C. G. *Chem. Soc. Rev.* **2010**, *39*, 189−227.

(9) (a) Xia, T.; Frankel, A.; Takahashi, T. T.; Ren, J.; Roberts, R. W. *Nat. Struct. Biol.* **2003**, *10*, 812−819. (b) Greenblatt, J.; Nodwell, J. R.; Mason, S. W. *Nature* **1993**, *364*, 401−406.

(10) Prasch, S.; Schwarz, S.; Eisenmann, A.; Wöhrl, B. M.; Schweimer, K.; Rösch, P. *Biochemistry* **2006**, *45*, 4542−4549.

(11) Low-energy transitions in lanthanide ions are symmetry forbidden, displaying very low excitation coefficients, and therefore they are usually excited through nonradiative energy transfer mechanisms from nearby organic fluorophore. This process is commonly known as *sensitization* and is highly sensitive to the distance between the organic antenna and the metal ion. (a) Bunzli, J.-C. G.; Piguet, C. *Chem. Soc. Rev.* **2005**, *34*, 1048−1077. (b) Nitz, M.; Sherawat, M.; Franz, K. J.; Peisach, E.; Allen, K. N.; Imperiali, B. *Angew. Chem., Int. Ed.* **2004**, *43*, 3682−3685. (c) Parker, D.; Williams, J. A. G. *J. Chem. Soc., Dalton Trans.* **1996**, 3613−3628. (d) Santos, dos, C. M. G.; Harte, A. J.; Quinn, S. J.; Gunnlaugsson, T. *Coord. Chem. Rev.* **2008**, *252*, 2512−2527.

(12) Cocozaki, A. I.; Ghattas, I. R.; Smith, C. A. *J. Bacteriol.* **2008**, *190*, 7699−7708.

(13) Cai, Z.; Gorin, A.; Frederick, R.; Ye, X.; Hu, W.; Majumdar, A.; Kettani, A.; Patel, D. J. *Nat. Struct. Biol.* **1998**, *5*, 203−212.

(14) Coin, I.; Beyermann, M.; Bienert, M. *Nat. Protoc.* **2007**, *2*, 3247−3256.

(15) (a) Thieriet, N.; Alsina, J.; Giralt, E.; Guibe, F.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 7275−7278. (b) Gomez-Martinez, P.; Dessolin, M.; Guibe, F.; Albericio, F. *J. Chem. Soc., Perkin Trans. 1* **1999**, 2871−2874.

(16) The DOTA tris(*t*-Bu)-ester was synthesized following known procedures in the literature: (a) Dadabhoy, A.; Faulkner, S.; Sammes, P. *J. Chem. Soc., Perkin Trans. 2* **2002**, 348−357. (b) Aarons, R. J.; Notta, J. K.; Meloni, M. M.; Feng, J.; Vidyasagar, R.; Narvainen, J.; Allan, S.; Spencer, N.; Kauppinen, R. A.; Snaith, J. S.; Faulkner, S. *Chem. Commun.* **2006**, 909−911.

(17) Sainlos, M.; Imperiali, B. *Nat. Protoc.* **2007**, *2*, 3201−3209.

(18) (a) Du, H.; Fuh, R. C. A.; Li, J.; Corkan, L. A.; Lindsey, J. S. *Photochem. Photobiol.* **1998**, *68*, 141−142. (b) Dixon, J. M.; Taniguchi, M.; Lindsey, J. S. *Photochem. Photobiol.* **2005**, *81*, 212−213.

(19) Curve-fitting was made using the Levenberg−Marquardt algorithm for nonlinear least-squares regression as implemented in the DynaFit program. Kuzmic, P. *Anal. Biochem.* **1996**, *237*, 260−273.

(20) Cilley, C. D.; Williamson, J. R. *RNA* **1997**, *3*, 57−67.

(21) Calculated as described in: Armbruster, D. A.; Pry, T. *Clin. Biochem. Rev.* **2008**, *29*, S49−S52.

(22) Draper, D. E. *J. Mol. Biol.* **1999**, *293*, 255−270.

(23) (a) Chen, Y. H.; Yang, J. S.; Chau, K. H. *Biochemistry* **1974**, *13*, 3350−3359. (b) Greenfield, N. J.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108−4116. (c) Rohl, C. A.; Chakrabartty, A.; Baldwin, R. L. *Protein Sci.* **1996**, *5*, 2623−2637.