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Hybridization of DNA and PNA Molecular Beacons to Single-Stranded and Double-Stranded DNA Targets

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Abstract: Molecular beacons are sensitive fluorescent probes hybridizing selectively to designated DNA and RNA targets. They have recently become practical tools for quantitative real-time monitoring of single-stranded nucleic acids. Here, we comparatively study the performance of a variety of such probes, stemless and stem-containing DNA and PNA (peptide nucleic acid) beacons, in Tris-buffer solutions containing various concentrations of NaCl and $MgCl_2$. We demonstrate that different molecular beacons respond differently to the change of salt concentration, which could be attributed to the differences in their backbones and constructions. We have found that the stemless PNA beacon hybridizes rapidly to the complementary oligodeoxynucleotide and is less sensitive than the DNA beacons to the change of salt thus allowing effective detection of nucleic acid targets under various conditions. Though we found stemless DNA beacons improper for diagnostic purposes due to high background fluorescence, we believe that use of these DNA and similar RNA constructs in molecular-biophysical studies may be helpful for analysis of conformational flexibility of single-stranded nucleic acids. With the aid of PNA "openers", molecular beacons were employed for the detection of a chosen target sequence directly in double-stranded DNA (dsDNA). Conditions are found where the stemless PNA beacon strongly discriminates the complementary versus mismatched dsDNA targets. Together with the insensitivity of PNA beacons to the presence of salt and DNA-binding/processing proteins, the latter results demonstrate the potential of these probes as robust tools for recognition of specific sequences within dsDNA without denaturation and deproteinization of duplex DNA.

Introduction

Synthetic DNA and RNA hybridization probes carrying various reporter groups are attractive biomolecular tools, which make it possible to directly pinpoint harmful genes or damaging mutations, as well as to identify infectious pathogens.[1a] In this connection, fluorescent probes have been receiving a great deal of attention due to the simplicity and robustness of detection of their binding to target nucleic acid sequences.[1] Well-known molecular beacons[2] belong to this category. Such probes carry a fluorophore and a quencher at their termini and, when not hybridized, form a closed structure that brings these labels in juxtaposition thus quenching fluorescence. Upon their binding to complementary targets, molecular beacons adopt an open conformation in which the labels are spatially separated. As a result, molecular beacons brightly fluoresce in the presence of nucleic acid target sequences.

To keep a quencher near a fluorophore in a free "dark" state, molecular beacons were initially designed as hairpin-shaped molecules featuring a stem-and-loop structure.[2a] Later, it was realized that this structure is not obligatory for the functioning of molecular beacons, and consequently their stemless constructs and alike were developed.[3] Besides the originally employed DNA beacons,[2a] chimeric DNA-PNA (peptide nucleic acid[4]) and purely PNA beacons were used to provide stronger binding of the probe.[3b,5] Thus, a variety of different molecular beacons now exists. However, a comparative analysis of their capabilities

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(1) (a) Tyagi, S. In *Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation*; Meyers, R. A., Ed.; John Wiley & Sons Ltd.: Chichester, UK, 2000; Vol. 6, p 4911. (b) Jordan, J. A. *Trends Microbiol.* **2000**, *12*, 61.

(2) (a) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303. For reviews, see: (b) Tan, W.; Fang, X.; Li, J.; Liu, X. *Chem. Eur. J.* **2000**, *6*, 1107. (c) Fang, X.; Li, J. J.; Perlette, J.; Tan, W.; Wang, K. *Anal. Chem.* **2000**, *72*, 747A.

(3) (a) Gildea, B. D.; Coull, J. M.; Hyldig-Nielsen, J. J.; Fiandaca, M. J. WO A-9921881, 1999. (b) Seitz, O. *Angew. Chem., Int. Ed.* **2000**, *39*, 3249. (c) Ranasinghe, R. T.; Brown, L. J.; Brown, T. *Chem. Commun.* **2001**, 1480.

(4) Abbreviations, acronyms, and symbols used (alphabetically): a.u., arbitrary units; DABCYL, (4-(4′-dimethylaminophenylazo)benzoic acid); DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*′-dimethylformamide; dsDNA, double-stranded DNA.; eg1, the bis-PNA linker unit 8-amino-3,6-dioxaoctanoic acid; Flu, fluorescein; Fmoc, fluorenylmethyloxycarbonyl; Glu, glutamic acid; HATU, N-[(dimethylamino)-1H-2,3,-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminiumhexafluorophosphate; HOAt, 3-hydroxy-3H-1,2,3,-triazolo-[4,5-*b*]pyridine; J, pseudoisocytosine; Lys, lysine; MB, molecular beacon; PD- or PP-loop, looped complex formed inside dsDNA by PNA openers in concert with oligonucleotide or PNA probes, respectively; PEG, poly(ethylene glycol); PNA, peptide nucleic acid; PS, polystyrene; ssDNA, single-stranded DNA; TFA, trifluoroacetic acid.

(5) (a) Ortiz, E.; Estrada, G.; Lizardi, P. M. *Mol. Cell. Probes* **1998**, *12*, 219. (b) Coull, J. M.; Gildea, B. D.; Hyldig-Nielsen, J. J. WO A-9922018, 1999. (c) Kuhn, H.; Demidov, V. V.; Gildea, B. D.; Fiandaca, M. J.; Coull, J. C.; Frank-Kamenetskii, M. D. *Antisense Nucleic Acid Drug Dev.* **2001**, *11*, 265. (d) Armitage, B.; Danith, L.; Koch, T.; Frydenlund, H.; Ørum, H.; Schuster, G. B. *Biochemistry* **1998**, *37*, 9417.

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under different conditions has not been performed yet. Also, current applications of molecular beacons are limited by their binding to single-stranded (ss) or denatured nucleic acids.[6]

In the present work, we study the performance of stemless and stem-containing DNA and PNA beacons in terms of kinetics of their hybridization to the complementary oligonucleotide and corresponding signal-to-background ratios in buffer solutions differing by concentration of salt. In addition, we demonstrate an approach allowing the hybridization of molecular beacons directly to dsDNA sites, which are locally exposed by PNA "openers". Conditions are found for strong discrimination of the complementary target within dsDNA by the stemless PNA beacon against the mismatched ones. A part of our experiments pertinent to the data reported here, i.e., hybridization of PNA beacons to complementary dsDNA target, has recently been reported by us in a form of brief communication.[5c] Our findings extend the potential of molecular beacons for DNA diagnostics and have implications for studies regarding the structure of ssDNA in solution.

Experimental Section

DNA and PNA Beacons. All molecular beacons we used (see Figures 1−3 for their structures and Figure 5 for shapes) contain fluorescein (Flu) as the fluorophore and DABCYL (4-(4′-dimethylaminophenylazo)benzoic acid) as the quencher. In DNA beacons, Flu was conjugated to the probe oligomer via a N-thiourea link, while in the PNA beacon, Flu was conjugated to the probe oligomer via an amide link. HPLC-purified DNA beacons were purchased from Operon Technologies (Alameda, CA). The PNA beacon[7] was synthesized by solid-phase methods (see the Supporting Information I on the Internet) on a 2 μmol scale using commercially available reagents and protocols supplied by Applied Biosystems (Foster City, CA). The HPLC-purified major product of this synthesis (λ_{max} 258.6 and 507.4 nm) was analyzed by MALDI-TOF mass spectrometry: calculated mass 4022.7; found mass 4022.9. For stability, stock solutions of all molecular beacons were kept in the dark at −20 °C: stocks of DNA beacons were dissolved in 20 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA, while the stock of the PNA beacon was kept in 1:1 (v/v) DMF/H_2O.

DNA Targets. Duplex DNA targets, plasmids pHIV, pB1MM, pB2MM, and pOP1MM, were constructed by cloning the following oligonucleotides (purchased from MWG-BIOTECH (High Point, NC); only one of the two complementary oligonucleotides is shown) into the *Bam*H I site of pUC19:

pHIV: 5'-GATCAGAGGAAGCTACTGGAGGAGAC,
pB1MM: 5'-GATCAGAGGAAGCTTCTGGAGGAGAC,
pB2MM: 5'-GATCAGAGGAAGATACCGGAGGAGAC,
pOP1MM: 5'-GATCAGAGCAAGCTACTGGAGGAGAC.

The pHIV insert contains a 21-bp-long sequence from the coding region of the HIV-1 *nef* gene (strain 92ug037 of HIV-1 subtype A). The same oligonucleotides were used as ssDNA targets in the corresponding experiments.

PNA Openers. Bis-PNA openers, $HLys_2$-$TCTC_2TC_2$-$(eg1)_3$-J_2TJ_2-TJT-$LysNH_2$ and $HLys_2$-$TJTJ_2T_2J$-$(eg1)_3$−CT_2C_2TCT-$LysNH_2$ (Lys denotes lysine, eg1 stands for the linker unit 8-amino-3,6-dioxaoctanoic acid, and J is pseudoisocytosine), were obtained commercially from PE Biosystems and were given to us by P. E. Nielsen as a gift. Preopened dsDNA/bis-PNAs complexes were prepared and analyzed as previously described.[5c]

Fluorescent Measurements. Fluorescence was monitored on a Photon Technology Intl. spectrofluorometer equipped with an external waterbath, using a semimicrofluorometer cell with a Teflon stopper. For fluorescent monitoring of hybridization kinetics, a solution of DNA or PNA beacon was preequilibrated in buffer until no change in fluorescence with time was detected. After that, the DNA target was added followed by vigorous mixing for 5−10 s. The fluorescent response was then recorded every 5 s until the detected signal reached a plateau. All samples were excited at 493 nm, and the emission was measured at 520 (DNA beacons) or 527 nm (PNA beacon), if it is not stated otherwise. Steady-state fluorescence spectra were measured at 2 nm intervals in the emission range 500−650 nm. Signal-to-background ratio, R, was calculated as

$$R = (F_s - F_i)/(F_b - F_i) \quad (1)$$

where F_i, F_b, and F_s correspond to the intrinsic fluorescence of a buffer solution alone and to the steady-state fluorescence responses of a molecular beacon in the absence (background) or in the presence (signal) of the DNA target, respectively.

Kinetic Analysis. Second-order rate constants, k_h (M^{-1} s^{-1}), for hybridization of DNA and PNA beacons to complementary ssDNA (oligonucleotide) or dsDNA (PP/PD-loops) targets at different ionic conditions were calculated by using the linearization procedures for a second-order reaction with either stoichiometric (equimolar) or non-stoichiometric proportions of reactants.[8] In the first case, the following equation was used:

$$1/[\text{free MB}]_t = \text{constant}' + k_h t \quad (2)$$

where the current concentration of a free, nonhybridized molecular beacon (MB) was determined by measuring the fluorescence of a corresponding solution at a time t. Here, a plot of $1/[\text{free MB}]_t$ versus t is linear with the slope being equal to k_h.

In the more general second case, we used another equation:

$$\ln([\text{free oligo}]_t/[\text{free MB}]_t) = \text{constant}'' + ([\text{free oligo}]_0 - [\text{free MB}]_0)k_h t \quad (3)$$

where the current concentration of a free, nonhybridized oligonucleotide was determined by taking into account its expense on 1:1 hybridization with molecular beacon. Thus, for a hybridization reaction in which the initial concentrations of oligonucleotide and molecular beacon were not in stoichiometric proportions, a plot of $\ln([\text{free oligo}]_t/[\text{free MB}]_t)$ versus t is linear with the slope being equal to $([\text{free oligo}]_0 - [\text{free MB}]_0)k_h$.

Results and Discussion

Comparative Study of the Performance of Molecular Beacons with ssDNA Target. Three molecular beacons have been studied: one stemless and one stem-forming DNA beacon, and one stemless PNA beacon. The stemless PNA beacon with an 11-mer probe sequence was chosen by us because two similar PNA beacons with longer probe sequences had some drawbacks: the one with a 15-mer probe sequence gradually aggregated and another one with a 13-mer probe sequence, though it did not aggregate, exhibited a fluorescence response upon hybridization that leveled off rather slowly. Since PNA

(6) After our paper was submitted, an article was published reporting on the triplex-type hybridization of a G-rich DNA molecular beacon to a complementary site on dsDNA: Antony, T.; Thomas, T.; Sigal, L. H.; Shirahata, A.; Thomas, T. J. *Biochemistry* **2001**, *40*, 9387. Notwithstanding the value of this new approach for studying DNA triplexes, note that its diagnostic usefulness is significantly restricted by the requirement for long homopurine and similar sites, which rarely occur within natural DNA sequences.

(7) PNA beacons (so-called LightSpeed probes) are now commercially available from Boston Probes, Inc. (http://www.bostonprobes.com).

(8) Silbey, R. J.; Alberty, R. A. *Physical Chemistry*, 3rd ed.; Wiley: New York, 2001; p 969.

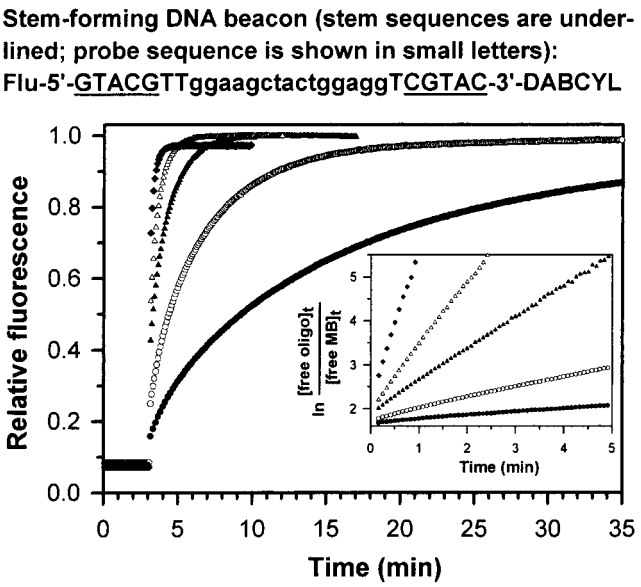

Figure 1. Kinetic responses of the stem-forming DNA beacon (170 nM) upon hybridization to complementary ssDNA target (850 nM) at 25 °C in 20 mM Tris-HCl buffer (pH 8.0) containing $MgCl_2$ at different concentrations: 1 (●), 2.5 (○), 5 (▲), 10 (△), and 50 mM (♦). Inset: Linearized semilogarithmic plots of the kinetic data. The slopes are proportional to the second-order rate constants for DNA beacon hybridization (see eq 3 in the Experimental Section).

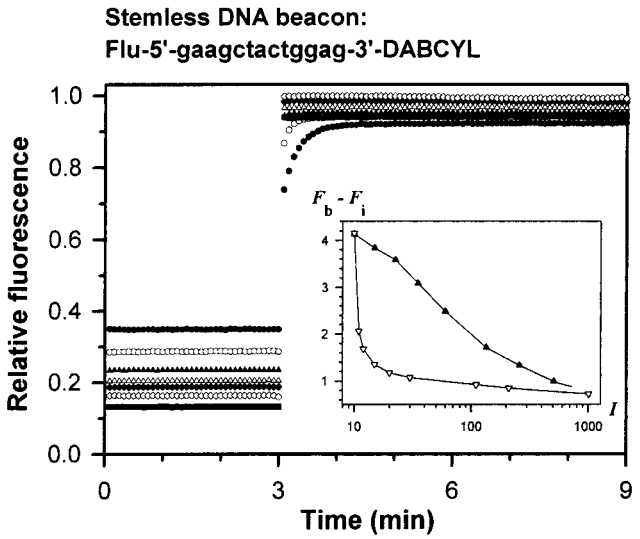

Figure 2. Kinetic responses of the stemless DNA beacon upon hybridization to complementary ssDNA target at 25 °C in 20 mM Tris-HCl buffer (pH 8.0) containing $MgCl_2$ at different concentrations: 0.5 (●), 1 (○), 2.5 (▲), 5 (△), 10 (♦), 50 (◊), and 500 mM (■). Concentrations of DNA beacon and DNA target are the same as in Figure 1. Inset: Dependence of the background fluorescence intensity, F_b (arbitrary units), of this molecular beacon on the ionic strength of solution, *I*, varied by addition of NaCl (▲) or $MgCl_2$ (▽). F_i denotes the intrinsic fluorescence of the buffer solutions. Data are taken from the experiments presented in the main figure and in the Supporting Information IIB.

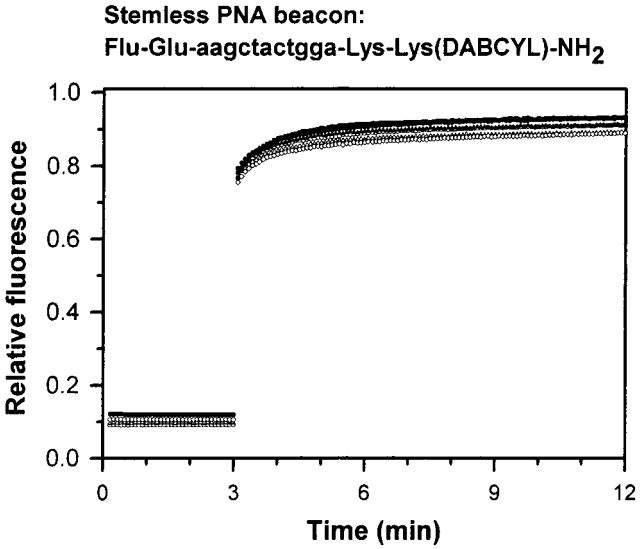

Figure 3. Kinetic responses of the stemless PNA beacon upon hybridization to complementary ssDNA target at 25 °C in 20 mM Tris-HCl buffer (pH 8.0) containing $MgCl_2$ at different concentrations: 0.5 (●), 1 (○), 2.5 (▲), 10 (△), 50 (♦), and 500 mM (◊). Concentrations of molecular beacon and DNA target are the same as in Figure 1.

hybridizes to ssDNA with very high affinity,[9] even a short, 11-mer PNA probe sequence should warrant the strong binding of the corresponding molecular beacon. Longer probe sequences, 13- and 15-mer for stemless and stem-forming constructs, respectively, were chosen for DNA beacons to provide them with satisfactory binding affinity. Note that a 15-mer probe sequence of the stem-forming DNA beacon is typical for the probes of that type.[2]

Figures 1−3 show the normalized kinetic responses of molecular beacons upon their hybridization to complementary ssDNA sequences within the same 26-nt-long oligodeoxynucleotide at different concentrations of $MgCl_2$. The corresponding second-order rate constants for DNA beacons at various ionic strengths are presented in Table 1.[10] One can see that the traditionally used stem-forming DNA beacon hybridizes relatively fast (within few minutes) to the complementary ssDNA target at high salt concentration (Figure 1). However, its hybridization is slow at low salt concentration. The signal-to-background ratio for this molecular beacon is high (>10) and it grows with increasing salt concentration (Figure 4). Hybridization of the stemless DNA beacon is very fast at any salt concentration (Figure 2), but the signal-to-background ratio is low (Figure 4). We therefore did not use this probe in any further hybridization experiments. In contrast to the stemless DNA beacon, the stemless PNA beacon hybridizes to the complementary oligodeoxynucleotide both rapidly (Figure 3) and with a high (∼10) signal-to-background ratio (Figure 4). Moreover, this molecular beacon is essentially insensitive to the change of salt thus allowing effective detection of nucleic acid targets under various conditions.

It is quite reasonable to assume that interactions between a quencher and a fluorophore are mostly of hydrophobic nature. Therefore, the different behavior of molecular beacons with respect to the response of their hybridization rate and signal-to-background ratio to the change of salt concentration could be attributed to the differences in their backbones and constructions. The insensitivity of the stemless PNA beacon to the ionic

(9) (a) Egholm, M.; Behrens, C.; Christensen, L.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Chem. Soc., Chem. Commun.* **1993**, 800. (b) Ratilainen, T.; Holmén, A.; Tuite, E.; Haaima, G.; Christensen, L.; Nielsen P. E.; Nordén, B. *Biochemistry* **1998**, *37*, 12331.

(10) The absolute values of maximal fluorescent hybridization responses of different molecular beacons were of comparable values. For similar experiments with NaCl, see the Supporting Information II on the Internet. We did not quantitate the rate constants for hybridization of PNA beacons under these conditions (see Table 2 for k_h values under other conditions) because binding of this probe to the complementary ssDNA sequence proceeds too fast, essentially within a minute, to be determined without a specialized stopped-flow equipment.

Table 1. Second-Order Rate Constants for Hybridization of DNA Beacons to Complementary ssDNA Target at Different Ionic Conditions[a,b]

molecular beacon	$MgCl_2$ (mM)	k_h ($M^{-1}s^{-1}$)	NaCl (mM)	k_h ($M^{-1}s^{-1}$)
stem-forming	1.0	1.6×10^3	250	2.0×10^3
DNA beacon	2.5	5.3×10^3	500	5.0×10^3
	5.0	1.8×10^4	1000	8.4×10^3
	10	3.5×10^4		
	50	8.0×10^4		
stemless	0.0	2.9×10^3	10	1.3×10^4
DNA beacon[c]	0.5	8.0×10^4	25	4.4×10^4
	1.0	1.6×10^5	50	9.8×10^4

[a] 20 mM Tris-HCl buffer (pH 8.0) with addition of $MgCl_2$ or NaCl, as indicated; 25 °C; 170 nM of molecular beacon; 5-fold excess of ssDNA target. [b] Rate constants were calculated based on the kinetic data presented in Figures 1 and 2 and in Supporting Information IIA,B by using the linearization procedure for a second-order reaction with nonstoichiometric proportions of reactants.[8] [c] For the stemless DNA beacon, k_h could not be measured for $[MgCl_2] > 1$ mM and [NaCl] > 50 mM due to a very fast response.

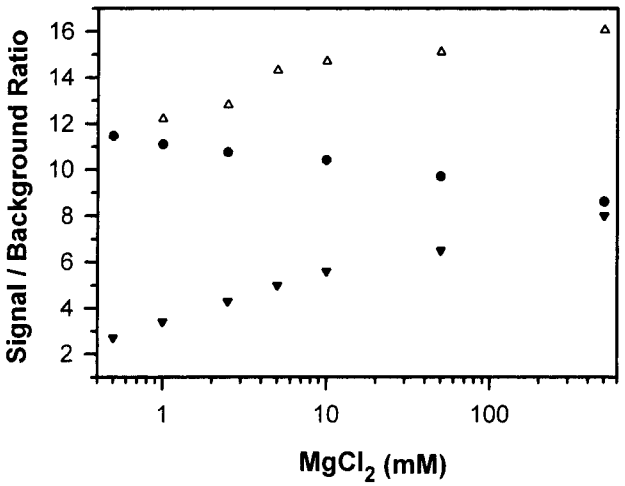

Figure 4. Effects of ionic strength on the signal/background ratio for the stem-forming DNA beacon (△), the stemless DNA beacon (▼), and the stemless PNA beacon (●). Conditions: 0.5−500 mM $MgCl_2$ in 20 mM Tris-HCl (pH 8.0). Data are taken from the experiments presented in Figures 1−3.

strength variations supports this assumption. Indeed, assuming hydrophobic quencher-fluorophore interactions, one could expect the PNA beacon to exhibit salt-independent hybridization properties because of the neutrality of the PNA backbone.[11] By contrast, the stemless DNA beacon will tend to stretch out at low salt concentration due to the polyanionic nature of its backbone[12] thus yielding significant background fluorescence (Figure 2). Furthermore, the ability of the DNA beacon to stretch out at low salt concentration is obviously limited in the stem-containing construct, which results in substantial decrease in its background fluorescence but, at the same time, decreases the hybridization rate, as compared to the stemless DNA beacon (Figure 1). Nonetheless, both DNA beacons hybridize faster at high salt concentrations, as it is normally observed for formation of DNA (and RNA) duplexes[13] and is consistent with similar observation by others.[14,15]

Why does the stemless PNA beacon work so well? One explanation was provided by Seitz.[3b] He concluded that such constructs form due to the neutrality of the PNA backbone and hydrophobic interactions between nucleobases, compact globules by themselves. As a result, a fluorophore becomes closer to a quencher and its fluorescence is quenched, as Seitz assumed, via collisional quenching and long-distance fluorescence resonance energy transfer (FRET). Though rather reasonable for the particular case, this interpretation could not generally be applied to our study of stemless molecular beacons. We believe that (1) compactization does not happen for stemless DNA constructs, especially at moderate-to-low concentrations of salt, and (2) the energy transfer from the fluorophore (fluorescein) to the quencher (DABCYL) used in our molecular beacons is not due to FRET but takes place via direct contact of fluorophore and quencher[5d,16] because of a poor overlap of the fluorescein emission spectrum and the DABCYL absorption spectrum.[17] Therefore, the workability of stemless DNA and PNA beacons can be attributed to the high flexibility of both sugar−phosphate and polyamide (pseudopeptide) backbones,[18] and to strong hydrophobic interactions between quencher and fluorophore, which essentially act as a lock for closing the stemless constructs.

Our explanation is supported by the data presented in Figure 2, which demonstrate a strong dependence of the background fluorescence intensity, F_b, of the stemless DNA beacon on the ionic strength of the solution, I. In the inset of this figure, one can see that at high ionic strengths the background fluorescence of this molecular beacon exponentially decreases with increasing salt concentration (the term F_i takes into account the intrinsic fluorescence of the buffer solutions):

$$(F_b - F_i) \sim I^{-n} \qquad (4)$$

with the exponent n equal to 0.4 for Na^+ and 0.2 for Mg^{2+}. This is what one could expect considering the salt-induced increase in ssDNA flexibility due to the screening effect of counterions on the repulsion of negatively charged phosphates of the DNA backbone.[12] The increased bending flexibility of ssDNA, which at high salt concentration is determined only by the intrinsic DNA rigidity, allows the quencher-fluorophore interactions to more effectively close the stemless constructs. This qualitative consideration demonstrates the potential of stemless DNA beacons for studies of the ssDNA flexibility responses to the change of experimental conditions.

Hybridization of Molecular Beacons to Duplex DNA. We have recently developed an approach that allows probe hybridization to dsDNA.[19] Our approach is based on the local opening of dsDNA by a pair of PNA openers yielding an extended strand-displacement complex (see Figure 5A). This structure is

(11) Tomac, S.; Sarkar, M.; Ratilainen, T.; Wittung, P.; Nielsen, P. E.; Nordén, B.; Gräslund, A. *J. Am. Chem. Soc*. **1996**, *118*, 5544.
(12) (a) Odijk, T. *J. Polym. Sci*. **1977**, 477. (b) Barrat, J. L.; Joanny, J. F. *Europhys. Lett*. **1993**, *24*, 333. (c) Tinland, B.; Pluen, A.; Sturm, J.; Weill, G. *Macromolecules* **1997**, *30*, 5763.
(13) (a) Wetmur, J. G.; Davidson, N. *J. Mol. Biol*. **1968**, *31*, 349. (b) Pörschke, D.; Eigen, M. *J. Mol. Biol*. **1971**, *62*, 361. (c) Orosz, J. M.; Wetmur, J. G. *Biopolymers* **1977**, *16*, 1183. (d) Sabahi, A.; Guidry, J.; Inamati, G. B.; Manoharan, M.; Wittung-Stafshede, P. *Nucleic Acids Res*. **2001**, *29*, 2163.
(14) Liu, X.; Tan, W. *Anal. Chem*. **1999**, *71*, 5054.
(15) Note that the stem-forming DNA beacon hybridizes several orders of magnitude slower than common oligonucleotides under similar conditions, whereas the hybridization of the stemless DNA beacon proceeds with rates comparable to those of oligonucleotides.[13b,d]
(16) Tyagi, S.; Marras, S. A. E.; Kramer, F. R. *Nature Biotechnol*. **2000**, *18*, 1191.
(17) In Seitz's case, FRET could also contribute to the quenching process since he used EDANS as a fluorophore whose fluorescence emission spectrum nicely overlaps with the DABCYL absorption spectrum.
(18) Recent MD simulations indicate that PNA oligomers can adopt a more compact coiled structure than DNA oligomers of the same size: Sen, S.; Nilsson, L. *J. Am. Chem. Soc*. **2001**, *123*, 7414.

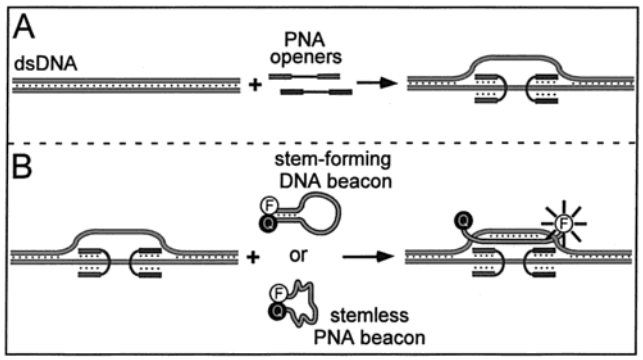

Figure 5. Schematics of the procedure for hybridization of molecular beacons to dsDNA. (A) Duplex DNA is pre-opened at an internal site by a pair of PNA openers hence exposing the target sequence located on the displaced DNA strand for binding the DNA and PNA beacons. (B) Binding of molecular beacons to the thus exposed dsDNA target sequence yields a fluorescence response due to spatial separation of fluorophore (F) and quencher (Q) within unfolded hybridization probes.

able to accommodate DNA and PNA probes by formation of PD-loop or PP-loop complexes, respectively. As a result, affinity capture[19a,20] and "topological" labeling[21] of dsDNA and highly selective detection of specific sequences[22] within linear DNA duplexes have become possible.

Here, we demonstrate that such an internally open site in duplex DNA serves as a target for molecular beacons, thus enabling applications of the molecular beacon technology to duplex DNA. Figure 5 schematically shows our procedure. First, we locally expose the target site for molecular beacons within dsDNA using a pair of PNA openers (Figure 5A). The PNA openers form triplexes with homopurine stretches that are closely located on one of the two dsDNA strands thus flanking the beacons target site (see the Experimental Section for its sequence). This results in the displacement of the opposite DNA strand, which becomes available for Watson−Crick pairing with a probe. Then we bind to pre-opened dsDNA target either the stem-containing DNA beacon or the stemless PNA beacon, both of which have complementary sequences to the displaced region of dsDNA. As a result, the PD-loop or PP-loop complex is formed, which yields a strong fluorescent signal (Figure 5B).

Note that in the absence of the target both molecular beacons show very weak fluorescence and are therefore predominantly in the closed conformation. Thus, it was by no means obvious that our procedure would yield PD-/PP-loop complex and the corresponding fluorescence enhancement.[5c] In fact, to form this complex, a molecular beacon must open up and thread itself in the open form through the pre-opened intermediate needle-eye-like structure making about one turn or more around the DNA target strand.[23,24] Apparently, both DNA and PNA beacons

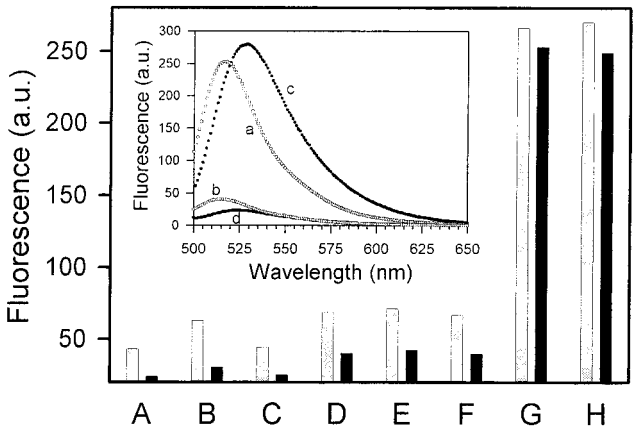

Figure 6. Steady-state fluorescence emissions of 200 nM stem-forming DNA beacon (gray bars) or 200 nM stemless PNA beacon (black bars) upon addition of equimolar target or control samples (a.u.: arbitrary units). Columns A−F correspond to control measurements: (A) molecular beacons in the absence of target; (B) after addition of both PNA openers; (C) after addition of dsDNA target (complementary insert within pHIV) without openers; (D and E) after addition of pHIV bound to a single PNA opener; (F) after addition of unrelated dsDNA (pUC19) prior incubated with both PNA openers. Columns G correspond to the pHIV target exposed by both PNA openers, while columns H represent measurements with the ssDNA target (complementary oligonucleotide). Conditions: 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 25 °C, excitation at 493 nm, emission at 520 nm. Inset: Steady-state emission spectra of the DNA (a, b) or PNA (c, d) beacons in the presence of pre-opened (a and c, respectively) or native (b and d, respectively) pHIV target.

"breath" extensively to perform the above-mentioned operations without serious problems, as the data in Figures 6 and 7 indicate.

Indeed, after mixing the pre-opened dsDNA target with molecular beacons, we observed a strong and rapid increase in fluorescence signal, which leveled off within a few minutes. We found that both the stemless PNA beacon and the stem-containing DNA beacon hybridize with very similar rates to the pre-opened dsDNA target (Table 2). Interestingly, the fluorescent response of the stem-containing DNA beacon develops significantly slower in the case of its hybridization to ssDNA target, whereas the fluorescence of the stemless PNA beacon increases equally fast for ssDNA and dsDNA targets (see Table 2).[25]

Figure 6 demonstrates that the steady-state fluorescence intensities observed as a result of DNA and PNA beacons binding to the dsDNA target are very similar for both molecular beacons (columns G) and are significantly higher than those for control samples (columns A−F). Since the fluorescence responses with dsDNA and ssDNA targets were virtually identical (compare columns G and H), we concluded that binding of both molecular beacons to dsDNA target was essentially complete. The signal-to-background ratios for both

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(25) Our PNA beacon contains no stem while the DNA beacon we studied here carries a stem, which significantly reduces its hybridization rate in case of ssDNA target, as compared to a stemless construct (see Table 1 and Footnote 15). We assume that the accelerated hybridization of the negatively charged DNA beacon to dsDNA by comparison with ssDNA is caused by electrostatic effects of the positively charged PNA openers, the components of the PD-loop. This effect is evidently absent in the case of hybridization of the essentially neutral PNA beacon.

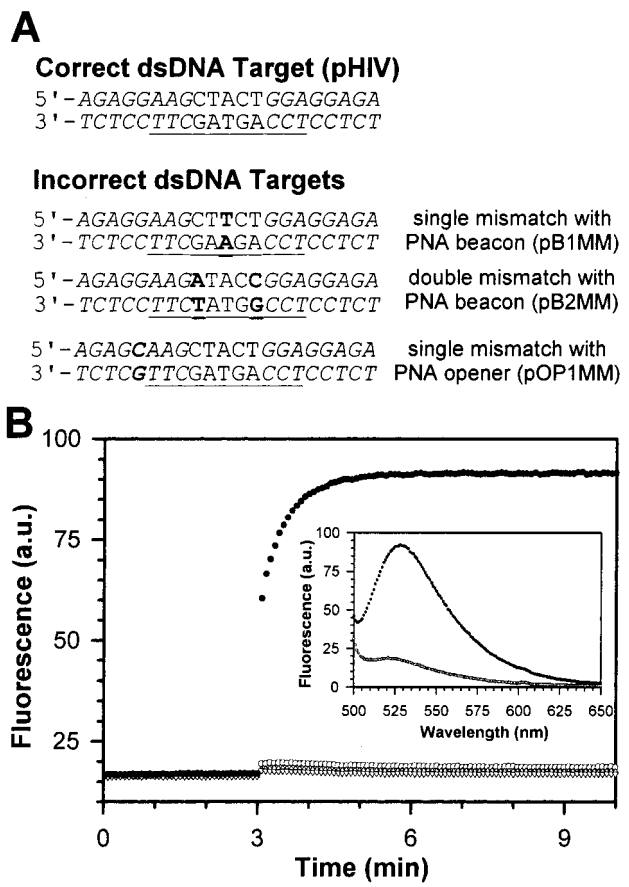

Figure 7. Discrimination of correct against incorrect dsDNA targets by the stemless PNA beacon. (A) Target sequences: binding sites for PNA openers are italicized, binding site for the PNA beacon is underlined, mismatches are shown in a bold type. (B) Kinetic responses of the PNA beacon (50 nM) upon hybridization to equimolar amounts of dsDNA targets pretreated with PNA openers: correct target, pHIV (●); incorrect targets, pB1MM (○), pB2MM (△), and pOP1MM (▽). Measurements were performed at 46 °C in 20 mM Tris-HCl (pH 8.0) containing 2.5 mM $MgCl_2$. Inset: Steady-state emission spectra of the PNA beacon in the presence of pre-opened pHIV (●) and pB1MM (○) targets.

molecular beacons, when measured at fluorescence emission maxima, were close to 10.

Though the same fluorophore (Flu) is used in all molecular beacons we studied and their fluorescence excitation spectra were very similar, exhibiting the major maximum at 493 nm, we observed different fluorescence emission maxima for DNA and PNA beacons: 520 and 527 nm, respectively. Such a difference was seen in the absence of DNA target (background fluorescence) as well as in the presence of either ssDNA or dsDNA targets. We attribute this feature to different chemistries in fluorophore conjugation to DNA and PNA probes (see the Experimental Section).

Discrimination of Mismatches within dsDNA Target by PNA Beacon. To study how the stemless PNA beacon will discriminate mismatches within dsDNA target, we cloned three incorrect inserts: two of them contain a single or double mismatch with the PNA beacon while the third insert contains a single mismatch with one PNA opener (Figure 7A). As could be expected, we found that discrimination of correct targets against the mismatched ones by the PNA beacon was better at elevated temperatures: the match-to-mismatch signal ratio increased from less than 1.5 at 25 °C to about 5 at 37 °C and to more than 20 at 46 °C. Figure 7B demonstrates that at 46 °C the PNA beacon yields a quick and strong response to the complementary dsDNA target pre-opened by PNA openers. However, even a single mismatch, let alone a double mismatch, prevents hybridization of the PNA beacon yielding a fluorescent signal indistinguishable from the background fluorescence. Note that the single mismatch within the PNA opener binding site also prevents hybridization of the PNA beacon, apparently because the corresponding target is not exposed. Similarly strong discrimination of mismatches by the PNA beacon were observed with incorrect ssDNA targets (data not shown). This is what one should surmise considering the enhanced sequence specificity of PNA versus DNA probes.[26]

Table 2. Second-Order Rate Constants, k_h (M^{-1} s^{-1}), for Hybridization of PNA and DNA Beacons to Complementary ssDNA (Oligonucleotide) or dsDNA (PP/PD-loops) Targets[a,b]

molecular beacon	ssDNA target	dsDNA target
stemless PNA beacon	2.5×10^5	3.2×10^5
stem-forming DNA beacon	2.9×10^3	1.9×10^5

[a] 50 mM Tris-HCl buffer (pH 8.0); 1 mM $MgCl_2$; 25 °C; 200 nM molecular beacon; 200 nM DNA target. [b] Rate constants were calculated by using the linearization procedure for a second-order reaction with stoichiometric/equimolar proportions of reactants.[8]

Such a study was not performed with the stem-containing DNA beacon. We are confident that there are certainly conditions at which strong mismatch discrimination by this molecular beacon will also be observed (see gray column F in Figure 6 demonstrating that the DNA beacon does not respond to the presence of unrelated dsDNA pre-targeted with PNA openers), but these conditions may vary from those we found for the PNA beacon.

Concluding Remarks. Several important conclusions can be drawn from this study. First, stemless PNA beacons may be advantageous in some aspects as hybridization probes over "classical" stem-containing DNA beacons. The simplicity of stemless constructs, stability of PNA oligomers against proteases and nucleases,[27] insensitivity of stemless PNA beacons to the presence of DNA-binding proteins,[5c] which normally affects the performance of DNA beacons,[28] and rapid and highly selective response of stemless PNA beacons to the presence of nucleic acid targets at low salt concentrations and elevated temperatures makes this new variety of molecular beacons very attractive for the future development of various DNA and RNA biosensors, which can operate both with crude nucleic acid extracts and under those conditions when any secondary structures within nucleic acids will be avoided.

Second, although stemless DNA beacons are not appropriate for diagnostic purposes due to high background fluorescence, these DNA (and RNA) constructs of different lengths and sequences could be useful tools for studying the conformational flexibility of single-stranded nucleic acids under a variety of experimental conditions. Likewise, stemless PNA beacons and

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similar constructs with other protein-like backbones can serve as convenient models for protein folding, e.g., to imitate the formation of closed loops[29] caused by interactions between hydrophobic amino acids.

Third, nondenaturing hybridization of molecular beacons to specific targets within dsDNA locally exposed by PNA openers is possible. We expect this approach to be useful in diagnostic applications in which denaturation of DNA analytes is unfavorable or undesirable. As in the case of other applications of the PD- and PP-loop strategy,[20−22] a major advantage of targeting dsDNA with molecular beacons may be a higher selectivity of probe binding. This is due to the inability of molecular beacon to hybridize with most of the mismatched binding sites because they will not be exposed to PNA openers being thus inaccessible to a probe. Also note here a much more rapid binding of DNA beacon to dsDNA target than to ssDNA target (Table 2).

Although the current protocol of nondenaturing detection of dsDNA targets with molecular beacons requires purification of the pre-opened dsDNA from PNA openers prior to detection by molecular beacon, this shortcoming is not unavoidable since it is due to the sequence overlap between probes and openers we chose for this study. Such an overlap causes partial binding of molecular beacons with PNA openers and, therefore, they should be removed after the pre-opening procedure.[30] We believe that choice of shorter probes and/or openers and use of other PD-loop forming DNA sequences with longer gap between the PNA openers binding sites will allow us to simultaneously target dsDNA with both openers and a probe (and our preliminary data support this expectation), thus avoiding the need for such an additional purification step.[31]

The use of molecular beacons for nondenaturing DNA diagnostics via PD-like loops is currently limited by the need for two short closely located oligopurine sites. However, these limitations are not severe since DNA sites featuring such sequences should occur quite frequently, statistically every four-to-five hundred base pairs of a random DNA sequence, on average.[19a,20,22b] The sequence limitations on the PD-loop-forming sites can be additionally relaxed by the use of recently developed pseudocomplementary PNAs (pcPNAs), pairs of which are able to invade mixed purine-pyrimidine dsDNA sequences.[32] We therefore expect that one of two pyrimidine bis-PNA openers can be replaced by a pair of pcPNAs consisting of all four nucleobases. Then, only one short oligopurine site, which should occur ca. every hundred base pairs, will be required within the designated dsDNA target for its recognition with a molecular beacon. Our preliminary data indicate that less sequence-restricted PP-loops of that kind can be formed by PNA beacons with the aid of pyrimidine bis-PNA and pcPNAs openers.

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Supporting Information Available: Protocol of the PNA beacon synthesis plus plots of kinetic responses and signal/background ratios of DNA and PNA beacons upon hybridization to complementary ssDNA target under various NaCl concentrations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(30) Partial binding of molecular beacons with PNA openers due to a sequence overlap results in increase in background fluorescence (see columns B in Figure 6). Much higher background is caused by PNA openers in the case of the DNA beacon (5 bp overlap with openers) than in the case of the PNA beacon (3 bp overlap).

(31) For PNA beacons, the optimal conditions for probe hybridization are compatible with those required for binding of PNA openers. In the case of DNA beacons, synchronal use of both openers and a probe may require addition of extra salt for optimal performance of this probe after some lag period needed for effective binding of PNA openers at low salt concentration.

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