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DNA assembly using bis-peptide nucleic acids (bisPNAs)

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ABSTRACT

DNA nanostructures are ordered oligonucleotide arrangements that have applications for DNA computers, crystallography, diagnostics and material sciences. Peptide nucleic acid (PNA) is a DNA/RNA mimic that offers many advantages for hybridization, but its potential for application in the field of DNA nanotechnology has yet to be thoroughly examined. We report the synthesis and characterization of tethered PNA molecules (bisPNAs) designed to assemble two individual DNA molecules through Watson–Crick base pairing. The spacer regions linking the PNAs were varied in length and contained amino acids with different electrostatic properties. We observed that bisPNAs effectively assembled oligonucleotides that were either the exact length of the PNA or that contained overhanging regions that projected outwards. In contrast, DNA assembly was much less efficient if the oligonucleotides contained overhanging regions that projected inwards. Surprisingly, the length of the spacer region between the PNA sequences did not greatly affect the efficiency of DNA assembly. Reasons for inefficient assembly of inward projecting DNA oligonucleotides include non-sequence-specific intramolecular interactions between the overhanging region of the bisPNA and steric conflicts that complicate simultaneous binding of two inward projecting strands. These results suggest that bisPNA molecules can be used for self-assembling DNA nanostructures provided that the arrangement of the hybridizing DNA oligonucleotides does not interfere with simultaneous hybridization to the bisPNA molecule.

INTRODUCTION

Nanotechnology involves assembly of small molecules into complex architectures for higher function (1). The canonical Watson–Crick base pairing of adenine to thymine and guanine to cytosine is ideal for organizing biomolecules in a highly predictable fashion. Numerous reports on using oligonucleotides to build higher order structures include DNA matrices based on subunits of fixed Holliday junctions (2), streptavidin–DNA fragment nanoparticle networks (3) and DNA dendrimer formations for drug delivery (4; for reviews see 5–8).

Peptide nucleic acids (PNAs) (Fig. 1A) are a promising connector for the assembly of DNA-based nanostructures. PNAs are synthetic DNA analogs containing a neutral 2-aminoethylglycine backbone (9) and hybridize sequence specifically to complementary DNA and RNA oligonucleotides (10). Binding occurs with high affinity (10,11), high sensitivity to mismatch discrimination (12) and is unaffected by the ionic strength (10). Because PNAs possess a neutral amide backbone they bind less to proteins than do oligomers with negatively charged linkages (13,14) and are nuclease and protease resistant (15). PNAs have an exceptional ability to hybridize to sequences within duplex DNA by strand invasion, suggesting that PNA molecules should also be superior agents for binding to single-stranded DNA at regions that contain intramolecular structure (16,17). An important practical advantage is that methods for PNA synthesis are compatible with peptide synthesis, allowing PNAs to be easily modified with amino acids and other moieties (18,19). High affinity binding by PNAs has already been used for nanostructure assembly, with applications for labeling of DNA (20,21) and strand invasion into DNA hairpins and tetraloop motifs (22; for reviews see 23,24).

Here we describe the synthesis of bisPNAs that contain spacer regions that differ in length and amino acid substitution. We characterize the ability of bisPNAs to assemble DNA and observe that the capacity of a bisPNA molecule to hybridize to two oligonucleotides is primarily dependent on the arrangement of the DNA oligonucleotides being assembled. These results suggest that bisPNA molecules with simple chemical modifications can be used in generating DNA:bisPNA:DNA ‘units’ for nanotechnology and DNA nanostructure assembly, but that proper orientation of the assembled DNA oligonucleotides is essential.

MATERIALS AND METHODS

PNA synthesis

PNA monomers (Fig. 1), 2-aminoethoxy-2-ethoxy acetic acid (AEEA) and other reagents for PNA synthesis were obtained from PE Biosystems (Foster City, CA). Fmoc-amino acids were obtained from Advanced Chemtech (Louisville, KY) or Calbiochem-Novabiochem Corp. (La Jolla, CA). PNAs were

prepared by automated synthesis using an Expedite 8909 synthesizer (PE Biosystems) as previously described (25).

PNAs were analyzed and purified by reverse phase high performance liquid chromatography (RP-HPLC) and mass spectral analysis by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) as previously described (25). Briefly, RP-HPLC analysis and purification of bisPNAs involved a running buffer of dH_2O/0.1% trifluoroacetic acid and an elution buffer of acetonitirile/0.1% trifluoroacetic acid from a C_{18} column inside a water jacket maintained at 55°C. Elution of PNA was monitored at 260 nm on a Dynamax UV-1 absorbance detector (Varian, Walnut Creek, CA). After purification, PNAs were lyophilized and resuspended in dH_2O. Mass spectrometry was performed by MALDI-TOF on a Voyager-DE Workstation (Applied Biosystems, Foster City, CA) using either α-cyano-4-hydroxycinnamic acid or sinapinic acid solution as a matrix (Sigma-Aldrich, St Louis, MO). The concentration of PNA solutions was measured using a Cary 100Bio UV-Visible spectrophotometer (Varian) at 260 nm and room temperature, using an extinction coefficient of 241.4 ml μmol^{-1} cm^{-1} (all of the bisPNA sequences are the same). The extinction coefficients of PNA 24 and PNA 26 are 127.8 ml μmol^{-1} cm^{-1} and 113.6 ml μmol^{-1} cm^{-1}, respectively.

Preparation of DNA oligonucleotides

DNA oligonucleotides (Invitrogen-Life Technologies, Carlsbad, CA) were radiolabeled using [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase (Sigma-Aldrich). Unincorporated [γ-^{32}P]ATP was removed by passing the oligonucleotides through a Bio-Spin 6 column (Bio-Rad Laboratories, Hercules, CA) that had been pre-equilibrated with distilled water. Equal amounts of unlabeled oligonucleotides were treated similarly and purified by Bio-Spin 6 column in parallel. These unlabeled oligonucleotides were used to estimate the concentration of the radiolabeled oligomers that had been treated similarly. Unlabeled oligonucleotides were also used for gel shift experiments. The concentration of each unlabeled DNA oligonucleotide was calculated on a Cary 100Bio UV-Visible spectrophotometer (Varian) at 260 nm using the extinction coefficient given by the manufacturer. For each 40mer and 50mer oligonucleotide the nucleobases were randomized, except for the complementary 12mer target sequences, to minimize the potential for formation of specific secondary structures.

Melting temperature (T_m) analysis

Melting temperature (T_m) experiments were performed on a Cary 100Bio UV-Visible spectrophotometer (Varian) at 260 nm. BisPNA and DNA oligonucleotides were suspended in Na_2HPO_4 buffer (100 µM, pH 7.5) at 2 µM each. The temperature was ramped from 95 to 15°C and back up to 95°C at a rate of 5°C/min with a 12 s hold at each reading. Cary WinUV software was used to determine the T_m for each combination.

Polyacrylamide gel analysis of bisPNA:DNA hybridizations

PNAs tend to aggregate upon storage, so each working solution of bisPNA was heated to 80°C for 5 min and then cooled to room temperature prior to use. bisPNA (150 nM final concentration) was mixed with ^{32}P-radiolabeled DNA oligonucleotide (150 nM final concentration) and unlabeled DNA

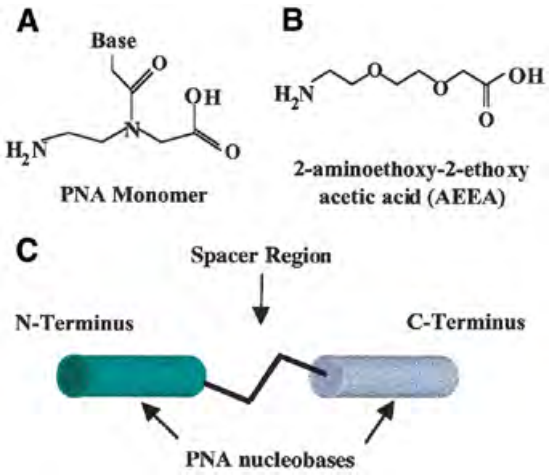

Figure 1. (**A**) Structure of a PNA monomer. (**B**) Structure of AEEA, the linker molecule used to join PNA strands. (**C**) Model of a bisPNA showing the N- and C-terminal PNAs connected by a spacer region that may include amino acids or one or more AEEA molecules.

oligonucleotide (0–25 µM) in a 50 µM Tris–HCl buffer (pH 8.0) in a 20 µl reaction. Oligonucleotide strands were annealed using a PE 9600 Thermocycler (Perkin Elmer, Norwalk, CT) (95°C for 5 min, cooled to 4°C over a period of 60 min). The samples were then flash frozen in an ethanol and dry ice bath and stored at –20°C until use. On ice, 10 µl of a 30% glycerol tracking dye (0.05% bromophenol blue, 0.05% xylene cyanol and 0.05% orange G) was added, followed by a quick spin, before loading into a non-denaturing 10% (19:1) polyacrylamide gel (Bio-Rad). The gel was run at 4°C and 250 V for 3.5 h. The gel was analyzed by autoradiography using a Molecular Dynamics model 425F phosphorimager (Sunnyvale, CA).

RESULTS AND DISCUSSION

Design of DNA oligonucleotides and bisPNAs

We designed bisPNA molecules containing two PNA strands linked by spacer regions of varied lengths (Fig. 1A–C). Previous reports have demonstrated that polypyrimidine bisPNAs can form four-stranded complexes capable of invading duplex DNA (26). The PNA sequences used in our studies, however, contained mixed purine and pyrimidine sequences because they were designed to bind and assemble two different single-stranded DNA oligonucleotides.

To test the effects of linker length and amino acid substitution within the spacer region between the two PNA strands we varied the number of AEEA linker molecules (Fig. 1B and Table 1) and number and identity of amino acids. Each AEEA molecule is ~11 Å in length, water soluble and highly flexible. Amino acids were included in some of the spacer regions to increase the distance between the PNA sequences and to test the effect of amino acid charge and steric bulk on the ability of the bisPNA to assembly both DNA oligonucleotides.

We designed DNA oligonucleotides to hybridize to bisPNAs in four different arrangements (Fig. 2A–D). The oligonucleotides were either exactly complementary to the PNA strands (Fig. 2A), or longer, so that overhanging single-stranded

Table 1. bisPNA sequences, expected and observed masses, and melting temperature values (T_m) for hybridization to oligonucleotide complements

bisPNA	Sequence (N→C)	Mass (Da) (found/calculated)	T_m[a] (°C)
PNA 1	tcttcacctaga-lys	3332.70/3332.60	50.4
PNA 2	gatacatatttg-lys	3412.19/3411.62	47.8
$AEEA_1$	tcttcacctaga-$(aeea)_1$-gatacatatttg-lys	6738.01/6744.42	52.7/48.3
$AEEA_3$	tcttcacctaga-$(aeea)_3$-gatacatatttg-lys	7032.80/7034.82	49.5/45.6
$AEEA_6$	tcttcacctaga-$(aeea)_6$-gatacatatttg-lys	7476.20/7470.42	49.1/46.3
$AEEA_6asp_5$	tcttcacctaga-$(aeea\text{-}asp)_5$(aeea)-gatacatatttg-lys	8023.45/8028.92	48.4/46.7
$AEEA_6lys_5$	tcttcacctaga-$(aeea\text{-}lys)_5$(aeea)-gatacatatttg-lys	8092.93/8093.42	49.1/47.8
$AEEA_6phe_5$	tcttcacctaga-$(aeea\text{-}phe)_5$(aeea)-gatacatatttg-lys	8495.29/8496.35	50.3/50.5

[a]For bisPNAs, the temperature on the right is the T_m of the N-terminal half of the bisPNA (analogous to PNA 1), while the temperature on the left is the T_m of the C-terminal half of the bisPNA (analogous to PNA 2).

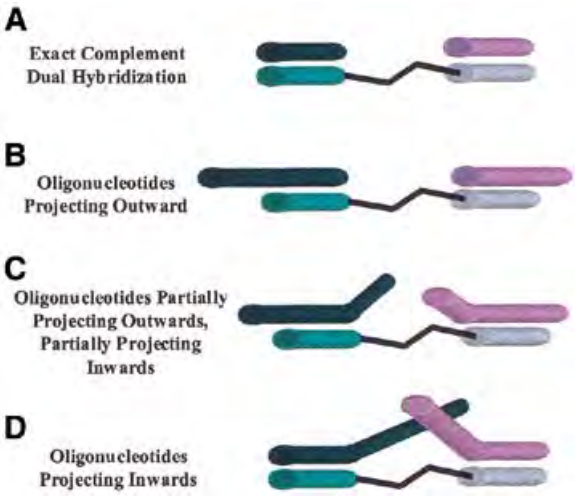

Figure 2. Models of bisPNA hybridized to DNA oligonucleotides. (**A**) DNA oligonucleotides that are exactly complementary to the PNA and do not have overhanging regions. (**B**) DNA oligonucleotides that have overhanging regions projecting outwards. (**C**) DNA oligonucleotides that have overhanging regions partially projecting inwards and partially projecting outwards. (**D**) DNA oligonucleotides that have overhanging regions projecting inwards.

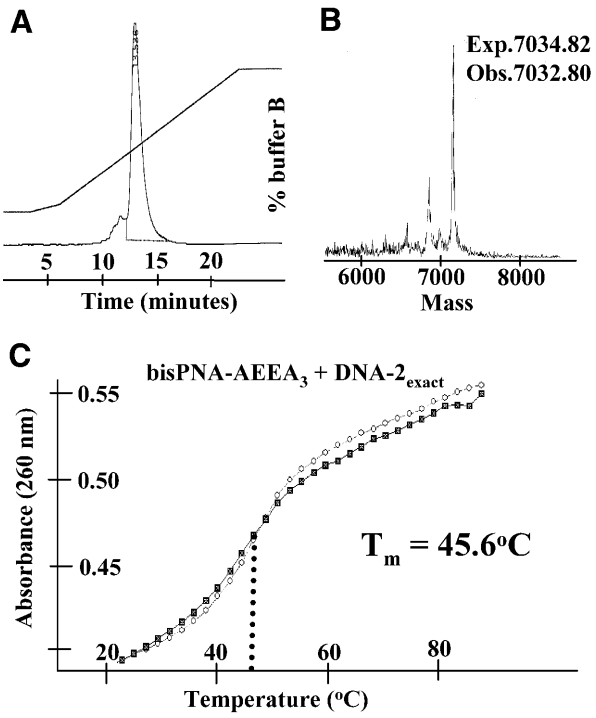

Figure 3. (**A**) RP-HPLC purification of bisPNA-$AEEA_3$. Purification conditions are described in Materials and Methods. (**B**) MALDI-TOF analysis of bisPNA-$AEEA_3$ with expected and observed molecular weights noted. (**C**) T_m analysis of bisPNA-$AEEA_3$ reversibly hybridizing to complementary DNA-2_{exact}. Closed squares represent association during decreasing temperatures. Open circles represent dissociation during increasing temperatures.

regions were created (Fig. 2B–D). The longer oligonucleotides could project outwards (DNA-$1_{outward}$ and DNA-$2_{outward}$), project partially outwards and partially inwards (DNA-1_{mid} and DNA-2_{mid}) or project inwards (DNA-1_{inward} and DNA-2_{inward}) relative to the bisPNA (Fig. 2B–D and Table 1). Except for the target sequence, each base of the DNA oligonucleotide was randomized to minimize secondary structure and DNA:DNA interactions. We studied the ability of PNAs to assemble DNA oligomers with overhanging bases because such overhangs can be used to bind additional nucleic acids and permit complex structures to be built up.

Synthesis and characterization of PNA and bisPNA molecules

The automated synthesis of bisPNA molecules involving at least 25 couplings required additional precautions to ensure complete synthesis of the molecule. Typically, when a PNA sequence contains three or more consecutive purines or pyrimidines synthesis efficiency decreases. To minimize this problem we repeated coupling steps for addition of the third consecutive base and any that followed. We found that the spacer molecule AEEA was difficult to couple efficiently, so each of these couplings was repeated prior to addition of the next molecule. Repeated coupling was also done for each amino acid. With these precautions, automated synthesis of bisPNAs with extensive spacer regions was routine. We analyzed and purified bisPNAs by C_{18} RP-HPLC (Fig. 3A). Full-length PNA was retained on the C_{18} column longer than

Table 2. Sequences of exact complement and overhanging oligonucleotides, melting temperatures (T_m) with bisPNA-AEEA$_3$ and differences in T_m between exact complements and overhanging oligonucleotides

	Name	Sequence (5′→3′)	T_m (°C)	ΔT_m (°C)
Exact complements	DNA-1$_{exact}$	tctaggtgaaga	49.5	-
	DNA-2$_{exact}$	caaatatgtatc	45.6	-
Projecting outward	DNA-1$_{outward}$	tctaggtgaaga(n)$_{38}$	49.3	–0.2
	DNA-2$_{outward}$	(n)$_{28}$caaatatgtatc	48.1	2.5
Partial inward/partial outward	DNA-1$_{mid}$	(n)$_{19}$tctaggtgaaga(n)$_{19}$	54	4.5
	DNA-2$_{mid}$	(n)$_{14}$caaatatgtatc(n)$_{14}$	52.8	6.2
Projecting inward	DNA-1$_{inward}$	(n)$_{38}$tctaggtgaaga	54.4	4.9
	DNA-2$_{inward}$	caaatatgtatc(n)$_{28}$	51.9	5.4

n, a randomized nucleotide.

truncated products from failed syntheses. Correct synthesis was confirmed by mass spectrometry (Fig. 3B). Both HPLC and mass spectral analysis routinely indicated that the main product was the desired one.

Melting temperature (T_m) analysis of exact complement DNA oligonucleotides to bisPNAs

We determined the melting temperature (T_m) values of the bisPNAs and complementary DNA oligonucleotides to characterize their potential for stable and selective hybridization (Fig. 3C). The T_m values for hybridization of bisPNAs with exactly complementary oligonucleotides were nearly the same as that of the individual 12 base PNAs corresponding to each half of the bisPNA (Table 1). This similarity in T_m values demonstrates that neither the molecules that make up the spacer region nor the unbound half of the bisPNA molecule significantly affects the temperature dependence of hybridization of the PNA and DNA oligonucleotides that lack an overhanging region. As we describe below, the interactions of bisPNAs with DNA oligonucleotides that do contain overhanging regions increase the T_m values in some cases (Table 2).

Assembly of short, complementary oligonucleotides by bisPNA

We analyzed the hybridization of bisPNAs to DNA oligonucleotides by monitoring the ability of the PNAs to shift the mobility of ^{32}P-radiolabeled DNA upon non-denaturing polyacrylamide gel electrophoresis. In all of the experiments described below, we established the mobility of labeled DNA alone, the mobility of labeled DNA bound to one PNA strand and the mobility of a mixture of two labeled DNA oligonucleotides directed to different PNA strands. ^{32}P-labeled and unlabeled oligonucleotides were added to bisPNAs at the same time.

A bisPNA containing three AEEA linkers (PNA-AEEA$_3$, Table 1) readily assembled short complementary DNA oligonucleotides, DNA-1$_{exact}$ and DNA-2$_{exact}$ (Table 2). Surprisingly, when bisPNA-AEEA$_3$ hybridized to both 12mer DNA oligonucleotides migration was faster than a single hybridized bisPNA-AEEA$_3$ (Fig. 4). The faster mobility of the DNA:bisPNA:DNA [illegible] with the longer DNA

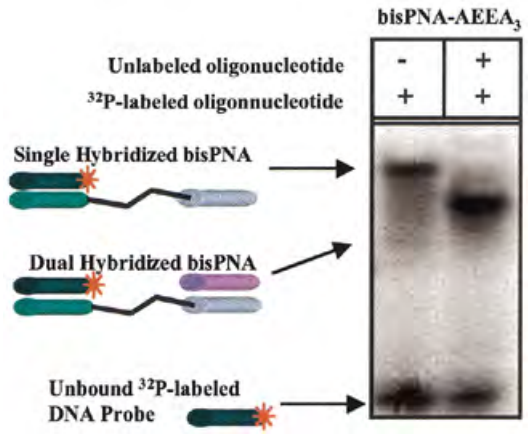

Figure 4. Non-denaturing polyacrylamide gel electrophoresis of bisPNA-AEEA$_3$ with ^{32}P-labeled DNA-1$_{exact}$ and unlabeled DNA-2$_{exact}$. Left lane, bisPNA-AEEA$_3$ and ^{32}P-labeled DNA-1$_{exact}$ are present in a 1:1 ratio (150:150 nM). Right lane, bisPNA-AEEA$_3$ and ^{32}P-labeled DNA-1$_{exact}$ and unlabeled DNA-2$_{exact}$ are present in a 1:1:3.3 ratio (150:150:500 nM).

sequences used in these studies. The increased mobility of the larger complex may be due to the dual hybridized bisPNA-AEEA$_3$ having twice the negative charge (from the second oligonucleotide phosphate backbone) and to formation of a more compact structure than the single hybridized bisPNA.

Assembly of oligonucleotides that project beyond the bisPNA

We next tested the ability of bisPNA-AEEA$_3$ (Table 1) to hybridize longer DNA oligonucleotides that extended past one or both PNA termini. Characterizing assembly of these longer oligonucleotides is important because the extended DNA sequences provide the potential for additional base pairing necessary for formation of higher order structures. When bisPNA was incubated with DNA oligonucleotides designed to project outwards we observed that the bisPNA readily assembled both DNA strands with similar results regardless of which DNA strand was labeled with ^{32}P (Fig. 5). The efficiency of hybridization was not affected by increasing the concentration of the unlabeled strands. In contrast to the ability of bisPNAs to successfully assemble short DNA oligonucleotides or DNA oligonucleotides with overhangs that project outwards (Figs 5 and 6, lanes 2), assembly of DNA oligonucleotides that project inwards upon hybridization was less apparent (Fig. 6, lanes 4 and 6). To improve binding of inward facing oligomers, we varied annealing conditions, but our attempts to bind two DNA oligonucleotides with inward projecting overhanging sequences invariably yielded only a small fraction of bisPNA bound to both DNA strands.

DNA assembly by bisPNAs connected by spacers of differing lengths

Inefficient assembly of the inward facing DNA oligonucleotides suggested that the first oligonucleotide bound to the bisPNA blocked binding of the second. One solution for overcoming this obstacle was to increase the length of the spacer region between the two PNA strands. In theory, this would [illegible]

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