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Continuous Solid-Phase Synthesis and Disulfide Cyclization of Peptide–PNA–Peptide Chimeras

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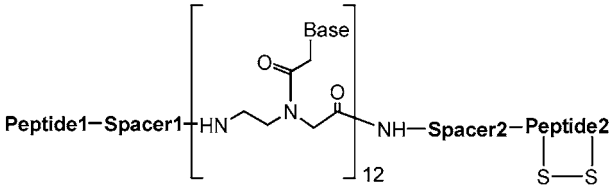
ABSTRACT



Chelator peptides were extended from the N-terminus of peptide nucleic acid (PNA) dodecamers, which in turn were extended from the N-termini of disulfide-bridged peptide ligand analogues, using Fmoc coupling for all residues. The cysteine thiols were cyclized on a solid support, either before or after PNA extension. This simplified synthetic approach might allow preparation of a variety of multipeptide disulfide-bridged PNA chimeras.

Peptide nucleic acids (PNAs) are a DNA/RNA mimic in which the phosphate deoxyribose backbone has been replaced by uncharged *N*-(2-aminoethyl) glycine linkages.[1] PNAs hybridize very strongly and specifically to RNA and DNA[2] and resist both proteases and nucleases,[3] and the uncharged PNA backbone is unlikely to interact with cellular proteins that normally bind negatively charged macromolecules.[4] Unlike normal DNA or phosphorothioate DNA, PNA hybridization does not induce RNase H degradation of bound mRNA.[5] Hence, PNA as a probe does not destroy its analyte, providing an opportunity for diagnostic application.

One of the primary requirements for an oligonucleotide analogue to be successful as an antigene/antisense agent is for it to be taken up by the cells in reasonable quantity so that it can reach its target in sufficient concentration. Since PNAs suffer from poor cellular uptake,[5,6] they have not been well developed as an antigene/antisense therapeutic agent. To improve cellular uptake, PNAs have been conjugated with cell-penetrating positively charged peptides with homology to nuclear localization sequences such as transportan, penetratin, and TAR-binding peptides,[7] or the SV40 nuclear localization sequence itself.[8,9]

Cell penetration in these cases is not receptor-dependent and thus not cell-specific. To elevate cellular uptake of PNA by cancer cells that overexpress insulin-like growth factor receptor (IGF1R), a disulfide-cyclized D-peptide IGF1 analogue, D-(CysSerLysCys),[10] was extended from a solid support, followed by the PNA.

The introduction of disulfide bridges into peptides allows the creation of conformational constraints that can improve

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the recognition between a ligand and its receptor, therefore improving biological activity.[11] The specific PNA−peptide chimera displayed a 5−10 times greater uptake than control PNA or PNA with a control peptide D-(CysAlaAlaCys), but only in cells overexpressing IGF1R.[12] We intend to utilize the IGF1 analogue to concentrate oncogene PNA probes in transformed cells that overexpress IGF1R and a cyclic peptide L-(CysAsnGlyArgCys) that binds selectively to human breast cancer xenografts in nude mice.[13]

To visualize externally those cancerous tissues that take up the PNA−peptide probes and concentrate them by hybridization to oncogene mRNA targets, we designed trifunctional peptide−PNA−peptides to extend from the N-terminus of the PNA a tetrapeptide, Gly−D−Ala−Gly−Gly, that chelates ^{99m}Tc firmly and efficiently for scintigraphic imaging of γ-particles emitted by decay of ^{99m}Tc.[14,15]

PNA−peptide conjugates are usually prepared by fragment condensation,[16] which requires multiple steps of preparation and purification, causing significant loss in yield. In our first attempt at facile PNA−peptide synthesis without intermediate conjugation steps,[12] the peptide was assembled on a solid phase with Fmoc coupling, and then *t*-Boc-protected PNA monomers were coupled manually.

We then hypothesized that continuous solid-phase synthesis on a single-resin support could be extended to Fmoc coupling of all peptide1, PNA, and peptide2 residues during a single run, with Cys−Cys cyclization on a solid phase, yielding a chimera capable of radionuclide chelation for imaging of gene expression *in vivo* after a single purification step.

Synthesis of Peptide1−PNA Chimeras. 4-Aminobutyric acid (Aba) was selected as a spacer[14] to minimize steric hindrance between the chelator and the PNA. We assembled Gly−Aba−Ado (**1**) using Fmoc PNA coupling[17] on an Applied Biosystems 8909 DNA synthesizer as a model compound. Because PNA monomers couple more rapidly than amino acid monomers, three reaction conditions were tested for coupling between amino acid monomers to PNA and for coupling between amino acid monomers: (1) standard single coupling, (2) double coupling, (3) single long coupling. Preparative RP-HPLC of *N*-Gly−Aba−Ado (**1**) cleavage products performed on a 10 × 250 mm Alltima C_{18} column eluted with a gradient over 25 min from 5 to 70% CH_3CN in aqueous 0.1% CF_3CO_2H, at 1 mL/min, 50 °C, monitored at 260 nm, indicated that the long coupling cycle gave the best yield, 99%, vs double cycling, recommended by Applied Biosystems for amino acid monomers when using the PNA protocol, which gave only a 95% yield. MALDI-TOF mass spectra confirmed the identity of the main product peak at 435.5 Da. While the differences among cycle yields were not large, Gly and Aba have very weak steric hindrance. In the case of amino acid monomers having strong steric hindrance (aromatic side chains or large protecting groups), long cycles might provide an even greater advantage.

We then used the long cycle to synthesize Gly−D−Ala−Gly−Gly−Aba−GCATCGTCGCGG (**2**), a chelator-PNA specific for the human c-*MYC* mRNA initiation region, on a 2 μmol Fmoc-XAL-PEG-PS column in a single automated run. The overall yield following preparative RP-HPLC was 35.4%. Similarly, a K-*RAS* Val12 oncogene probe, Gly−D-(Ala)−Gly−Gly−Aba−CGCCAACAGCTC (**3**), gave 39.9% yield. MALDI-TOF mass spectra confirmed the identity of the main product peaks at 3613.8 Da (**2**) and 3526.5 Da (**3**).

Synthesis of Peptide1−PNA with a Targeting Peptide2. The peptide2 with a Gly_4 spacer was assembled by Fmoc coupling on PAL-PEG-PS resin (loading, 0.2−0.3 mmol/g) on an Applied Biosystems 430A peptide synthesizer. Then, PNA monomers and amino acid monomers were sequentially coupled to the resin on the 8909 DNA synthesizer, using the long cycle protocol for the peptide1 amino acids.

Both the IGF1 analogue D-(CysAlaAlaCys) and the breast cancer targeting peptide L-(CysAsnGlyArgCys) include a disulfide bridge between the terminal cysteines. Cyclization typically limits overall yield, in addition to the problem of block conjugation, because a peptide with a pair of cysteines to be cyclized is usually assembled on a solid phase, deprotected, cleaved, and purified by RP-HPLC. The reduced cysteine thiols are then oxidized nonspecifically by air in a basic solution (pH 8.5) at high dilution to minimize dimerization and oligomerization.[12]

Unfortunately, PNAs are less soluble when the pH is higher than 7, which leads to a lower yield. Deprotection/oxidation of the cysteines while the peptide chain is still on the support provides an alternative to the dilute aqueous route. Solid-phase cyclization is based on the assumption that, for polymer-bound reactants, intramolecular processes are preferred due to pseudodilution.[18] Under the latter conditions, deprotection and cyclization of cysteine residues are achieved simultaneously. Thus, the solubility problem of PNAs can be avoided, and the preparative steps can be simplified, improving the overall yield. We therefore tested different conditions of cyclization on a solid phase in order to find a simple and efficient cyclization method for peptide−PNA−peptide chimeras that would obviate the need for postsynthetic cyclization. We protected the two cysteine side chains with *S*-acetamidomethyl (Acm) instead of *S*-triphenylmethyl (Trt) because Acm gives a higher yield of cyclization in $(Me)_2NCHO$.[19]

We used Fmoc-PAL-PEG-PS (0.2−0.3 mmol/g) for assembling the targeting peptides because it is compatible with

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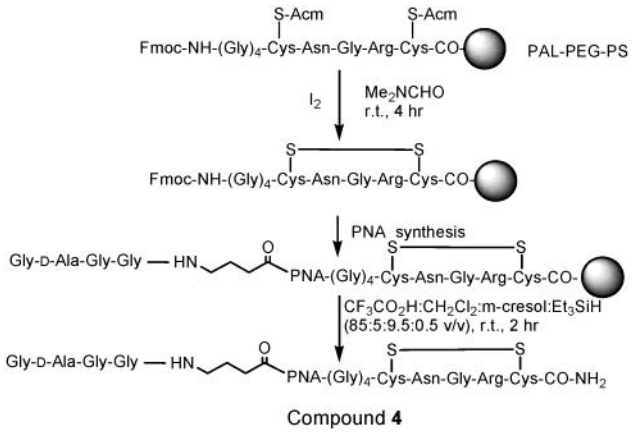
Scheme 1. Cysteine Cyclization on a Solid Phase before PNA Extension

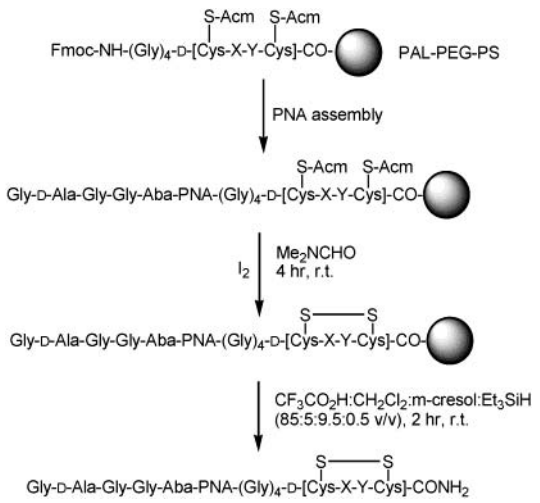


PNA synthesis. Our experimental results suggested that, if the loading of resin was higher than 0.3 mmol/g, cyclization on the resin would lead to complicated products due to cross-reaction between peptide chains. Three different reagents have been utilized for deprotection/oxidation of resin-bound peptide: (i) Tl $(tfa)_3$,[20] (ii) I_2 under acidic conditions,[21] and (iii) I_2 in $(Me)_2NCHO$.[22] $Tl(tfa)_3$ is expensive, highly toxic, and requires anhydrous conditions. In addition, it is possible that $Tl(tfa)_3$ would react with amino or hydroxyl groups on nucleobases during the cyclization of cysteine residues. CF_3CO_2H or HOAc could not be used as the solvent due to the acid lability of the PAL anchor. Hence, we tested two deprotection/oxidation conditions with $I_2/(Me)_2NCHO$: cyclization of cysteine residues prior to extending with peptide1-PNA, and after assembly of the complete peptide1−PNA−peptide2 sequences.

Cyclization on a Resin before Extension of Peptide1−PNA. Peptide2−resin was suspended in $(Me)_2NCHO$. Oxidation was carried out with I_2 (10 equiv) for 4 h at room temperature (Scheme 1). The resin was washed with $(Me)_2NCHO$ to remove excess iodine and dried in a vacuum. Two μmol equivalents of dry resin were placed in an empty column for PNA extension and chelator coupling. Cleaved and deprotected **4** was purified by preparative RP-HPLC at 50 °C as above and gave an overall yield of 19.5%. Over 26 coupling cycles and one deprotection/oxidation step, the average yield per cycle was therefore 94%. MALDI-TOF mass spectra gave the predicted molecular weight of 4372.2 Da. The result indicates that the intramolecular disulfide bond formed on the solid phase is stable under the conditions of PNA synthesis, and the cyclization of peptides prior to extending PNA is feasible.

Cyclization on a Resin after Assembly of Peptide1−PNA−Peptide2. In practice, it is more desirable to continue PNA synthesis immediately after the assembly of the peptide2−resin. Then the question arises whether the peptide1−PNA chain will interfere with the formation of intramolecular disulfide bonds while the chimera remains bound to the support. To address this, we synthesized five PNA 12-mer sequences with 5′- and 3′-peptides by programmed continuous solid-phase synthesis, with cyclization after assembly but before cleavage (Scheme 2). Compounds

Scheme 2. Cysteine Cyclization on a Solid Phase after PNA Extension



5−**7** are complementary to c-*MYC* mRNA (antisense, antisense with a control peptide, and mismatched sequences), compound **8** targeting cyclin D1 mRNA and compound **9** targeting *ERB2* mRNA. We cleaved and deprotected the chimeras in 85:5:9.5:0.5 CF_3CO_2H/CH_2Cl_2/*m*-cresol/Et_3SiH for 2 h at room temperature. Chimeras were purified by RP-HPLC as above, and the yields (Table 1) were higher than when cyclizing before PNA extension.

Table 1. Yields from Cyclization on a Resin after Assembly

compound	X	Y	PNA (5′→3′)	yield (%)
5	Ser	Lys	GCATCGTCGCGG	32.9
6	Ala	Ala	GCATCGTCGCGG	37.6
7	Ser	Lys	GCATGTCTGCGG	21.6
8	Ser	Lys	CTGGTGTTCCAT	39.1
9	Ser	Lys	CATGGTGCTCAC	57.6

Perhaps the coupling of the first PNA monomer to cyclic peptide2 (Scheme 1) was not as efficient as coupling to linear peptide2 (Scheme 2). On the other hand, it is possible that I_2 treatment of peptide2-bound resin created some byproducts that reduced the efficiency of the subsequent coupling reaction. In any case, the results imply that the long sequence of peptide1−PNA−peptide2 anchored to its support does not impair the formation of intramolecular disulfide bonds.

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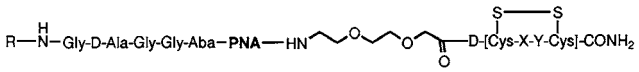
Replacement of the Gly_4 Spacer with an $(EtO)_2$ Spacer. To improve the specificity of ^{99m}Tc labeling, we then replaced the Gly_4 spacer, whose structure is similar to the chelator peptide1, Gly−D-Ala−Gly−Gly, with an $(EtO)_2$ spacer. Simultaneously, we solved the problem of Fmoc-Aba monomer decomposing to Fmoc and insoluble Aba by using the entire chelator peptide1 as a single synthon, instead of coupling the five amino acids sequentially. We expected that the two measures would improve the overall yields of peptide−PNA−peptide chimeras because the number of coupling cycles would be reduced from 25 to 17. Compounds **10**−**12** were prepared with cyclization on a solid phase after PNA extension (Table 2), but MALDI-TOF mass spectra

Table 2. Yields with an $(EtO)_2$ Spacer

compound	X	Y	R	PNA (5′→3′)	yield (%)
10	Ser	Lys	H	GCATCGTCGCGG	
11	Ala	Ala	H	GCATCGTCGCGG	
12	Ser	Lys	H	GCATGTCTGCGG	
13	Ser	Lys	Ac	GCATCGTCGCGG	27.2
14	Ala	Ala	Ac	GCATCGTCGCGG	26.8
15	Ser	Lys	Ac	CTGGTGTTCCAT	30.6
16	Ala	Ala	Ac	CTGGTGTTCCAT	34.0

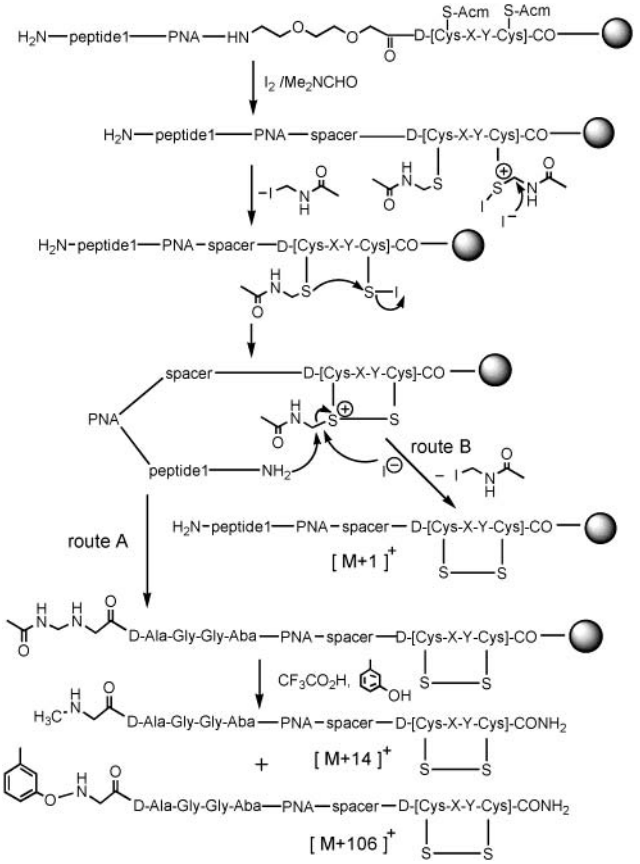


displayed peaks of $[M + 14]^+$ and $[M + 106]^+$ besides $[M + 1]^+$. The intensity ratio $[M + 1]^+/[M + 14]^+$ was about 3:2, while the intensity of the $[M + 106]^+$ peak was much lower. There were also many unidentified peaks.

When we tried to prepare fluorescein-labeled **10**, Fmoc-Lys(ϵ-fluorescein)-OH could not be coupled to the peptide1−PNA−peptide2. Instead, acetylated product **13** was obtained, which gave a sharp HPLC peak with a MALDI-TOF mass of $[M + 1]^+$. Thus it would appear that acetylation or a similar capping reaction is necessary prior to Cys cyclization with these particular chimeras. Based on related studies on the mechanism of deprotection/oxidation of cysteine residues,[21,22] a possible mechanism of formation of the two peaks $[M + 14]^+$ and $[M + 106]^+$ is proposed in Scheme 3. Perhaps the flexibility and hydrophilicity of the $(EtO)_2$ spacer, relative to that of the Gly_4 spacer, favored the postulated intramolecular S_N2 reaction through route A and contributed to the formation of the byproducts (Scheme 3). Compounds **13**−**16** were assembled, cyclized, acetylated by capping, cleaved/deprotected, and purified by RP-HPLC as above. In all cases, MALDI-TOF mass spectra indicated that acetylated full-length chimeras were the major products, and no methylated or *m*-cresolyl products were detected.

Melting Temperature Analysis was utilized to determine the influence of the peptide moiety on the hybridization efficiency of the antisense PNA for its complementary RNA strand. Measurements were carried out in triplicate with equimolar 2.5 μM PNAs and complementary 12-mer RNAs in 10 mM Na_2HPO_4, 1.0 M NaCl, 0.5 mM EDTA, pH 7.0. The T_m of the parent unmodified PNA GCATCGTCGCGG (**17**) duplex with RNA was found to be 79.0 ± 1.2 °C;

Scheme 3. Possible Mechanism of Byproduct Formation



mismatched chimera **7** showed no hybridization with its complementary RNA strand, and the T_m values of chimeras **2** (80.5 ± 0.5 °C), **5** (82.4 ± 0.3 °C), and **6** (81.3 ± 0.2 °C) were undiminished. These results indicate that the pair of peptide moieties at the N and C termini of the 12-mer PNA do not interfere with the hybridization efficiency.

In summary, automated, continuous solid-phase synthesis of peptide1−PNA−peptide2 was successful with a long coupling cycle for the amino acid residues. Two methods of introducing disulfide bonds into PNA−peptide chimeras in the solid phase were established, before or after PNA extension. Acetylation of the terminal amino group is a key prior step to obtain disulfide cyclized PNA−peptide chimeras in high purity and high yield when using EtO spacers. The melting temperatures of PNA:RNA duplexes implied that the two peptide moieties at the N and C termini of the 12-mer PNA stabilize the duplexes slightly whether the peptides are positively charged or not. Labeling and biological studies are in progress. The disulfide cyclization methods may be useful for a wide variety of peptides and peptide chimeras.

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