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Evidences for complex formation between L-*dab*PNA and *aeg*PNA

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ABSTRACT

Continuing our research on the development of nucleopeptides as ODN analogs for biomedical and bioengineering applications, here we report the synthesis and the chemical–physical characterization of a homoadenine hexamer based on a L-diaminobutyric acid (L-DABA) backbone (*dab*PNA), and its binding studies with a complementary *aeg*PNA. We demonstrated by CD and UV experiments that the L-*dab*PNA binds the *aeg*PNA forming a complex with good thermal stability, that we identified as a left-handed triplex.

The simple four-base recognition in nucleic acids has inspired for decades chemists and biologists to develop natural oligonucleotides (ODNs) as therapeutic and diagnostic tools or as new nanomaterials in biomedical and bioengineering applications.[1,2] However, the use of natural ODNs is limited by several factors including poor cellular uptake, a relatively short half-life in physiological conditions due to nucleases, and various non-specific effects relative to ODN–protein interactions.[1,2] In order to overcome these problems, the development of new ODN analogs has been widely explored for decades and many modified ODNs were designed and tested.[1–3] One of the most conservative ODN modification is contained in phosphorothioate (PS) ODNs, in which a non-bridging oxygen atom in the phosphate group is replaced by sulfur.[4] PS-ODNs showed high affinity to nucleic acid targets, good nuclease resistance, and remarkable ability to cross the lipid bilayer. Despite these positive characteristics, which allowed the entrance of PS-ODNs in advanced phases of clinical trials, the effect of their backbone stereogenicity on target binding and the toxicity connected with their non-specific binding to several proteins are still subjects of studies.[4,5] On the other hand, one of the most dramatic deviations from the natural DNA structure is represented by *aminoethylglycyl*PNAs (*aeg*PNAs), introduced in 1991 by Nielsen,[6a] which contain an achiral pseudopeptide backbone, instead of the sugar-phosphate one, on which nucleobases are anchored through a carboxymethylene linker.[6] These analogs were found to possess remarkable properties such as high nuclease resistance as well as good affinity and specificity for complementary natural nucleic acids and for complementary *aeg*PNAs themselves.[6] However, some drawbacks, such as low water solubility, tendency to aggregate, and costly precursors, limit their use in various applications.[6b,6c] In order to improve the water solubility and to decrease their aggregation, positive charges, generally coming from basic aminoacid moieties, were inserted in the pseudopeptide backbone of *aeg*PNAs.[6b,7] Furthermore, the introduction of chiral centers into *aeg*PNAs induced preferential orientation (parallel or antiparallel) of the PNA relative to the complementary strand increasing the specificity for the target.[6b,8]

In order to develop new nucleopeptides as ODN analogs for biomedical and bioengineering applications, recently we began to study chiral *dab*PNAs, reporting for the first time the synthesis of a new homothymine L-DABA-based dodecamer, its characterization, and hybridization studies with natural nucleic acids.[9] Continuing our research in this field, here we describe the synthesis and the characterization of a novel adenine L-DABA monomer suitably protected for solid phase assembly, its oligomerization to the corresponding hexamer, and binding studies with a complementary *aeg*PNA in order to explore the potential use of these nucleopeptides as new materials.

The synthesis of the adenine L-DABA monomer ($a_{L\text{-}dab}$, **2**, Scheme 1) was performed starting from the commercially available Boc-L-DAB(Fmoc)-OH diaminoacid with a procedure that allowed

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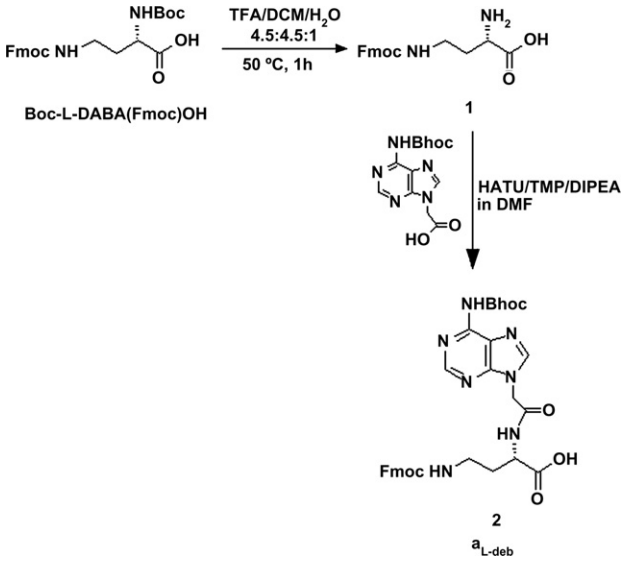


Scheme 1. Synthesis of $a_{L\text{-}dab}$ monomer **2**.

us to obtain the desired building block in only two synthetic steps (see Supplementary Material for details). Firstly, the Boc was selectively removed from the α-amino group of DABA by TFA treatment giving compound **1** in almost quantitative yield. The successive coupling of **1** with the Bhoc-protected (adenin-9-yl)acetic acid was performed using HATU/DIEA/TMP in DMF as the activating system, leading to the orthogonally protected $a_{L\text{-}dab}$ nucleoaminoacid **2** (Scheme 1). After removal of DMF followed by precipitation in cold water and centrifugation, the crude pellet was purified by RP-HPLC, using TFA-free eluents to prevent loss of the Bhoc group, and pure product **2** was obtained in 51% yield.

The new adenine DABA-based monomer was characterized by NMR and LC-ESIMS (Fig. 1a) and oligomerized on solid phase to the corresponding *dab*PNA hexamer **3** (Scheme 2), using a peptide-like protocol and Fmoc-chemistry (see Supplementary Material for details). Firstly, the solid support was functionalized with a L-lysine by reaction of deprotected Rink-amide resin (0.5 mmol NH_2/g) with Fmoc-L-Lys(Boc)-OH following a standard procedure (PyBOP/DIEA in DMF), reducing the resin functionalization to 0.25 mmol/g. This value is generally appropriate for the synthesis of PNAs in order to avoid aggregation effects during chain elongation. Then, we performed six coupling steps with *dab*PNA monomer **2** using a procedure, reported in the literature,[8d,9] that minimizes racemization (HATU/TMP without preactivation). Coupling yields were checked spectrophotometrically on solid phase by UV Fmoc test and found to be in the range of 70–75%. A glycine was added, as the last residue, at the N-terminus to prevent side reactions (*N*-acyl transfer or loss of last residue through cyclization) that occur, as for *aeg*PNAs, when the N-terminal amino group of *dab*PNAs is free in basic or neutral medium.[6b] A 20% overall yield for **3** was estimated on the final Fmoc test with respect to the initial functionalization of the Rink-Lys-NH_2 resin. The L-lysine and glycine residues, incorporated in the strand at C- and N-terminus, respectively, are useful to improve the solubility in water of the free homoadenine oligomer. Deprotection and cleavage from the solid support were achieved by TFA treatment, followed by precipitation in cold diethyl ether. Crude *dab*PNA was purified by reverse phase HPLC, and the pure product **3** was quantified by UV and characterized by LC-ESIMS (Fig. 1b).

Subsequently, we studied the CD behavior of the homoadenine L-*dab*PNA single strand. In particular, the CD spectrum of nucleopeptide **3** (Fig. 2, solid line) presented a profile similar to that we recently published for t_{12} L-*dab*PNA[9] (Fig. 2, dashed line) with a shift of the negative band minimum from 281 to 269 nm. It is interesting to underline that the observed CD profile is analogous to that reported for other peptide nucleic acids carrying L-amino-acid residues in the backbone, such as homothymine L-*orn*PNA oligomers.[8d]

In order to explore the ability of L-*dab*PNA to bind *aeg*PNA, we performed CD studies using a tandem cell. For this purpose, a t_{12} *aeg*PNA was assembled on an automatic synthesizer using stan-

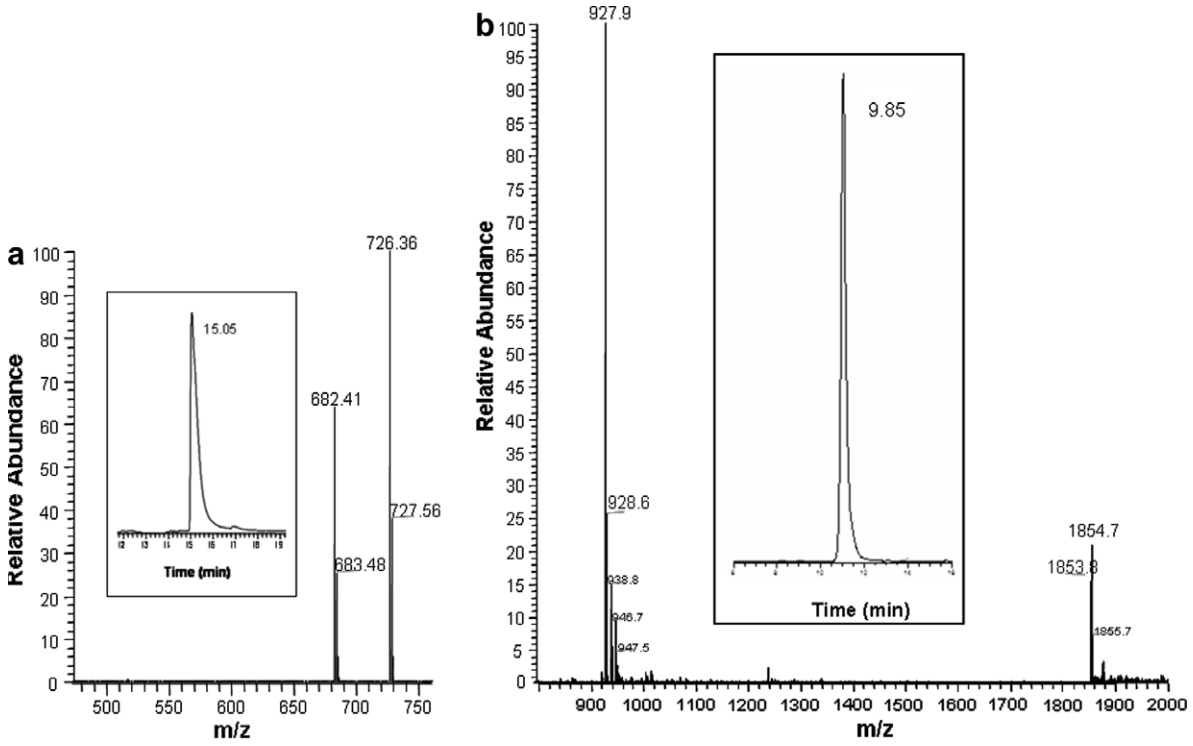
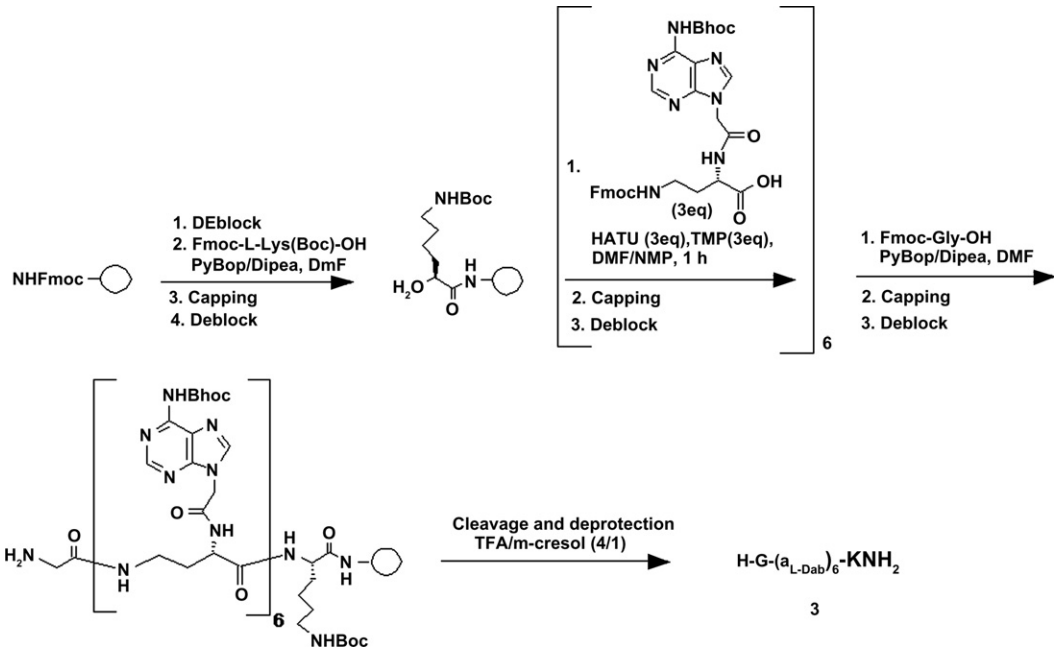


Figure 1. LC-ESIMS profiles for $a_{L\text{-}dab}$ monomer **2** (a) and hexamer **3** (b).



Scheme 2. Synthesis of the homoadenine DABA-based oligomer **3**.

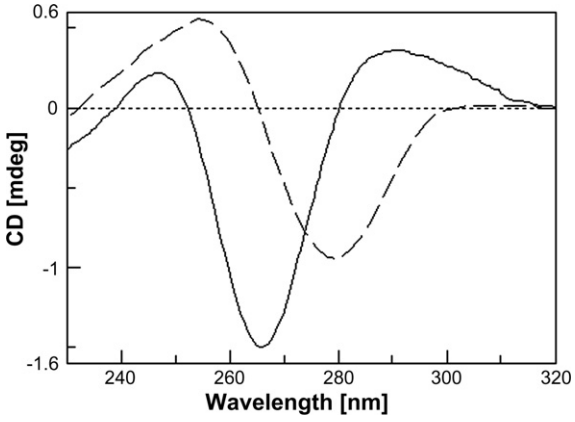


Figure 2. Overlapped CD spectra of $(a_{L\text{-}dab})_6$ **3** (solid line) and $(t_{L\text{-}dab})_{12}$ (dashed line), 8 μM in 10 mM phosphate buffer, pH = 7.5 (1 cm cell path length).

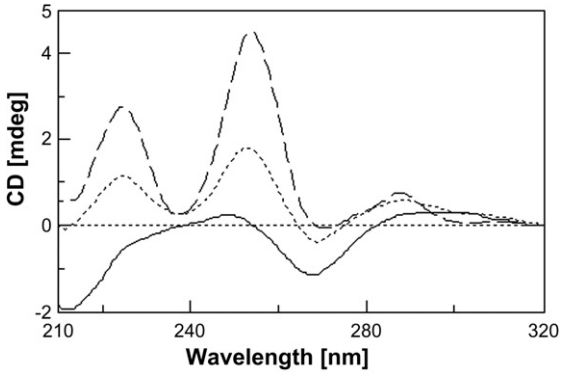


Figure 3. Overlapped *sum* (solid line), *mix* after 5 min (dotted line) and *mix* after 60 min (dashed line) CD spectra of 1:1 $(a_{L\text{-}dab})_6/t_{12}$ *aeg*PNA (8 μM each strand before mixing, 2 × 0.4375 cm cell path length).

dard protocol and Fmoc solid phase chemistry. The *sum* CD spectrum of t_{12} *aeg*PNA and nucleopeptide **3**, contained in two separated compartments of the tandem cell at the same concentration (2:1 ratio in bases) in phosphate buffer (pH = 7.5) at 20 °C, is reported in Figure 3 (solid line). As expected, the *sum* spectrum was essentially due to the contribution of the chiral nucleopeptide because the achiral *aeg*PNA, even with a L-lysine at its C-terminus, do not furnish a CD signal, as we verified by performing the CD spectrum of the t_{12} *aeg*PNA alone (data not shown) and in agreement with literature data.[8d] On the other hand, the *mix* CD spectrum, recorded 5 min after the two components were mixed (dotted line, Fig. 3), evidenced a significant change with respect to the *sum*, with the appearance of three main positive bands centered at 220, 260, and 280 nm, whose intensity increased and stabilized after approximately 60 min (dashed line).

In other words, the mixture of the complementary chiral *dab*PNA **3** and achiral *aeg*PNA strands gave a strong CD signal, suggesting the formation of a complex $(a_{L\text{-}dab})_6/t_{12}$ *aeg*PNA adopting a well-defined structure of stacked base pairs. More particularly, the CD spectrum of the complex showed a pattern very similar to that reported in the literature for an $a_{10}(t_{10})_2$ PNA–PNA–PNA triplex[10] presenting the same positive maxima at 220, 260, and 280 nm. Thus, we suggest the formation of a triple helix also in our case, of the type *aeg*PNA–*dab*PNA–*aeg*PNA based on T–A–T triplets. More particularly, the helix structure should have a left-handed winding in accord with previous studies in which it was shown that complexes between PNAs containing S stereocenters, or equivalently L-aminoacids, in one of the PNA strands were characterized by left-handed helices, predominant in solution.[8c,11] However, this hypothesis should be confirmed by NMR spectroscopy.

Thermal stability for the complex $(a_{L\text{-}dab})_6/t_{12}$ *aeg*PNA (1:1 in phosphate buffer, pH = 7.5) was then studied by UV spectroscopy. The UV melting curve, reported in Figure 4, exhibited a transition at 43.8 °C (T_m). Assuming the formation of an $a_6/(t_6)_2$ triplex, a contribution to the T_m of 7.3 °C *per* T–A–T triplet can be deduced. This value is essentially identical to that obtained for pure *aeg*PNA triplexes (7.6 °C *per* triplet).[10] The presence of only one transition in the melting curve suggests that no duplex intermediate was effectively formed, in perfect agreement to the reported behavior of PNA triplexes.[10] The sigmoidal profile of the UV melting curve

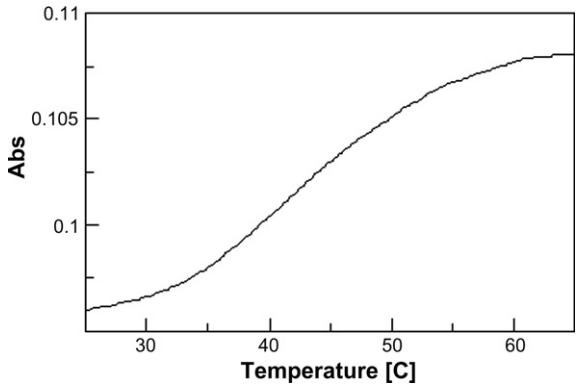


Figure 4. UV melting of $(a_{L\text{-}dab})_6/t_{12}$ aegPNA complex (0.55 μM each strand, A:T = 1:2) in phosphate buffer (pH = 7.5, heating rate 0.5 °C/min).

suggested the formation of a complex based on cooperative hydrogen bonds and base-stacking. The process was reversible and the pairing was completed over a temperature change of approximately 35 °C (70 min at 0.5 °C/min).

In this work we have demonstrated, for the first time, the formation of a complex between complementary achiral *aeg*PNA and chiral L-*dab*PNA molecules. In particular, the $(a_{L\text{-}dab})_6$ oligomer, synthesized starting from the Fmoc/Bhoc-protected adenine L-DABA-based monomer, and characterized by CD and LC-ESIMS, was shown to bind a t_{12} *aeg*PNA forming a complex of high thermal stability (T_m = 43.8 °C). On the basis of the CD spectrum profile, similar to those reported in the literature for (homoadenine)/(homothymine)$_2$ PNA triplexes, we suggest a triple helical structure also for the a_6 L-*dab*PNA/t_{12} *aeg*PNA system, hypothesis to be validated by further studies based on NMR spectroscopy. Moreover, UV melting experiments evidenced that this complex denaturates in one step without involving a duplex intermediate, as already reported also for other PNA triplexes.

From a biotechnological point of view, the results obtained in this work provide important direction for the development of novel self-assembling materials based on mixed structures of *aeg*/*dab*PNA. The chirality of these mixed systems, due to the presence of *dab*PNA, could confer remarkable properties to the resulting bio-inspired material, such as preferential strand orientation and helical handedness, providing highly ordered three-dimensional networks. Thus, we suggest the combined utilization of *dab*PNA and *aeg*PNA in order to realize chiral self-assembling systems, characterized by nucleobase-directed recognition, thermal stability, as well as chemical and enzymatic resistance, which could be useful tools, for example, as hydrogels for the controlled drug delivery.

Nevertheless, it is worth to underline that both *aeg*PNAs and *dab*PNAs were proposed as ancestors of ribonucleic acid during the evolution of life on Earth, firstly by Nielsen[12] in 1993 and, subsequently, by Meierhenrich[13a] in 2004 following the recovery of DABA,[13a] together with nucleobases,[13b] in the extraterrestrial soil of Murchison meteorite. Furthermore, both *aeg*PNA and *dab*PNA submonomeric units were obtained in simulated stellar conditions involving electric discharge reactions from CH_4, N_2, NH_3, and H_2O.[14] Thus, the formation of a complex *dab*PNA/*aeg*PNA, evidenced by this work, is a question that should be deeply investigated to ascertain if this recognition could have been involved in the already hypothesized prebiotic PNA world.[12,13a,15]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.005.

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