NEW PEPTIDE NUCLEIC ACIDS FOR BIOTECHNOLOGICAL APPLICATIONS

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A me e a chi mi è stato vicino

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Riassunto

Nell'ambito delle scienze biotecnologiche, gli analoghi oligonucleotidici occupano un ruolo importante. Essi, infatti, essendo in grado di legare targets nucleotidici naturali e non naturali contribuiscono allo sviluppo di strategie terapeutiche o diagnostiche e di nuovi materiali.
Tra gli analoghi oligonucleotidici di maggiore successo vi sono gli acidi peptido nucleici (PNA da Peptide Nucleic Acids), ideati da Peter Nielsen nel 1991.
I PNA (Figura 1) sono analoghi aciclici del DNA in cui lo scheletro zucchero-fosfato dei normali acidi nucleici è completamente sostituito da una struttura pseudopeptidica, in cui l'unità ripetitiva consiste in una N-(2-amminoetil)glicina (aeg) che porta la base eterociclica legata all'azoto glicinico mediante un ponte metilencarbonilico (Nielsen et. al, 1991) .

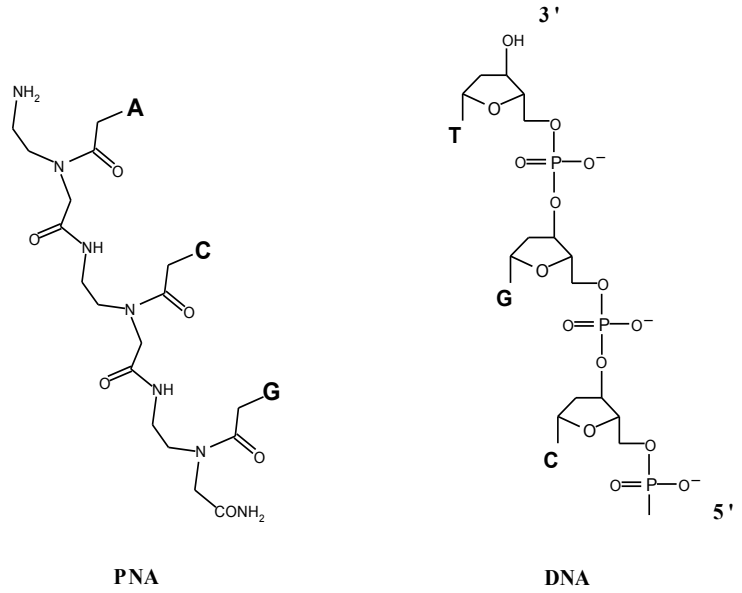


Figura 1: Confronto tra PNA e DNA.

Gli aegPNA riconoscono DNA e RNA complementari in maniera specifica. Essi formano, infatti, complessi molto stabili con DNA e RNA, obbedendo allo schema di legame ad idrogeno di Watson-Crick, con un'affinità maggiore rispetto ad oligonucleotidi convenzionali o analoghi (Egholm et al., 1993); riconoscono, inoltre, sequenze duplex omopuriniche di DNA a cui si legano formando stabili triplex PNA-DNA-PNA (Nielsen et al., 1994).
Gli aegPNA risultano chimicamente stabili e non suscettibili di degradazione enzimatica da parte di nucleasi o proteasi, sono, di conseguenza molto biostabili sia nel siero umano che in estratti cellulari (Uhlmann et al., 1998). Inoltre sono achirali e privi di carica. L'achiralità fa sì che possano essere sintetizzati senza nessuna procedura stereoselettiva, la natura non carica, invece, determina un aumento di stabilità delle duplex PNA-DNA(RNA) rispetto alle homoduplex naturali grazie alla perdita delle repulsioni elettrostatiche.
I PNA sono molto interessanti dal punto di vista biologico per lo sviluppo di strategie terapeutiche. Infatti, essi possono fungere da agenti antisenso (Nielsen et al., 1993), per la loro capacità di inibire la traduzione riconoscendo mRNA complementari, da

agenti antigene (Nielsen et al., 1994), per la modulazione della trascrizione e replicazione, oltre che da inibitori di enzimi quali telomerasi, data la loro capacità di legarsi alla subunità oligonucleotidica delle ribonucleoproteine. L'azione antisenso dei PNA è dovuta al blocco sterico nel complesso PNA-RNA del meccanismo di traduzione. Per quanto riguarda l'azione antigenica essa dipende dalla formazione di triple eliche del tipo PNA-DNA-PNA e più raramente DNA-PNA-DNA che blocca il processo di trascrizione. Tali complessi creano un ingombro strutturale tale da bloccare la normale funzione dell'RNA polimerasi.

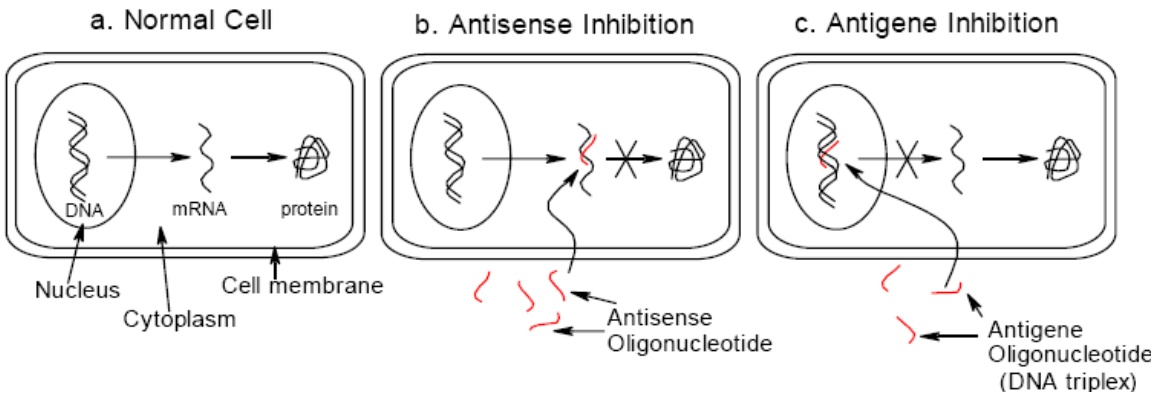
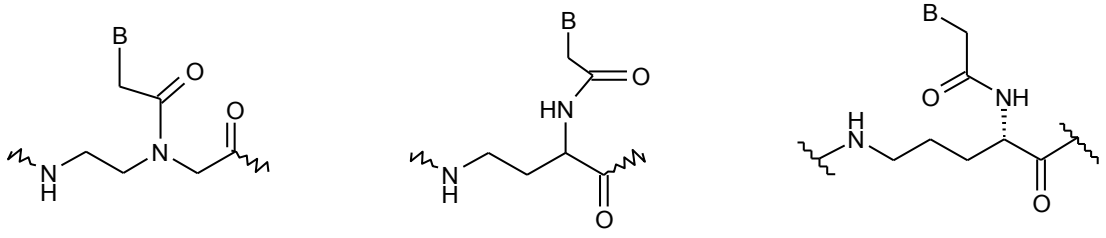


Figura 2: Illustrazione schematica di (a) Normale espressione genica, (b) inibizione antisenso, (c) inibizione antigenica.

Anche nel campo della diagnostica, i PNA hanno dato un notevole contributo.Oligomeri di PNA mostrano un'elevata specificità di legame con un DNA complementare (stringenza): ciò significa che gli accoppiamenti errati tra basi sono più destabilizzanti nel caso di duplex PNA/DNA (ma anche PNA/RNA) piuttosto che in duplex DNA/DNA. Grazie alla loro alta stringenza nel riconoscimento di target naturali, essi sono stati utilizzati con successo per rivelare la presenza di mutazioni geniche puntiformi, come nel caso del gene CFTR della fibrosi cistica (Carlsson et al., 1996), oggetto tuttora di studio di molti gruppi di ricerca internazionali.
Sin dall'introduzione degli aegPNA nel 1991, sono stati pubblicati molti studi su modifiche a PNA, effettuate allo scopo di ottenere oligomeri con caratteristiche e funzioni ottimizzate, quali la scarsa solubilità in soluzioni acquose, l'ambiguità di orientamento (parallelo/antiparallelo) nel legame al DNA, e lo scarso uptake cellulare (Uhlmann et al, 1998). Le modificazioni ai PNA più esplorate hanno riguardato lo scheletro amminoetilglicinico ed il linker metilencarbonilico (modifiche di 1^{a} generazione). Sicuramente una delle più importanti modifiche consiste nell'allungamento della struttura dell'aegPNA, mediante l'aggiunta di un gruppo metilenico al backbone poliammidico o al linker, come ad esempio nel caso dei PNA con unità ripetitive di N-(2-aminoetil)-β-alanina, o dei PNA in cui le nucleobasi sono legate tramite un ponte etilene-carbonilico. È stato riscontrato comunque che nelle modifiche di prima generazione l'unica sostituzione tollerata ha riguardato la posizione α della glicina.

Tra le modifiche di 2[a] generazione ai PNA, molti lavori sono stati orientati alla progettazione di molecole aventi un backbone ciclico (Gangamani et al., 1999 e Kumar et al., 2000). Questi tipi di modifiche, che implicano la connessione di differenti porzioni dello scheletro di PNA, introducono restrizioni conformazionali e/o stereocentri nel polimero risultante, conferendogli interessanti proprietà di ibridazione. In quest'ambito gli amminoprolil PNA (ampPNA) sono risultati i composti che hanno suscitato maggiore interesse. Oligomeri di aegPNA, contenenti una singola unità di amminoprolil PNA all'N-terminale, hanno mostrato una singolare capacità di discriminazione tra il legame antiparallelo rispetto a quello parallelo al DNA. Oligomeri formati interamente da monomeri di ampPNA invece, non hanno mostrato capacità d'ibridazione verso target nucleotidici naturali. Sorprendentemente, il polimero in cui i monomeri di aegPNA si alternano a quelli di ampPNA ha mostrato un'affinità di legame al DNA più alta rispetto all'oligomero formato interamente da aegPNA.
Un'altra interessante classe di analoghi di PNA è basata su uno scheletro diamminoacidico, in cui la nucleobase è legata attraverso un legame ammidico ad uno dei due ammino gruppi. In particolare, è stato recentemente dimostrato che l'analogo politiminico di PNA basato sulla D- o L-ornitina (3, Figura 3) lega RNA complementari formando strutture a tripla elica (Sforza et al., 2002).
Gli analoghi di PNA, finora presentati, vengono definiti nucleopeptidi. Con tale termine si indica una classe di molecole, di significativa importanza biomedica, contenti una struttura peptidica coniugata alle nucleobasi del DNA (o RNA) attraverso differenti linker (Roviello et al., 2010).
In questo contesto si inserisce la ricerca svolta dalla candidata durante il periodo di dottorato che ha previsto la sintesi, la caratterizzazione e gli studi di ibridazione di un nuovo analogo nucleopeptidico chirale, denominato dabPNA (2, Figura 3).

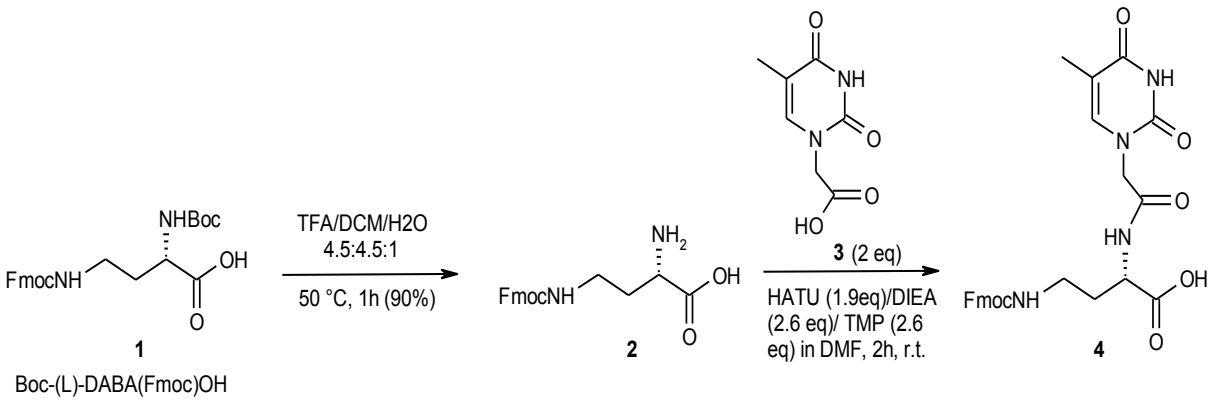


*aeg*PNA **1** | *dab*PNA **2** | *orn*PNA 3

Figura 3: Confronto tra aegPNA, dabPNA e ornPNA.

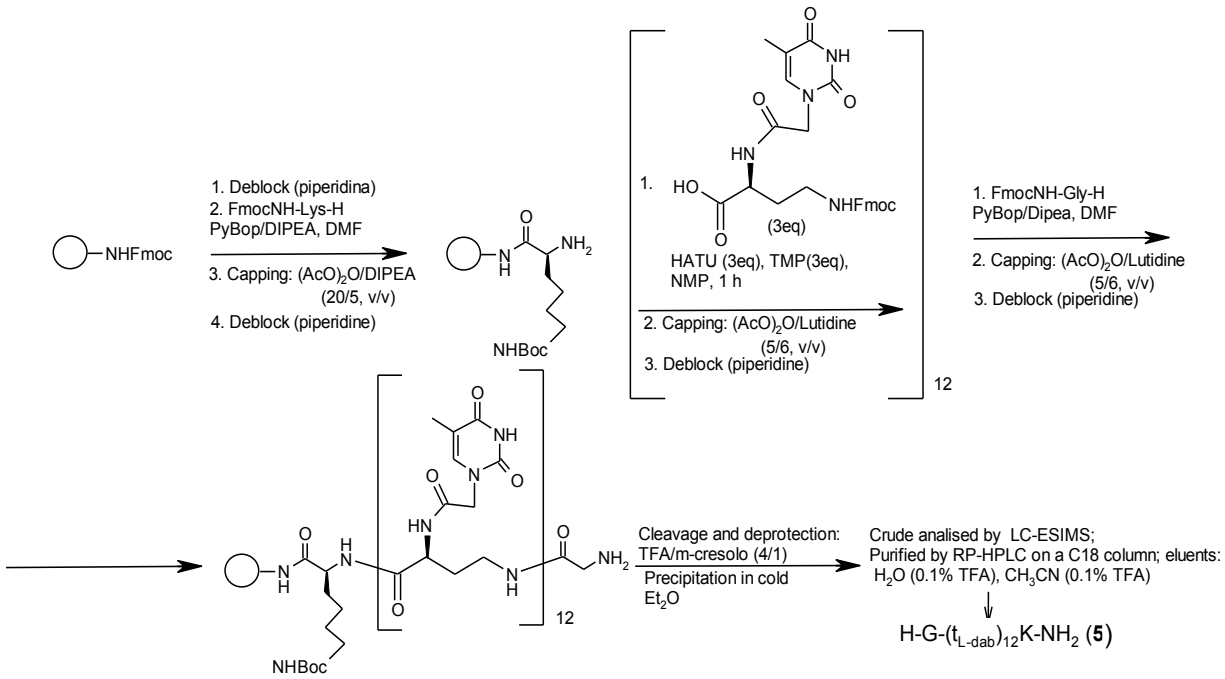
Il nuovo analogo possiede un'unità monomerica costituita dall' acido 2,4-diamminobutanoico (DABA) a cui è connesso la base eterociclica mediante un legame ammidico, mentre l'ammino gruppo in posizione γ è implicato nella polimerizzazione per dare lo scheletro poliammidico. Tale monomero, quindi, si può

considerare come l'analogo aciclico degli ampPNA, e si differenzia strutturalmente dagli aegPNA per la presenza di un atomo in meno nel backbone, che pertanto risulta più corto, e per una maggiore distanza tra la base azotata ed il backbone, dovuta alla presenza di un atomo in più.
La polimerizzazione di questo monomero porta ad una nucleopoliammide chirale con scheletro γ-peptidico (nucleo-γ-peptide), a differenza dello scheletro pseudopeptidico achirale degli aegPNA.
Sono vari i motivi che hanno spinto a basare il nuovo analogo sul DABA. Inanzitutto, l' acido 2,4-diamminobutanoico è un amminoacido non-proteico ritrovato in natura nei semi e nei tessuti vegetali di Lathyrus Silvestris L.: il DABA è distribuito per circa il 15% nei cloroplasti e per meno del 75% nei vacuoli intracellulari (Foster et al., 1987).
Un altro motivo che ci ha portato alla scelta del DABA è che l'acido poli(2,4-diaminobutanoico), PDBA, ottenuto mediante polimerizzazione del DABA, è stato recentemente utilizzato al posto della polilisina in un nuovo sistema di trasporto del gene dell'Interleuchina-12, permettendo di ottenere un'alta efficienza di trasfezione. In particolare, sono stati studiati in vivo gli effetti antitumorali dell'immunoterapia di una cellula dendritica, sfruttando il trasporto intratumorale, mediato dal PDBA, del gene dell'Interleuchina-12; da tali studi è stato evidenziato che questi sistemi sopprimono significativamente la crescita neoplastica (Iwashita et al., 2005).
Inoltre, il DABA insieme ad altri diamminoacidi ed alle nucleobasi, è stato rinvenuto nel suolo del meteorite caduto nel 1969 a Murchison (Meierhenrich et al., 2005) ed in virtù di questo nucleopeptidi basati su questi diamminoacidi sono stati proposti come costituenti del materiale genetico primordiale (Meierhenrich et al., 2004 e Nielsen, 2007). Da tale osservazione, si evince che la sintesi di polimeri basati su diamminoacidi, come il DABA, potrebbe contribuire allo studio del ruolo dei nucleopeptidi come materiale prebiotico in un "mondo a PNA" che, secondo recenti ipotesi scientifiche, potrebbe successivamente aver portato all'attuale mondo basato su DNA/RNA/proteine.
Altra osservazione che ci faceva propendere verso la scelta del DABA e che l'analogo basato su di esso è un nucleo-γ-peptide e da letteratura è nota la resistenza dei γ-peptidi alla degradazione enzimatica.
La prima fase della ricerca ha riguardato la sintesi dell'unità monomerica nucleoamminoacidica chirale $t_{L\text{-}dab}$ (4, Schema1) coniugando due bulding block : il diamminoacido Boc-L-DAB(Fmoc)-OH (1, Schema 1) e la timina carbossimetilata (3, Schema 1), entrambi presenti in commercio. In particolare la sintesi del $t_{L\text{-}dab}$ ha previsto la rimozione selettiva del gruppo Boc con acido trifluoroacetico a 50 °C dal composto ottenendo l'ammino gruppo in α libero. Il prodotto così ottenuto (2), caratterizzato mediante ^{1}H-NMR ed LC-ESIMS, è stato successivamente coniugato con l'acido timinil-1-acetico in differenti condizioni sintetiche. I migliori risultati sono stati ottenuti utilizzando HATU come attivante, DIPEA come base e DMF come solvente (resa 72%, Schema 1).



Schema 1: Sintesi dell' unità monometrica $t_{L\text{-}dab}$.

Il monomero $t_{L\text{-}dab}$, purificato mediante HPLC semipreparativa e caratterizzato mediante $^{1}H/^{13}C$ NMR ed LC-ESI-MS, è stato, successivamente oligomerizzato per ottenere il dodecamero politiminico $(t_{L\text{-}dab})_{12}$ (5, Schema 2). In particolare, la sintesi del nuovo nucleopeptide è avvenuta manualmente in fase solida mediante strategia Fmoc utilizzando HATU/TMP come sistema attivante in NMP/DMF (1:4, v/v), condizione che assicura la conservazione della chiralità durante ogni step sintetico (Corradini et al.,2001).



Schema 2: Sintesi dell' oligomero $(t_{L\text{-}dab})_{12}$

L'oligomero $(t_{L\text{-}dab})_{12}$ ottenuto è stato rimosso dal supporto solido mediante trattamento acido, precipitato in etere etilico freddo e recuperato dopo centrifugazione. In seguito, è stato purificato mediante RP-HPLC semipreparativa e successivamente caratterizzato mediante LC-ESIMS.
L'abilità del nuovo polimero sintetizzato nel legare acidi nucleici complementari è stata studiata al fine di esplorare il suo possibile utilizzo nelle strategie terapeutiche antisenso/antigene o in applicazioni diagnostiche.
Esperimenti di dicroismo circolare (CD), associati a curve di denaturazione termica UV, hanno mostrato che l'omopolimero $(t_{L\text{-}dab})_{12}$ non lega un dA_{12} complementare.
Inoltre, si è osservato che l'introduzione di un monomero di dabPNA nella parte centrale o all' N-terminale di un filamento t_{12} di aegPNA ha portato ad oligomeri caratterizzati da una minore affinità di legame verso il DNA complementare rispetto ad un filamento interamente costituito da aegPNA.
E' stata poi esplorata la capacità del nuovo omopolimero di ibridare l'RNA (A_{12}), ma anche in questo caso esperimenti CD hanno evidenziato che per il filamento $(t_{L\text{-}dab})_{12}$ non si ha formazione di una struttura duplex con il target complementare.
A questo punto è stato sintetizzato il monomero $t_{D\text{-}dab}$ che è stato oligomerizzato all'esamero $(t_D\text{-}dab)_6$ utilizzando le stesse procedure illustrate per ottenere $t_{L\text{-}dab.}$e l'oligomero corrispondente $(t_{L\text{-}dab})_{12}$. Anche in questo caso attraverso studi CD e UV non si evidenzia capacità del $(t_D\text{-}dab)_6$ di legame verso DNA e RNA. Poichè dabPNA basati su L- e D-DABA non legano acidi nucleici naturali, è stata esplorata la possibilità che nucleopeptidi complementari basati su D- o L-DABA si leghino fra loro. Questa capacità è di grande interesse per lo sviluppo di sistemi dendrimerici, come nuovi materiali, e anche per la realizzazione di nuovi strumenti diagnostici, come per esempio le sonde molecolari, in analogia a quanto è stato già riportato per gli alanilPNA (Diederichsen et al.,1996). In particolare, questi analoghi, che non legano acidi nucleici naturali, come verificato nel caso dei dabPNA, ma hanno la capacità di legare se stessi in filamenti complementari con chiralità alternata e per tale motivo sono stati proposti come costituenti della porzione in duplex degli stem presenti nei molecular beacon, lasciando l'oligonucleotide naturale nel loop della struttura a forcina (Figura 4).

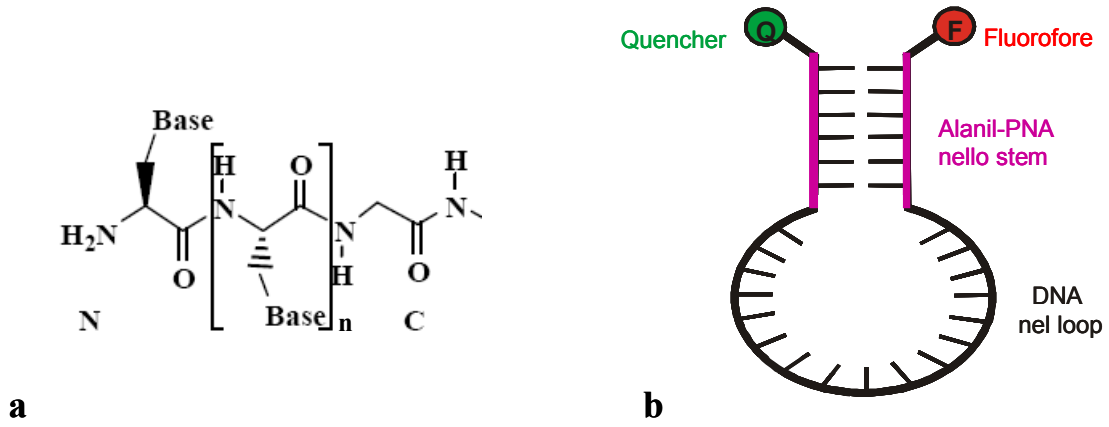


Figura 4: (a) filamento di L-alanilPNA; (b) molecular beacon con stem a base di alanilPNA.

Il vantaggio di utilizzare una molecola così strutturata risiede nel fatto che se la parte dello stem in doppia elica non riconoscesse target naturali, potrebbe aumentare la specificità della sonda nei confronti del target cellulare; in aggiunta la porzione dello stem conferirebbe una maggiore resistenza della sonda all'attacco delle nucleasi.
Per i nostri scopi sono state sintetizzate, utilizzando le stessa procedura sintetica illustrata nello Schema 1, i monomeri $a_{L\text{-}dab}$ e $a_{D\text{-}dab}$ dove ovviamente per entrambe come base azotata carbossimetilata si utilizza quella adeninica e rispettivamente L- e il D-DABA come diamminoacido.
Con i monomeri $t_{L\text{-}dab}$, $t_{D\text{-}dab,}$ $a_{L\text{-}dab}$ e $a_{D\text{-}dab}$ ottenuti si sono sintetizzati i seguenti sistemi oligomerici:

- H-G-$(a_{L\text{-}dab})_6$K-NH_2;
- H-G-$(a_{L\text{-}dab})_6$K-NH_2 ;
- H-G-$(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$K-$NH_2$;
- H-G-$(a_{L\text{-}dab}\text{-}a_{D\text{-}dab})_3$K-$NH_2$;
- H-G-$(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$K-$NH_2$;
- H-G-$(a_{L-dab}\text{-}t_{D-dab})_3$K-$NH_2$.

A questo punto sono stati effettuati studi di ibridazione tra i sistemi costituiti da:

- filamenti con stessa chiralità : H-G-$(a_{L\text{-}dab})_6$K-NH_2 / H-G-$(t_{L\text{-}dab})_6$K-NH_2;
- filamenti con opposta chiralità : H-G-$(a_{L\text{-}dab})_6$K-NH_2 / H-G-$(t_{D\text{-}dab})_6$K-NH_2;
- un filamento con chiralità alternata e un filamento omochirale: H-G-$(a_{L\text{-}dab})_6$K-NH_2/H-G-$(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$K-$NH_2$
- filamenti con chiralità alternata: H-G-$(a_{L\text{-}dab}\text{-}a_{D\text{-}dab})_3$K-$NH_2$/H-G-$(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$K-$NH_2$;
- filamenti di oligomero autocomplementare: H-G-$(a_{L-dab}\text{-}t_{D-dab})_3$K-$NH_2$.

Dagli esperimenti CD e UV, si ha evidenza di legame nel caso di oligomeri con chiralità alternata a_6D,L-dab/ t_6D,L-dab, anche se la stabilità del complesso formato non è molto elevata (T_m 11°C).
Inoltre, da studi preliminari di DLS relativi all'oligomero autocomplentare H-G-$(a_{L-dab}\text{-}t_{D-dab})_3$K-$NH_2$ emerge che vi è la formazione di un aggragato multimerico per questo sistema.
Questi risultati, insieme all'alta stabilità in siero osservata per gli oligomeri basati sul DABA, suggeriscono l'utilizzo di polimeri di dabPNA nello sviluppo di nuovi nanomateriali o di nuovi strumenti biotecnologici per applicazioni biomediche e della bioingegneria.

Summary

Aminoethylglycyl peptide nucleic acids (aegPNAs) emerged more than a decade ago as strong and specific DNA/RNA binding agents, triggering much research interest into the development of PNA based antisense/antigene therapeutics (Nielsen et al, 1993 and Nielsen et al., 1994) or diagnostics (Carlsson et al., 1996) thanks to their remarkable properties.
Many efforts have mainly been directed towards the refinement of aegPNA properties such as binding affinity to DNA/RNA, water solubility, discrimination between parallel and antiparallel binding modes and cellular uptake (Uhlmann et al, 1998). Different PNA modifications are widely described in the scientific literature, having met in some cases with success.
The most explored PNA modifications regarded the aminoethylglycine backbone and the methylene carbonyl linker (1^{st} generation modifications). In the 1^{st} generation modifications the only tolerated substitution regards the α-position of the glycine. For example, substitution of the glycine with an alanine (D or L) resulted in a slightly lower binding affinity towards DNA. Introduction of a D-lysine resulted in a slight increase of T_m in the binding to DNA, while the L-isomer had the opposite effect.
In addition, much work has been devoted to investigate various cyclic backbone-based PNAs (Gangamani et al., 1999 e Kumar et al., 2000) (2^{nd} generation modifications) with the goal of increasing the selectivity or strength of interaction with natural nucleic acids. This class of modifications, by connecting different parts of the PNA backbone, introduces conformational restrictions and stereocenters into the polymer which can present remarkable hybridization properties. Among these kind of modifications aminoprolyl PNA containing oligomers are very interesting. aegPNAs with a single aminoprolyl PNA unit at N-terminus showed discrimination of antiparallel vs parallel binding to DNA. Another class of interesting PNA analogues is based on a diaminoacid backbone carrying the nucleobase by means of an amidic bond to one amino group. In particular, it was recently reported that an oligo thymine PNA analogue based on D- or L-ornithine binds to RNA by forming triple helices (Sforza et al., 2002).
In this context we report our investigation about novel chiral PNA analogues, focusing the attention not only to the synthetic strategies but also to the binding studies towards natural and artificial targets.
The new analogue designed and realized is a chiral nucleo-γ-peptide, dabPNA, isomer of the aegPNA unit and characterized by a 2,4-diaminobutyric acid (DABA)-based backbone carrying the carboxymethylated nucleobase on its α amino group.
Differently from aegPNA monomers, dabPNA presents a shorter backbone (3C between the nitrogen atom and the carbonyl) and an enhanced distance between the nucleobase and the backbone, i.e. 3 atoms (1N+2C) instead of 2C in aegPNAs. Furthermore, in comparison with ornPNA that binds to RNA forming a stable triplex, daba-based PNA has a methylene group less in the backbone.
To achieve dabPNA oligomers, the new monomers $t_{L\text{-}dab}$, $t_{D\text{-}dab}$, $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ were synthesized in good yield using suitable protected building blocks and characterized by NMR and ESI-MS techniques.
Initially, the homothymine oligomers $(t_{L\text{-}dab})_{12}$ and $(t_{D\text{-}}dab)_6$ were synthesized, using a synthetic strategy that ensured the maintenance of chirality during the coupling steps, and tested for hybridization towards natural nucleic acids. No binding evidence with both DNA (dA_{12}) and RNA (A_{12}) was revealed by CD and UV experiments. Furthermore, the insertion of a single $t_{L\text{-}dab}$ unit in the middle or at N-terminus of a

homothymine aegPNA chain leads to a decreased binding efficiency to the target DNA in comparison to full aegPNA.
Since dabPNAs based on L- and D-DABA don't bind natural nucleic acids, we explored the possibility that complementary nucleopeptides based on D or L-DABA could bind between themselves. This property would be interesting in order to develop novel DABA-based dendrimeric systems, as new materials, and also to realize new diagnostic tools, as for example new molecular beacon probe. To verify this interesting possibility, the monomers $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ were oligomerized to the homoadenine hexamers $(a_{L\text{-}dab})_6$ and $(a_{D,L\text{-}dab})_6$. In order to find the correct combination of chirality suitable for obtaining the binding, also the $(t_{D,L\text{-}dab})_6$ oligomer were realized to perform the hybridization studies.
From CD and UV experiments, binding evidence was revealed in the case of the complementary oligomers with alternate chirality, $(a_{D,L\text{-}dab})_6$ and $(t_{D,L\text{-}dab})_6$, even if the stability of the complex formed was not so high (Tm 11°C).
Furthermore, some interesting properties relative to the self-complementary oligomer $(a_{L-dab}\text{-}t_{D-dab})_3$ emerged from preliminary DLS experiments that evidenced the formation of multimeric aggregates for this system.
These results, together with the high serum stability of the DABA-based oligomers, suggest further studies on dabPNAs as new self-recognizing bio-inspired polymers, with the potentiality to develop new nanomaterials or new biotechnological tools in bioengineering and biomedical applications.

1. Introduction

Oligodeoxyribonucleotide (ODN)-like molecules have been widely investigated as therapeutic or diagnostic tools for their ability to bind natural nucleic acids with complementary base sequence, but recently also for the possibility to develop new selfassembling materials based on hydrogen-bond pairing to be used in bioengineering (nanosensor devices) and biomedical (hydrogels for drug delivery) fields.

1.1 Oligonucleotides (ODN) for biotechnological applications

The recognition of nitrogenous bases of nucleic acids is of fundamental importance in biological systems because it ensures the conservation of genetic information and its transfer in living systems. The hybridization in nucleic acids is based on hydrogen bonding of Watson-Crick type between the complementary nucleobases: A-T (A-U) and G-C. Based on this simple system of pairing, several research groups have developed systems based on DNA or similar in view of new therapeutic applications (antisense/antigen) and diagnostics, or for the development of new materials.
An oligonucleotide is a fragment of DNA or RNA with a number of less than 100 nucleotide residues. The oligodeoxynucleotides (ODN) can selectively recognize the genetic material of the cell (for example, whether genes or mRNA) or specific proteins such as DNA binding protein (DBP), interfering with their normal functioning and in some cases blocking it. Therefore it can be easily understood the importance of strategies based on the use of these molecules in pharmacological approaches, such as when the gene is modulated or blocked viral DNA, an oncogene, or another gene involved in major diseases. An ODN is able to take early in a selective manner compared to traditional drugs that target proteins (Gewirtz et al., 1998) generally. The approach used is that antisense dell'ODN where the target is a messenger RNA (mRNA, Figure 1b). The theory of antisense oligonucleotides requires relatively small molecules, ie, synthetic ODN lengths ranging from 13 to 30 nucleotides, possess the specificity required, due to their mechanism of action based on the formation of a double helix structure by hydrogen bonding Watson-Crick with a stroke of mRNA chosen as a target. The formation of a duplex between the ODN el'mRNA causes the deactivation of the mRNA target and a block of translation in order to obtain the corresponding repression of protein synthesis.

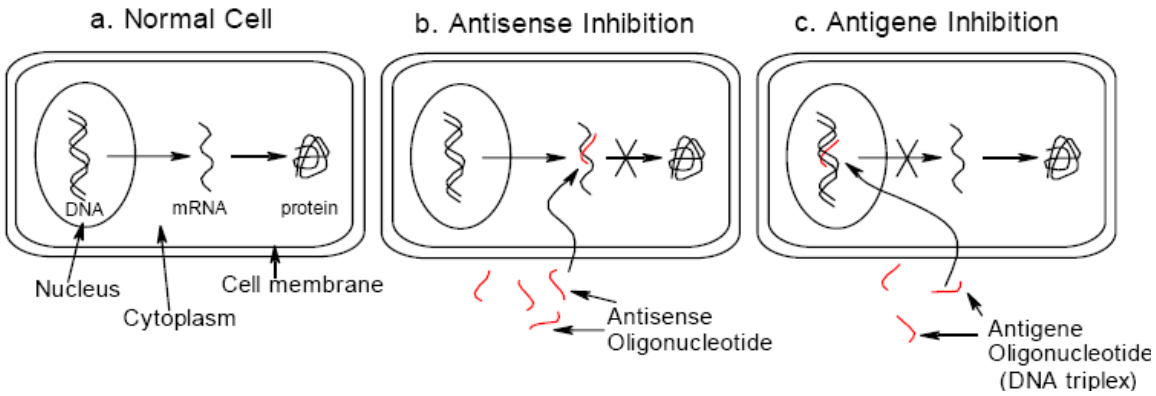


Figure 1 Schematic illustration of (a) Normal gene expression, (b) antisense inhibition, (c) Inhibition of antigen.

Following the formation of the hybrid RNA may lose its function in two distinct ways. The first type of event is the hydrolysis of the RNA target itself, after its hybridization antisense all'oligonucleotide by RNase H, which is an enzyme that degrades the RNA strand of an RNA-DNA hybrid. In the second case it is possible that the formation of the hybrid would result in a steric hindrance preventing the binding of proteins important for RNA processing (splicing) and interfering with the assembly of the ribosomal unit or the sliding of the ribosome along the messenger, or again with the nucleus-cytoplasm transport of the messenger. Besides antisense approach was developed further promising therapeutic strategies based on synthetic ODN, including the strategy is important to recall antigen, the aptamers (Wu CC et al., 2003), ribozymes (Qureshi et al., 2006) and RNA interference (Hannon, 2002).

The strategy is designed to inactivate an antigen gene (Figure 1c). In this case, the ODN is designed to pair up in a stretch of complementary dsDNA genome, forming a triple-helical stretch. Dell' ODN action, which occurs only in nuclear, achieves the same result as the antisense strategy, but with lower doses of medication and increased biological activity, as in the cell there are only two copies of the gene compared the hundreds or thousands of copies of messenger or protein.

The aptamers are an emerging class of molecules with promising diagnostic and therapeutic applications. They are oligomers of DNA or RNA capable of binding to certain proteins with high affinity and specificity based on their structural characteristics. It is in fact the structure of the oligo that allows the selective recognition of the target protein.

The natural ribozymes are RNA molecules capable of hydrolyzing specific sequences recognized mRNA. Unlike other molecules “tails blockers" with stoichiometric mechanism of action, have the advantage of being molecules with catalytic activity, in fact, a ribozyme molecule can hydrolyze more than one mRNA. By inserting this construct into an expression vector, transformed cells can be induced to synthesize the ribozyme that hydrolyze much faster then the target mRNA.

RNA interference is a form of post-transcriptional gene regulation mediated by a double-stranded RNA (*post* trascripional gene silencing or PTGS). In practice, a double-stranded RNA signals the degradation of complementary mRNA, and then encoded by the gene is silenced quell'mRNA. The dsRNA is recognized within the cell by RISC (RNA-induced silencing complex), a multimeric complex with endonuclease activity, which separates the two strands of this molecule, select the strand complementary mRNA targets and guide the interaction with the target will be degraded, in the case of perfect complementarity between the ssRNA and the messenger, or, if not complete complementarity, it will create a steric hindrance that prevents access of the ribosomal machinery for translation. Given their relative ease of synthesis and high sensitivity and specificity of the ODN play an important role in the field of molecular diagnostics *in vitro* (Demidov et al., 2003).

The diagnostic applications of ODN include the most commonly used DNA chip (DNA microarray), PCR-based techniques, methods of *in situ* hybridization (FISH) and detection of single nucleotide polymorphisms (SNPs), the molecular beacon, and much more. In particular, the molecular beacon probe is based on a single-stranded oligonucleotide that forms a “hairpin structure” (stem and loop). The loop contains a sequence complementary to the sequence of the target and the portion of the stem consists of two complementary treatments that flank the loop. A fluorophore and quencher are linked covalently to the end of the probe: the molecular beacon probe gives no fluorescence if it is in the form stem-and-loop, with a fluorophore and quencher spatially close, as it becomes fluorescent when it hybridizes to the target

sequence (Figure 2). Obviously the length of the loop and the stem must be such that the formation of a more stable probe-target compared to the one formed by two sections of the stem. The molecular beacons are extremely specific, it can discriminate between target sequences that differ from each other for even a single nucleotide (Tan et al., 2000).

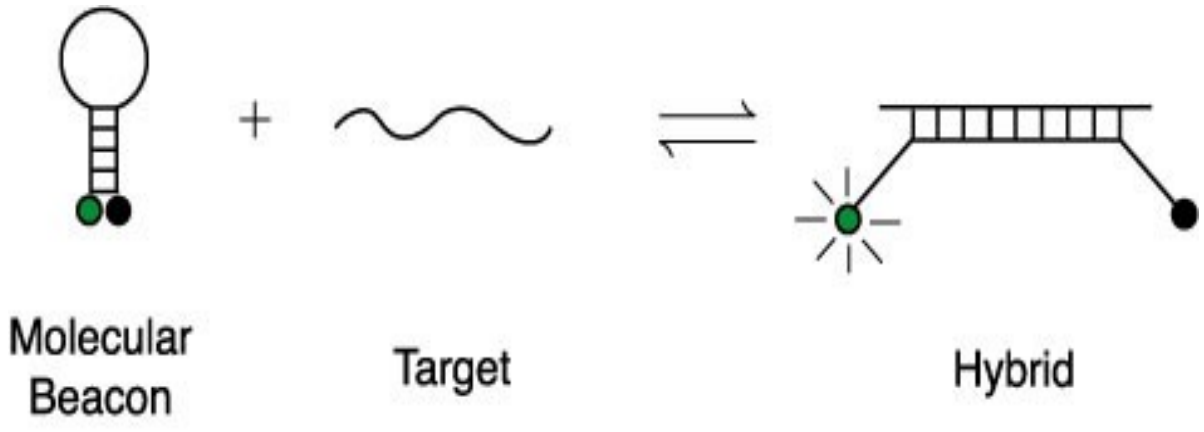


Figure 2: Mechanism of action of a molecular beacon probe.

Taking advantage of the four bases of DNA recognition in many chemists and physicists have turned their attention to building systems based on dendrimeric ODN to develop new nanomaterials (Seeman et al., 2003). In this way opens the chapter of biotechnology at the nanoscale, which combines biological molecules with chemical and physical principles and procedures to create nanoscale structures with specific properties and functions. The DNA molecule exhibits chemical, structural and electronic properties that make it suitable for the formation of nanomaterials, which find applications in many research fields ranging from molecular biology to chemistry, including materials science and physics course, to engineering mechanics and electronics. The microscopic size of the DNA molecule with a diameter of about 2 nanometers, its quick pace propeller 3.4-3.6 nm and its ability to self-assemble to form large three-dimensional structures, size, shape and density well-defined are essential for the construction of nanoscale devices. For example, the conjugation of gold nanoparticles with DNA allowed the construction of self-assembling nanowires, which are characterized in terms of structural and electronic products, which were measured the size and resistivity (Figure 3) (Mbindyo et al., 2001).There are two main approaches currently pursued in nanobiotechnology: one is a “bottom-up” approach where materials and devices are made starting from the molecular components that self-assemble through chemical bonds, using principles of molecular recognition and the other is the approach of “top-down", where nanoscale devices are made from materials from which macroscopic ″cut″ the nanostructures through a careful process control of miniaturization precisely at the atomic level.

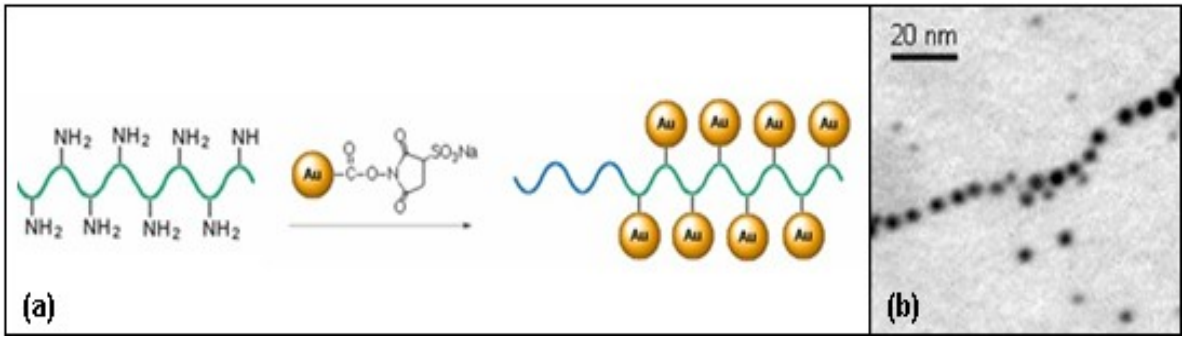


Figure 3: (a) Conjugation of ODN modified with Nanogold ®, (b) TEM image of a DNA duplex conjugated to gold nanocrystals.

1.1.a Limits on the use of natural ODN

The use of synthetic ODN for the regulation of gene expression, although it seems a valid and rational, in fact, presents practical difficulties, due to the difficulty of penetration of cell membranes and the short half-life of these molecules (Calogero, 1997).The cellular uptake problem stems from the fact that an oligonucleotide is by nature a polyanion, so do not have the ability to diffuse across cell membranes, consisting essentially of lipid structures.Today this problem seems less important, as the oligonucleotides can enter cells using active absorption mechanisms, mediated by receptors and channels on the cell surface. They can also be encapsulated within lipid particles called liposomes (cationic lipids) to improve the lipophilicity (Meyer et al., 1998), or can be conjugated to suitable carriers, exploiting the natural cellular uptake. In human serum, as well as in the cytoplasm of various cell systems, the nuclease responsible for rapid degradation of DNA and RNA, reducing the half-life in minutes. Consider also that only a process of hydrolysis could inactivate the oligo or even make it toxic.

1.1.b Oligodeoxyribonucleotide (ODN)-like molecules.

To overcome the limitations just mentioned, over the years, many researches have been focused on the introduction of chemical modifications in the structure of the ODN, while preserving the ability of molecular recognition (Chen et al., 2005).
In general, the features should have a similar synthetic oligonucleotides are: resistance to nucleases, good cellular uptake, high affinity for nucleic acid target, high binding specificity and low toxicity.
Clearly, any deviation from the original structure implies the often unexpected consequences, leading to alterations in the ability to recognize the complementary strand in duplex or triplex stability of the complexes formed in recognition of certain enzymes or proteins.
The possible sites of modification in an oligonucleotide can involve the sugar-phosphate backbone and / or nucleobases (Figure 4).

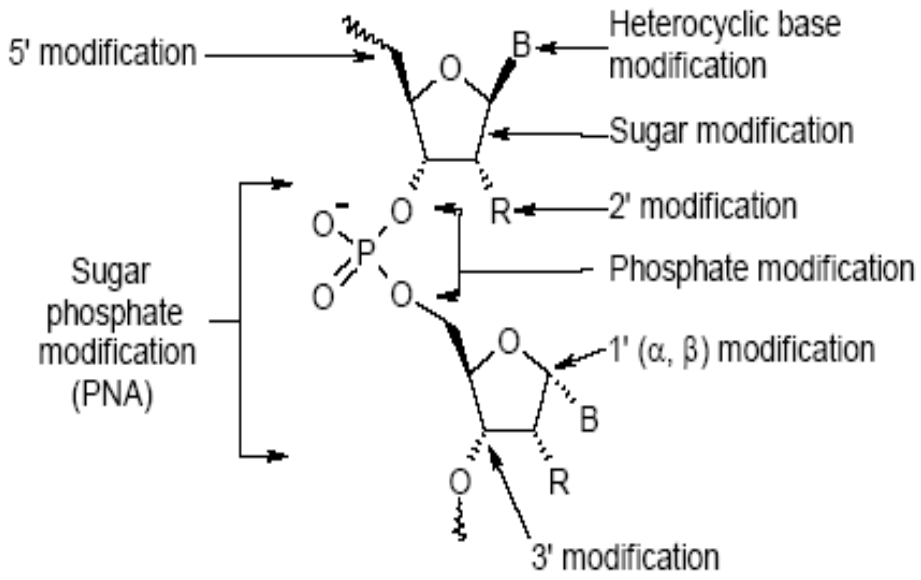


Figure 4: Possible sites of modification of DNA.

Amendments to the ODN can be grouped into three classes:
1) modifications of the first generation, which include analogues with alterations at the level of the phosphate;
2) changes in the second generation which have similar changes in blood sugar (mainly in position 2 ' of ribose) or the heterocyclic bases;
3) changes in third generation, which include analogues in which the changes relate to the ribose or the phosphodiester bond and also include molecules with a completely different place furanosic ring of sugar.
The first generation of changes designed to make the most internucleotide linkages resistant to nucleases, the backbone phosphodiester concern and led to the synthesis of a wide variety of analogues such as phosphorothioates (Agrawal et al., 1998) (a) and phosphorodithioate (b), methylphosphonates (Miller et al., 1991) (c) and hydroxymethylphosphonium (d), the phosphodiester (s) and phosphoramidates (Agrawal et al., 1998) (f) (Figure 5).

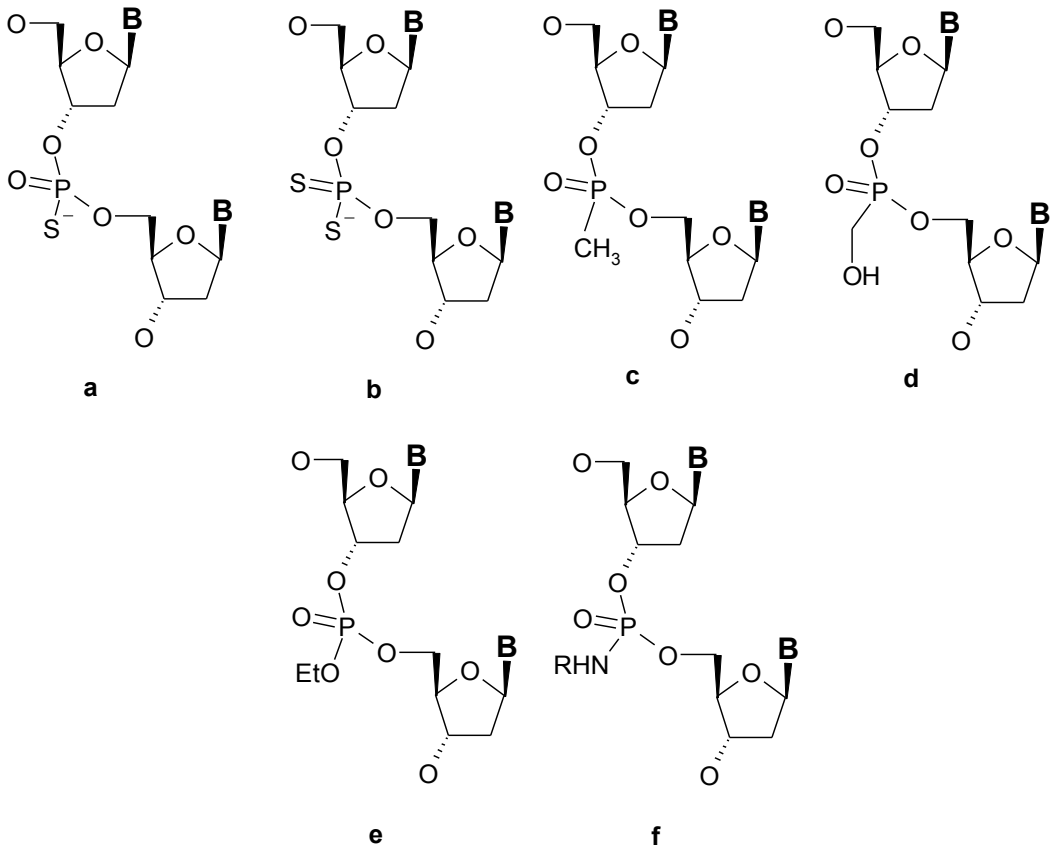


Figure 5: First-generation antisense oligonucleotides: (a) phosphorothioate, (b) phosphorodithioate, (c) methylphosphonates, (d) hydroxymethylphosphonium (e) phosphodiester, (f) phosphoramidates.

The phosphorothioate ODN, in which one of the oxygen does not bridge the phosphodiester bond is replaced by a sulfur atom, similar results are very promising as part of the antisense.
The Vitravene ®, a phosphorothioate used in local treatment of cytomegalovirus retinitis (CMV) in patients with acquired immunodeficiency syndrome (AIDS) was the first drug based on antisense oligonucleotide approved by the FDA (Anderson et al., 1998).
The 2'-O-alchilribonucleotidi (Majlessi et al., 1998) are similar to the second generation, derived from the RNA, in which the hydroxyl group 2 ' is etherified with an alkyl-group. Among the most representative are the 2'-O-methoxy-ethyl ribonucleotides (Figure 6a) and 2'-O-methyl (Figure 6b).

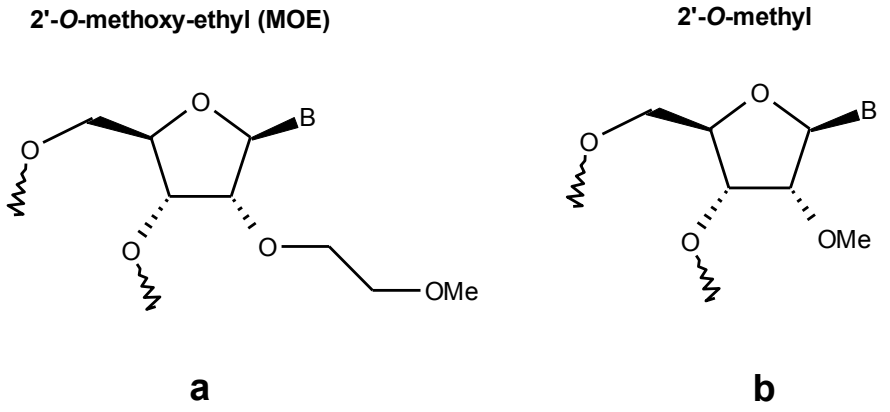


Figure 6: Second-generation antisense oligonucleotides.

These derivatives are comparable to the DNA from the viewpoint of chemical stability, are resistant to nucleases and, in particular, the resistance increases, as well as the lipophilicity, with increasing alkyl chain length. Given these positive features, the only disadvantage is that the 2'-O-methyl-RNA are not able to induce the enzymatic cleavage of mRNA target by RNase H.
Among the analogues of the third generation Morpholino phosphoramidates (MP, Figure 7a) (Corey et al., 2001), peptide nucleic acids (PNA, Figure 7b) (Nielsen et al., 1991) and locked nucleic acids (LNA, Figure 7c) (Wahlestedt et al., 2000) are those which have aroused the most interest.

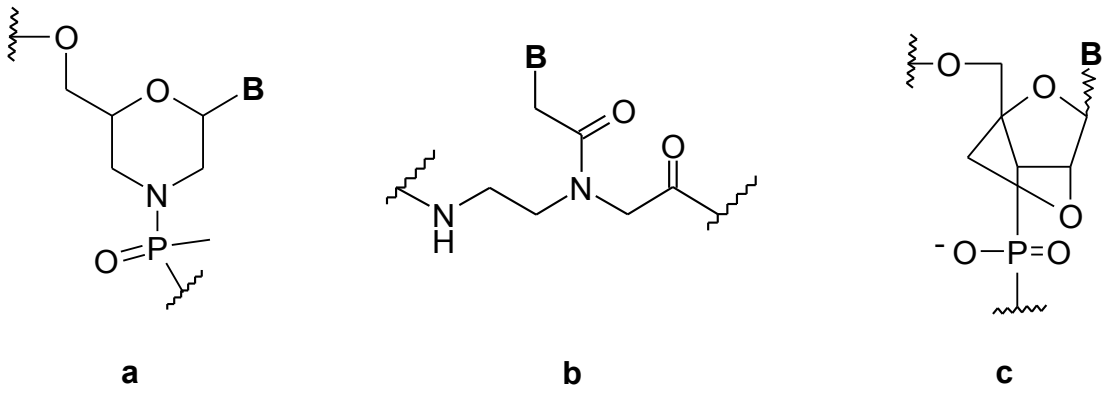


Figure 7: Similar ODN third generation: (a) MP, (b) PNA, (c) LNA.

These compounds are essentially resistant to nucleases and keep a good affinity for their complementary target.
In particular it has been demonstrated *in vivo* the potent knock-down of similar MPs, acting recognizing sequences within the target mRNA, resulting in a steric hindrance that prevents interaction with other molecular partners. Precisely for this reason many therapeutic compounds have been developed, one of which is directed against the oncogene *c-myc*, which is currently in phase I and phase II clinical trial, produced by AVI BioPharma (Portland, OR, USA).

The LNAs are a class of sugar analogues in which the ring is conformationally "locked" by a methylene bridge connecting carbon atoms 2' and 4'. The bridge locks the ribose conformation in the C3' endo (often found in the A form of DNA and RNA), this structural organization appears to be an important factor to ensure high affinity binding of these analogs with the natural nucleic acids, a thermal stability and high resistance to nucleases.
It was shown that these analogues inhibit tumor growth in a xenograft model, using an effective concentration of LNA is five times lower than that found for the corresponding phosphorothioate analogue.
Among the many similar oligonucleotide in the literature, peptide-nucleic acids (PNA), described for the first time in Nielsen et al. in 1991 have certainly enjoyed the most successful offering in both therapeutic and diagnostic prospects encouraging continuous development.

1.2 Peptide nucleic acids (PNA)

The PNAs are structural mimics of DNA, in which the sugar phosphate backbone is replaced by an entirely neutral and achiral polyamide backbone consisting of repeating units of N-(2-aminoethyl)glycine (aeg), joined by amide bond.
Each of the four nucleobases, adenine, cytosine, guanine and thymine, is linked to the backbone through a methylene carbonyl linker. The skeleton pseudopeptide is designed so that the bases keep the distance between them that in natural DNA and are able to recognize the complementary bases, obeying the Watson-Crick bonding pattern (Uhlmann et al., 1998) (Figure 8).

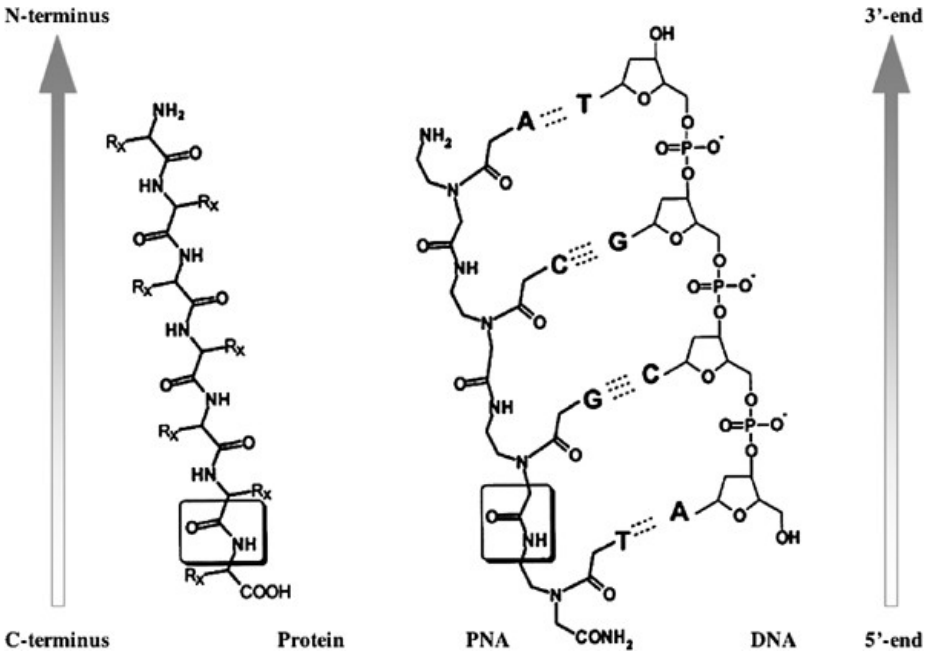


Figure 8: Comparison between the structure of a protein, and a duplex PNA / DNA.

Being achiral, the PNA binds to natural DNA and RNA in parallel or antiparallel (Figure 9), depending on whether the amino-terminal, respectively, PNA binds the 5' end or the 3' end of DNA between the two antiparallel orientation is slightly preferred to the parallel, and its kinetics of formation is much faster than that of the complex parallel.

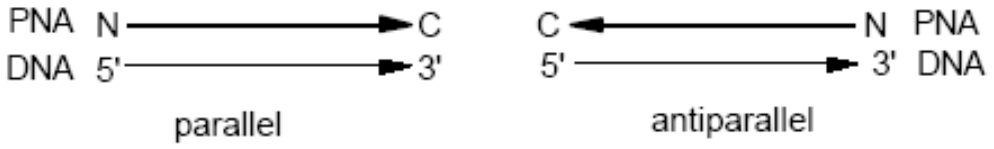


Figure 9: Schematic representation of orientation that may have a duplex PNA: DNA.

From numerous studies on these molecules is immediately revealed that the PNA specifically recognize complementary strands of DNA and RNA, and in particular, the PNA-DNA hybrids and PNA-RNA are more stable, respectively, for DNA-DNA and DNA RNA (due to less electrostatic repulsion), and also PNA-RNA duplex is more stable than the corresponding PNA-DNA duplex (Egholm et al., 1993).
PNAs with complementary sequences, also can hybridize with each other through the training Watson-Crick (W-C) hydrogen bonds highly specific, creating parallel or antiparallel duplex (preferred orientation), extremely stable (Eriksson et al., 1996).
The PNA is able to adopt both conformations A and B when they bind, respectively, RNA and DNA, while the PNA-PNA duplex are structured in a particular conformation, called P-form, characterized by a helix tour of 18 base pairs larger than the forms A and B.

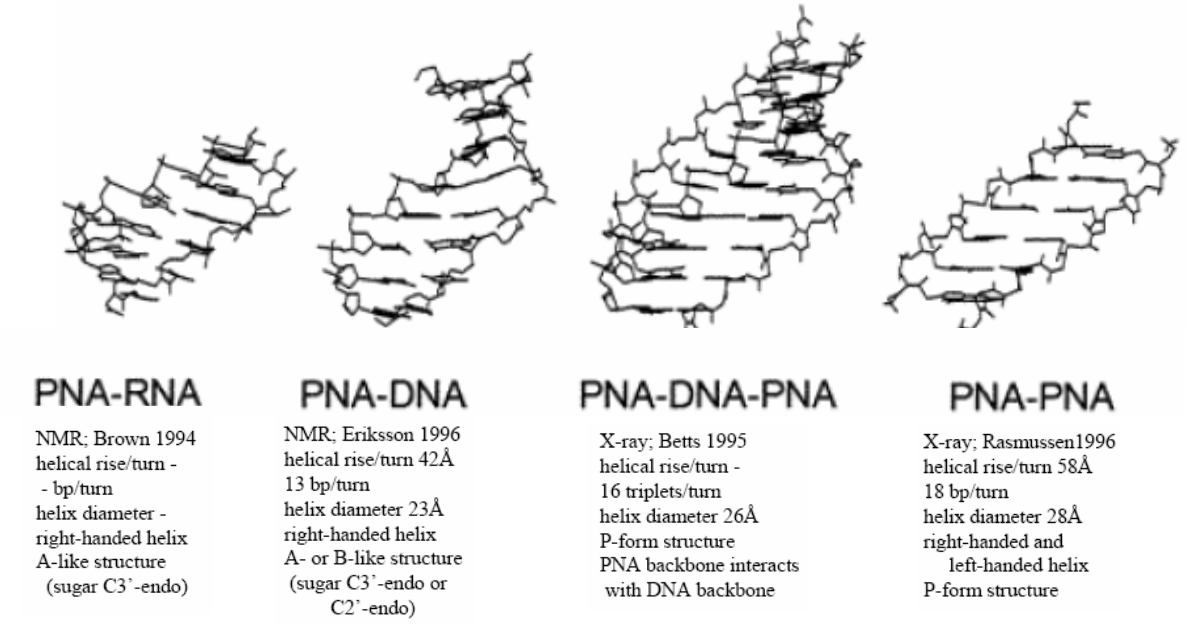


Figure 10: The conformation of the PNA-nucleic acid complex.

The PNAs are to be chemically very stable: in fact, unlike DNA, which undergoes depurination when treated with strong acids, are completely stable. The only chemical instability of the PNA is due to the amino group to N-terminus. In particular, in basic conditions can be a slow transfer to the nucleobase acetyl-N-terminal amino group of the backbone (Figure 11a) or the loss of monomer through the N-terminal cyclization (Figure 11b). These secondary reactions can be avoided by protecting the amino group to the N terminal, for example, with a group acetile (Uhlmann et al., 1998).

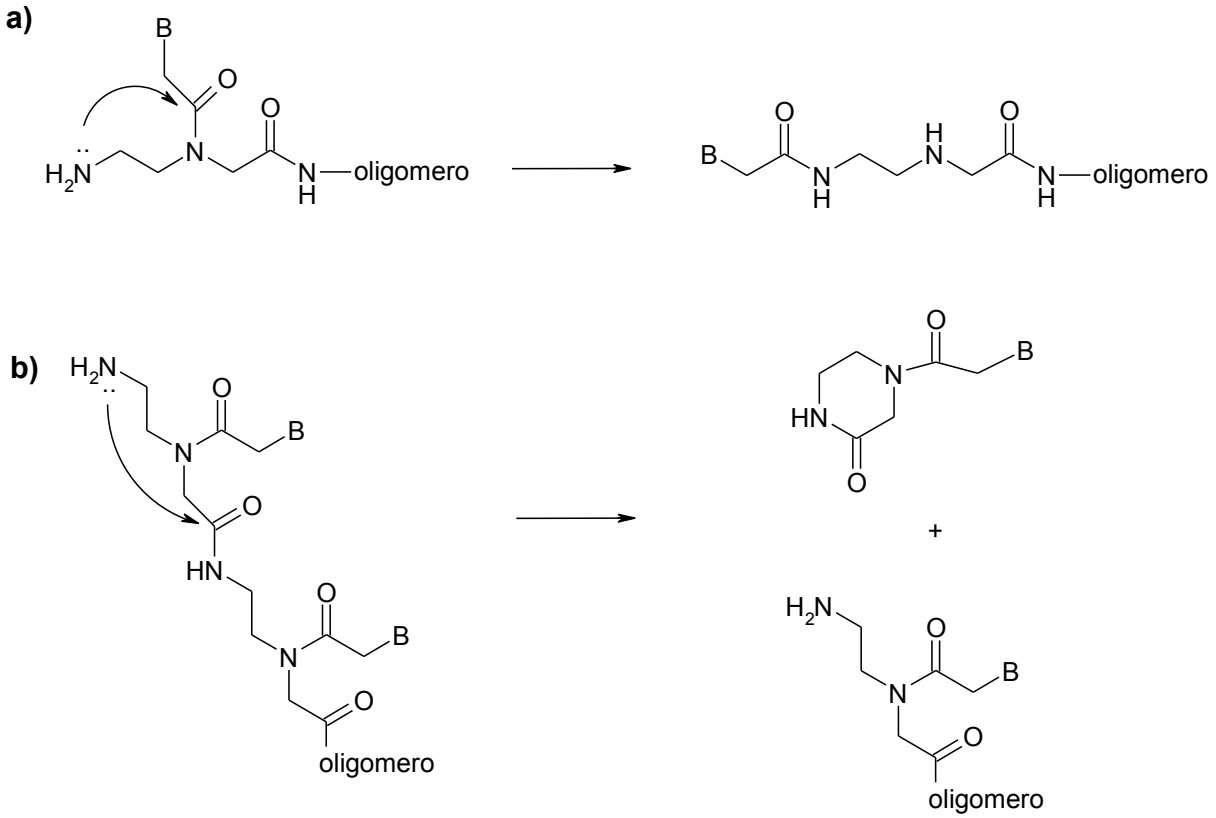


Figure 11: (a) N-acyl transfer reaction, (b) loss of the last residue N-terminal.

The PNA are not susceptible to enzymatic degradation by nucleases and proteases. Are therefore very biostable both in human serum and in extracts cellulari (Uhlmann et al., 1998).
The use of PNA has led to the development of numerous therapeutic applications. These polymers, in recognition of natural nucleic acids, can interfere in the processes of replication, transcription and translation device (Egholm et al., 1993).
A PNA can be designed to recognize and bind complementary sequences of a double helix of DNA, thus interfering with its replication and transcription (strategy antigen) (Eriksson et al. ,1996). The PNA is able to bind dsDNA, forming different types of complexes, as shown in Figure 12. The way in which the NAP interacts with target DNA double-strand depends on the base composition of the sequence involved. In general, the NAP applies a strand invasion mechanism, which involves the displacement of a DNA strand in the duplex. Where in the sequence of the target

dsDNA is present homopurinic. Suddenly, the PNA will bind to form two main types of triplex structures $(DNA)_2$-PNA (c) e $(PNA)_2$-DNA (d). In the latter form a PNA strand of Watson-Crick interactions and the other interactions with the Hoogsteen strand of complementary DNA (Buchardt et al., 1993).

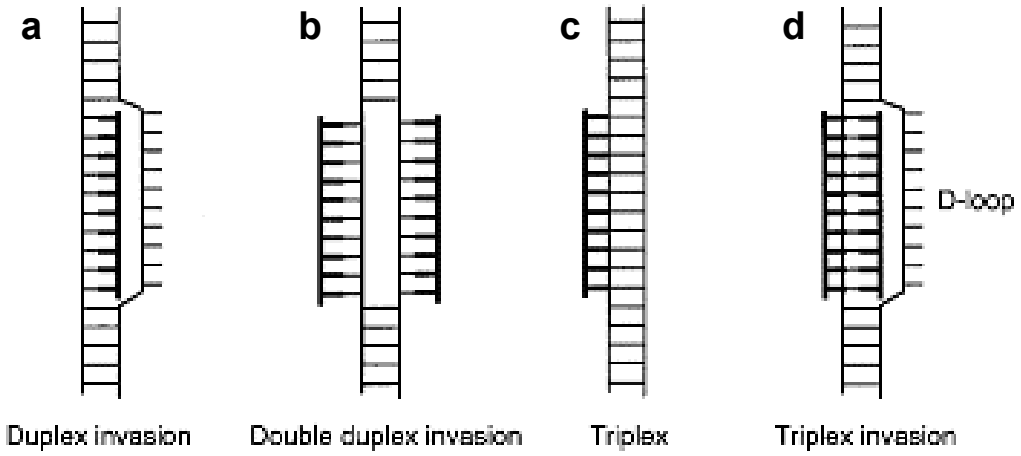


Figure 12: Representation of the mode of binding of a PNA and dsDNA. The PNA oligomers are shown in bold (**a**) Standard duplex invasion. (**b**) Double duplex invasion complex, very stable, but only possible with PNA containing modified nucleobases. (**c**) conventional triplex structure. (**d**) Triplex invasion by displacement of one strand of dsDNA.

A PNA that targets the promoter region of a gene can form a stable PNA-DNA complex that does not provide access to its DNA polymerase. *In vitro* studies have shown that these complexes are capable of influencing the process of transcription in both prokaryotes and eukaryotes.
Alternatively, a PNA can be designed to recognize and bind complementary sequences in mRNA (antisense strategy) (Nielsen et al., 1994).
Generally, the effect of PNA as antisense agents is based inhibition of process of maturation, transport and translation of RNA in the cytoplasm by forming complexes that cause steric hindrance, preventing interactions with other molecules that regulate these processes.
Even the PNA in diagnostics have made a significant contribution. PNA oligomers show high binding specificity with a complementary DNA (stringency), which means that the wrong pairing between bases are more destabilizing in the case of duplex PNA / DNA (as well as PNA / RNA) than in duplex DNA / DNA .
Due to their high stringency in the recognition of natural target, they have been used successfully to detect the presence of gene mutations point, as in the case of the CFTR gene for cystic fibrosis (Nielsen et al., 1994) are still subject of study of many international research groups.
In addition, aegPNAs have recently been proposed as hypothetical precursors of prebiotic systems based on RNA, which, as is now widely accepted, preceded the current world "DNA- RNA- proteins" (Nielsen et al., 1993 and Carlsson et al.,1996).

The implication of genetic aegPNA prebiotic evolution is supported by the following results:
1) aegPNA bind complementary RNA to form stable complexes;
2) can act as a template for the synthesis of RNA (transfer information from aegPNA to RNA has been demonstrated experimentally);
3) the constituents of aegPNA, in particular N-(2-aminoethyl)glycine and carboxymethylated nucleobases, can be obtained under prebiotic experiments. However, the synthesis of the whole molecule of PNA in simulated prebiotic conditions has not yet been implemented, so that we can not draw definitive conclusions about the importance of aegPNA nell'abiogenesis.

1.2.a Synthesis of PNA

The synthesis of PNA can be performed in analogy to peptides (Merrifield 1986), with solid phase strategies making use of a solid support, e.g. a (methylbenzhydryl) amine polystyrene resin (Christensen et al. 1995; Pothukanuri et al. 2008; Lee et al. 2007), and of Fmoc(9-fluorenylmethoxycarbonyl group)-protected PNA monomers in which the exocyclic amino groups of A, G and C bear Bhoc(benzyhydryloxycarbonyl) protecting groups which are removed at the end of the synthesis by treatment with trifluoroacetic acid. The primary amino group in the monomer backbone can be deprotected from the Fmoc-group by treatment with 20% piperidine in DMF (dimethylformamide) at the end of each coupling step. After the synthesis is accomplished, the PNA can be cleaved from the resin by treatment with trifluoroacetic acid and m-cresol (4/1 v/v) and precipitated by cold diethyl ether. Subsequently, HPLC purification can be performed and pure PNA oligomers are characterized by mass spectrometry (Christensen et al. 1995). Taking into account the importance of the orthogonality of PNA synthesis with other chemistries, Pothukanuri et al. performed a thorough investigation of six types of protecting groups for the terminal nitrogen atom and five protecting groups for the nucleobases of PNA monomers (Pothukanuri et al. 2008). Furthermore, the synthesis of self-activated PNA monomers as well as an efficient route to PNAs using a benzothiazole-2-sulfonyl group as an amine-protecting group and an acid-activating group were also reported by Lee et al.(2007). Since the synthesis of homothymine PNA segments, required for the construction of PNA-based triplex structures, is plagued by the occurrence of a significant amount of truncation products, Altenbrunn and Seitz investigated the use of novel allyl-protected thymine PNA monomers which provided significant improvements to the yields with the standard protocols used in the automated PNA synthesis (Altenbrunn and Seitz 2008). Interestingly, the synthesis of a Fmoc/Boc pseudoisocytosine monomer for peptide nucleic acid assembly was recently described by Hudson and Wojciechowski (2008).

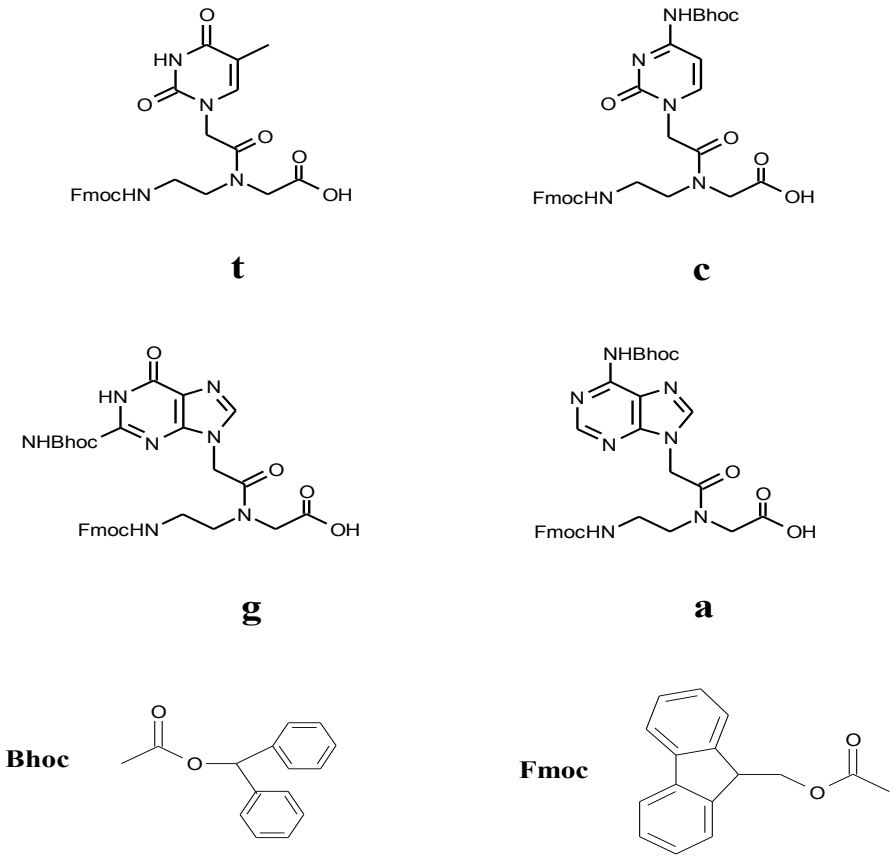


Figure 1.13: aegPNA protected monomers.

1.3 Modifications PNA: Nucleopeptides.

Besides the remarkable binding properties of aegPNAs, however, some drawbacks, such as low water solubility, poor cell permeability, costly precursors and also ambiguity in nucleic acids recognition (parallel/antiparallel) (Ray et al., 2000), are expected to be over come.
Different PNA modifications are widely described in the scientific literature, having met in some cases with success.
The type of changes reported in the literature aim to:

1. To introduce chirality in an achiral molecule selectively in order to guide the direction parallel / antiparallel hybridization with target DNA;
2. To produce molecules that have less conformational freedom in the backbone, pre-established structure in order to govern the hybridization to the target;
3. Add a cationic functional groups directly into the backbone of the PNA, or ends N-and C-terminal to improve its solubility in water;
4. Modular pairing between the nucleobases, by modifying or the methylene carbonyl linker or the bases;
5. Combining with the PNA molecules that act as carriers to optimize their entry into the cell.

The most explored PNA modifications regarded the aminoethylglycine backbone and the methylene carbonyl linker (1st generation modifications).
Surely one of the most important consists in stretching the structure dell'aegPNA by the addition of a methylene group to the polyamide backbone or linker, such as in the case of PNA with repeating units of N-(2-aminoethyl)- β-alanine (Figure 15a), or PNA in which the nucleobases are linked through an ethylene bridge carbonyl (Figure 15b).

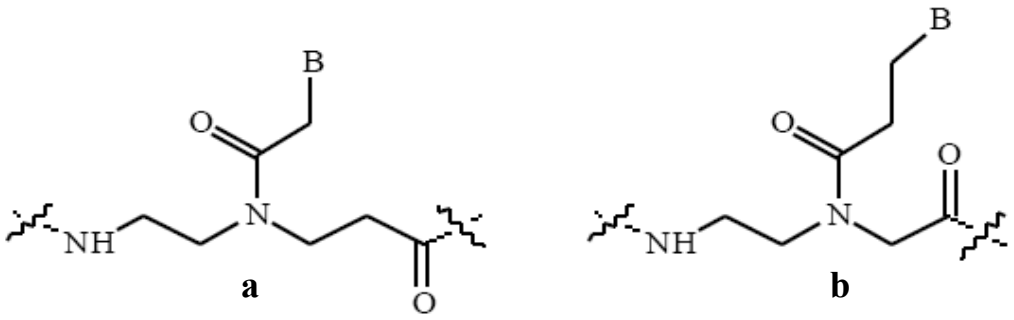


Figure 15: PNA with (a) backbone of N-(2-aminoethyl)-β-alanine and (b) carbonyl linker ethylene.

In the 1st generation, it was found, however, that changes in the first generation the only substitution tolerated focused on the α-position of glycine.
For example, substitution of the glycine with an alanine (D or L) resulted in a slightly lower binding affinity towards DNA.
Among the changes to the PNA of 2st generation, many works have been oriented to the design of molecules with a cyclic backbone (Kumar et al, 2002 and Gangamani et al., 1999). These types of changes, involving the connection of different portions of the skeleton of PNA, introduce conformational restrictions and / or resulting stereocenters in the polymer, giving interesting properties of hybridization. In this context, the PNA amminoprolil (ampPNA, Figure 16) were the compounds that have attracted most interest.

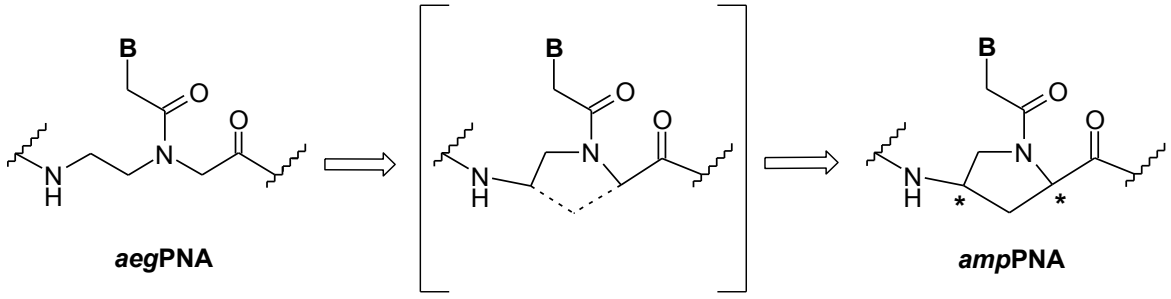


Figure 16: Comparison between aegPNA and ampPNA.

Oligomers formed entirely of monomers ampPNA however, showed no ability to target nucleotide natural hybridization. Surprisingly, the polymer where the aegPNA monomers alternate with those of ampPNA showed DNA binding affinity higher than oligomer format entirely aegPNA. The aegPNA oligomers containing a single unit of the N-terminus amminoprolil PNA, have shown a remarkable ability to discriminate between the bond than parallel to antiparallel DNA.
In particular, the possibility to discriminate between parallel/antiparallel binding modes is an important feature for a nucleotidic analogue to achieve the best specificity in the detection of complementary strands of natural targets. To introduce this favourable characteristic into PNAs, different approaches were reported in which aminoacidicmoieties, especially lysines or arginines, were added to the aegPNA ends (Ray et al., 2000 and Silvester et al., 2007). In this way the presence of the stereogenic centres allowed for improved binding mode discrimination, while the positive charge of the aminoacidic side chain favoured the solubility of the PNA oligomers. Furthermore, lysine units were also inserted in the aegPNA backbone in place of the glycine unit creating a chiral box inside the PNA oligomer (Sforza et al., 2000).
As can be seen, artificial nucleic acids illustrated, unlike the common aegPNA, whose backbone is pseudo-peptide, possess a peptide skeleton, they are referred to as nucleopeptides.
This class of molecules, nucleobase-containing peptides, has gained a considerable attention in the scientific community for the possibility of achieving novel powerful molecules able to :

(a) interact with natural nucleic acids;
(b) form materials, in particular, molecular networks based on hydrogen-bonding schemes between complementary (or self-complementary) nucleopeptide strands. These networks are useful for complexation and release in cell genes and drugs.

1.4 Diaminoacid-based nucleopeptides

In 1996, two different research teams reported on nucleopeptides based on a chiral natural diaminoacid, i.e. ornithine (6a, Figure 6), which was functionalized with thymine nucleobase through a methyl carbonyl linker by using thymine acetic acid.
More in detail, Lioy and Kessler prepared three chiral nucleopeptides by making use of both L- and D-ornithine nucleoamino acids by a Fmoc solid phase synthetic strategy (Lioy and Kessler 1996), whereas Petersen et al. reported the synthesis and the evaluation of the RNA-binding properties of a decamer based solely on the L-enantiomer (Petersen et al. 1996).
Subsequently, Korshunova et al. (1997) reported the design and the synthesis of several ‘‘oligonucleopeptides’’, i.e. δ-nucleopeptides based on ornithine with an ethyl linker to the nucleobase (6b, Korshunova et al. 1997). Ornithine-containing δ-nucleopeptides based on repeating unit 6a were also the object of researches performed by Inaki et al. (1998), which demonstrated by CD and NMR the presence of hydrogen-bonding between thymine and ornithine moieties (Inaki et al. 1998) in such nucleopeptides, and by van der Laan et al., who in the same year prepared several chirally-pure ornithine nucleopeptides (Van der Laan et al. 1998). Four years later Mandrugin et al. reported the synthesis of ‘‘new hetero organic nucleopeptides’’, whose base sequence was complementary to specific viral mRNA regions

(Mandrugin et al. 2002). Such nucleopeptides were characterized by a δ–ornithine backbone, with some residues carrying also free amino groups in the linker to nucleobase (6c).

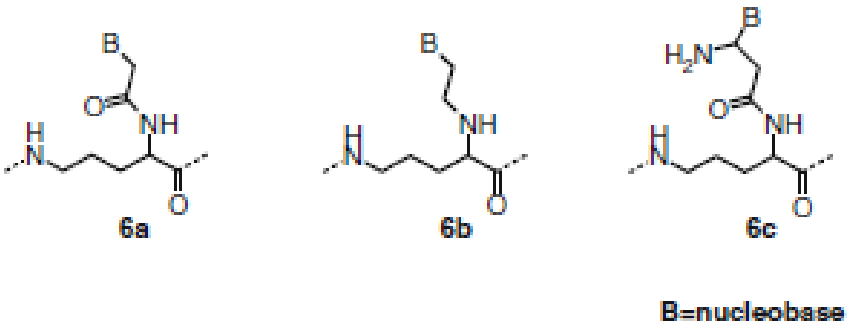
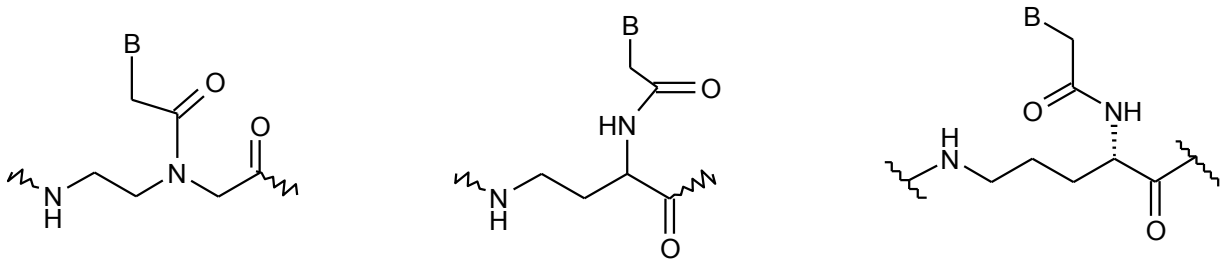


Figure 6: Molecular representation of ornithine-based repeating units in some literature δ-nucleopeptides.

In the same year a study by Sforza et al. demonstrated the need of modified coupling conditions to obtain by solid phase synthesis optically pure D- or L-ornithyl δ-peptides (based on 6a moieties) which presented novel binding characteristics towards nucleic acids, as they demonstrated by CD spectroscopy (Sforza et al., 2002). More in detail, the use of HATU as a coupling agent, and collidine (TMP) as a base, to be added in portions and without preactivation of the nucleoamino acid monomer, led to the lowest degree of epimerization, as reported by Corradini et al. (2001) for the synthesis of ornithine-containing nucleopeptides. It was reported that an oligo thymine PNA analogue based on D- or L-ornithine binds to RNA by forming triple helices (Sforza et al., 2002).
In this context we report our investigation about novel chiral PNA analogues, focusing the attention not only to the synthetic strategies but also to the binding studies towards natural and non-natural targets.

2. Aim of the thesis

In our research for the development of new ODN-like drugs or diagnostics and new materials, we set as its objective the realization and binding studies with natural and non natural targets of new chiral nucleo-γ-peptide, dabPNA (2, Figure 17), isomer of the aegPNA unit (1, Figure 17) and characterized by a 2,4-diaminobutyric acid (DABA)-based backbone carrying the carboxymethylated nucleobase on its α amino group.



*aeg*PNA **1** | *dab*PNA **2** | *orn*PNA 3

Figure 17: Comparison between aegPNA(1), dabPNA (2), ornPNA(3).

Differently from aegPNA monomers, dabPNA (2, Figure 17) presents a shorter backbone (3C between the nitrogen atom and the carbonyl) and an enhanced distance between the nucleobase and the backbone, i.e. 3 atoms (1N+2C) instead of 2C in aegPNAs. Furthermore, in comparison with ornPNA (3) that binds to RNA forming a stable triplex, daba-based PNA has a methylene group less in the backbone.
Our interest to the presented dabPNA arises from the possibility to obtain new molecules able to:
1)bind target DNA and RNA for therapeutical or diagnostic applications;
2) form molecular networks.
DABA is a nonprotein amino acid found in the seeds and vegetative tissues of flatpea (Lathyrus sylvestris L.) distributed intracellularly in mature leaves with about 15% of the cellular DABA contained in chloroplasts and at least 75% in vacuoles(Foster et al., 1987).Interestingly, poly(2,4-diaminobutyric acid), (PDBA), was recently used in a novel nonviral cytokine gene delivery system, which makes use of PDBA instead of polylysine resulting in high transfection efficiency.
In particular, *in vivo* antitumor effects of dendritic cell immunotherapy with poly(D,L-2,4-diaminobutyric acid)-mediated intratumoral delivery of the interleukin-12 gene were evaluated with the conclusion that this system suppresses tumor growth significantly (Iwashita et al., 2005).
Furthermore, this research was supported by the known stability of γ-peptides to enzymatic degradation and also by the proposal formulated by Meierhenrich et al.

(2004) DABA-based PNAs as prebiotic material in a primordial “PNA world” that would have preceded our present-day “two-polymer world”.
This hypothesis followed the recovery of DABA and other diaminoacids in the extraterrestrial soil of the Murchison meteorite (figure 18) together with the DNA purine and pyrimidine bases (Meierhenrich et al., 2004 and Nielsen et al., 1993).

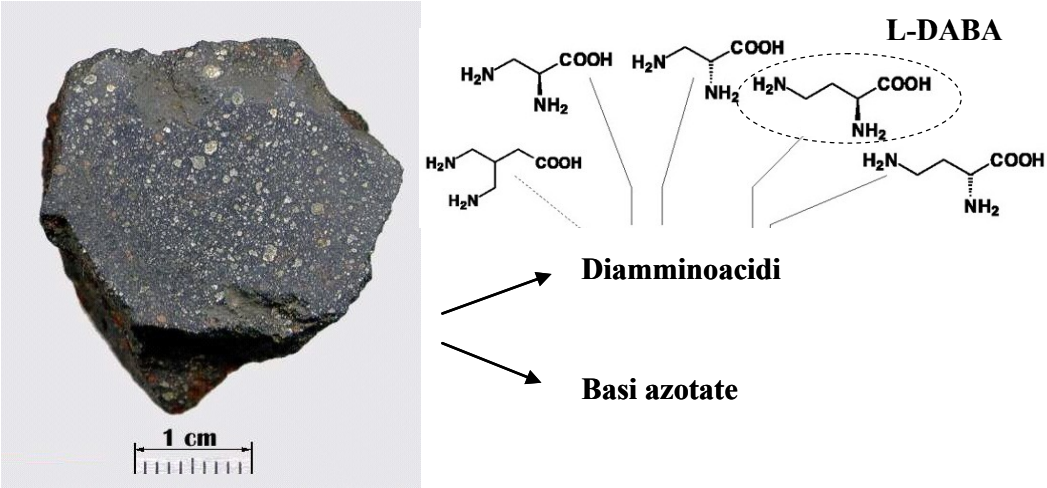


Figure 18: Fragment of the Murchison meteorite and some of the molecules found in the soil meteorite.

3. Results and discussion

3.1 Synthesis and oligomerization of $t_{L\text{-}dab}$ monomer, and binding studies on $(t_{L\text{-}dab})_{12}$ oligomer

The synthetic strategy adopted to obtain the new monomer $t_{L\text{-}dab}$ is based on the conjugation of two building blocks, 2,4-diaminobutyrric acid adequately protected (A, Figure 3.1) and carboxymethylated nucleobase (B, Figure 3.1).

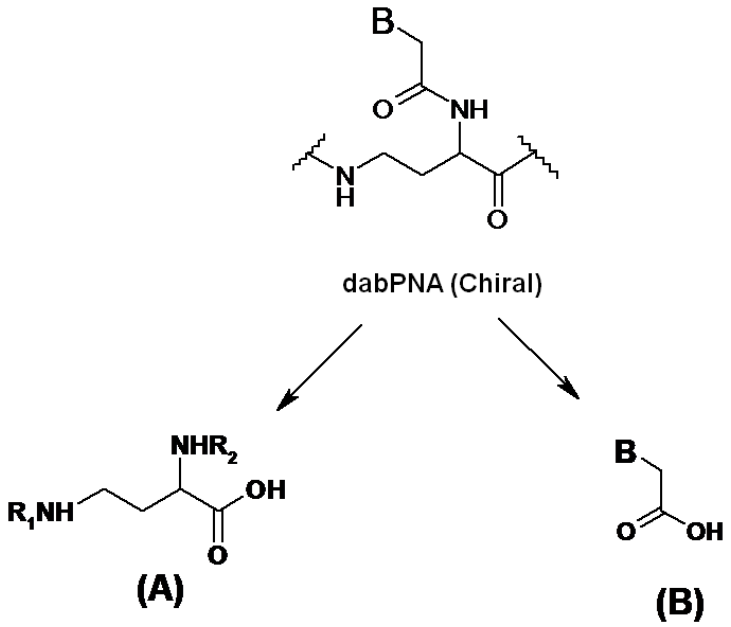
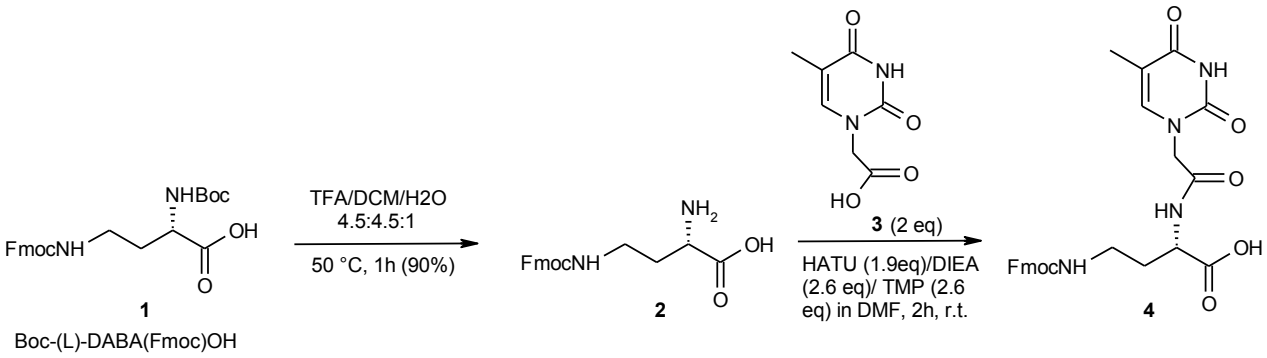


Figure 3.1: Molecular basis for the synthesis of a monomer dabPNA:
(A) 2,4-diaminobutirric acid protected and (B) carboxymethylated nucleobase.

The L-enantiomer of the natural diaminobutyric acid, was chosen, as starting material for the synthesis of the new monomer, in analogy to the L-ornithine-based PNAs, proposed by Petersen et al. (1996).
In particular, the thymine containing monomer was synthesised starting from the commercially available Boc-(L)-DAB(Fmoc)-OH diaminoacid (**1**, Scheme 1). In the first synthetic step the Boc group was selectively removed with trifluoroacetic acid (TFA) to give the free amino group in α-position. The obtained product 2, characterized by ^{1}H NMR and LC-ESIMS, was coupled with the thymine-1-acetic acid using HATU/DIEA/TMP as activating system in DMF leading to the compound **4** (Figure 3.1) in 72 % yield.



Scheme 1: Synthesis of $t_{L\text{-}dab}$ monomer.

The Fmoc-protected monomer **4** was purified by precipitations and characterized by $^{1}H/^{13}C$ NMR and LC-ESIMS, shown in Figure 3.2.

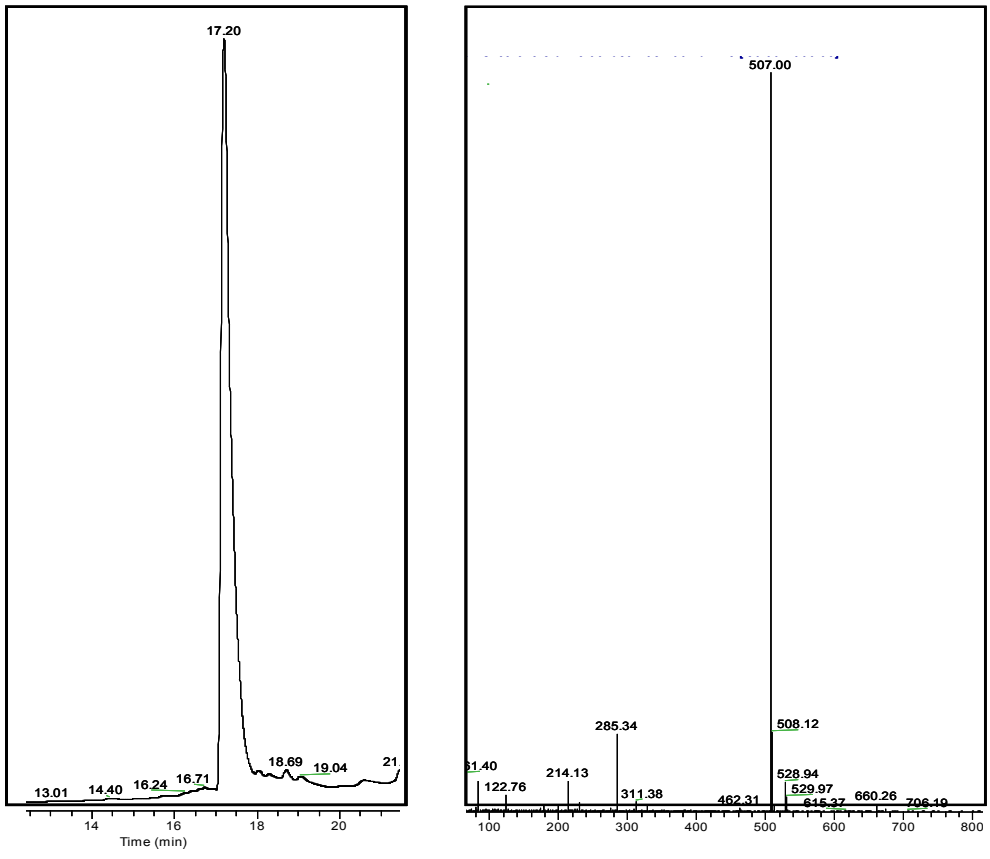


Figure 3.2: LC-ESIMS of $t_{L\text{-}dab}$ monomer.

The new monomer **4** was oligomerized manually on solid phase to the corresponding dabPNA dodecamer (**5**, Scheme 2) as showed in scheme 2.
The synthetic substrate used was polystyrene Rink amide-MBHA resin (Figure 3.3) as a linker containing a derivative of benzidrilammina (MBHA) and having a cross-linking of divinylbenzene (DVB, 1%).

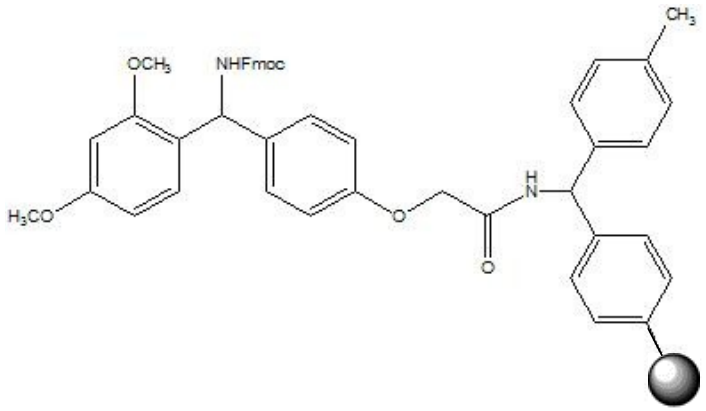
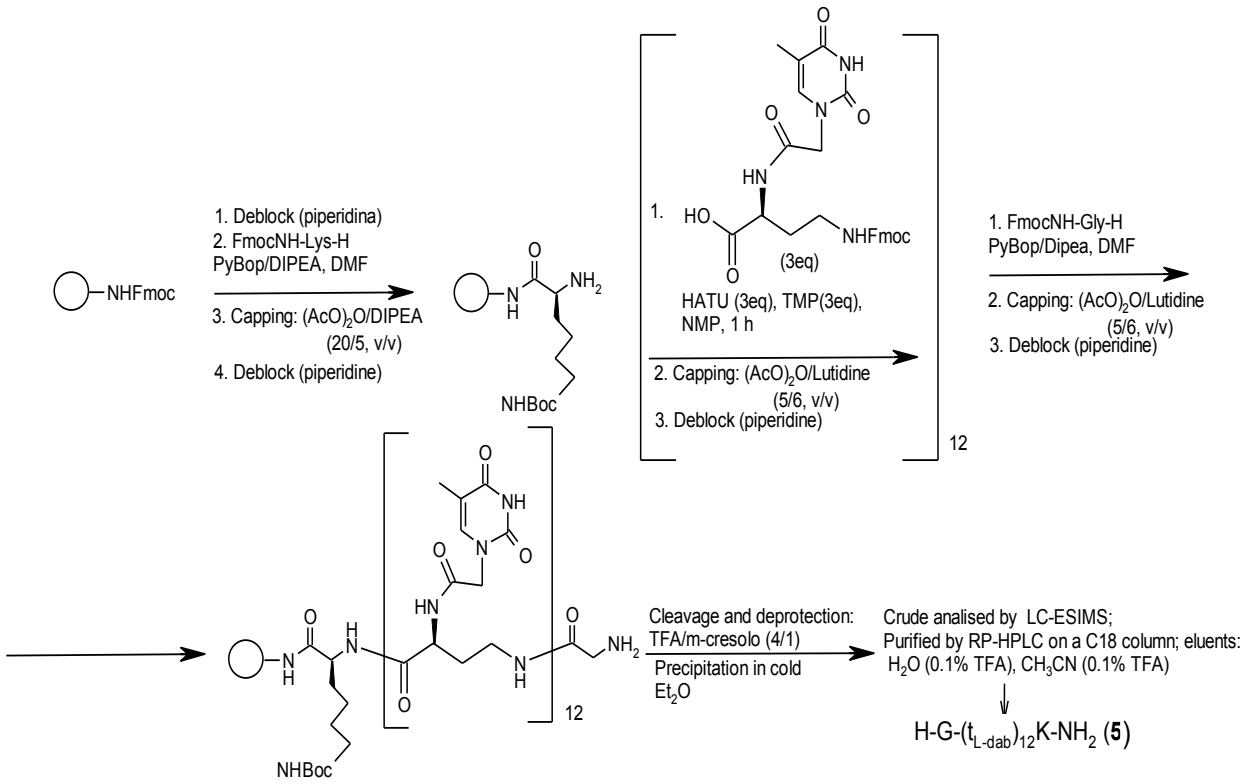


Figure 3.3: Rink amide-MBHA polystyrene resin.

This support was initially treated with a solution of 20% piperidine in DMF to leave the protection of Fmoc amino functions.
Subsequently, the deprotected support was reacted with a unit of lysine, sull'ammino-protected side-chain group with the Boc group and the one linked to C_α with the Fmoc group.
The reaction is being done in anhydrous DMF in the presence of PyBop/DIPEA, such as activating the carboxyl function of lysine against nucleophilic attack by the free - NH_2 group on the resin (Scheme 2). Any remaining free amino groups on solid support were blocked with a solution of acetic anhydride / DIPEA (20 / 5, v / v) in DMF. The yield of incorporation of lysine on the solid support was calculated by UV spectroscopy (UV Fmoc test).
On the resin, so functionalized, is assembled the dabPNA dodecamer (**5**, Table 1) using for coupling HATU / TMP as activating system in NMP / DMF (1:4, v / v), as shown in Scheme 2.
The choice of activator for the amidation of a carboxyl group having a chiral carbon in α is very critical because of the possibility of loss of chirality. In particular, since the racemization is promoted by deprotonation of the carboxylic group, the choice of the base, the activator, and pre-activation time points are essential to minimize this problem. In analogy to the synthetic strategy adopted for the coupling of L-ornithine, which is given to only 4% of epimerisation, we used for the coupling of the new monomer $t_{L\text{-}dab}$, the weak base TMP, added in four aliquots over 1h and the activator HATU, without the pre-activation (Corradini et al., 2001).



Scheme 2: Synthesis of H-G-($t_{L\text{-}dab}$)$_{12}$K-NH_2 oligomer.

The manual synthesis of the oligomer **5** allowed us to check, by UV Fmoc test, the coupling efficiency which was high in the first six steps (about 96%) and decreased gradually from 96% to 70% in the last six steps, probably because of the enhanced aggregation tendency of the increasing homo thymine chain. L-lysine and glycine residues were incorporated in the strand at C and N termini, respectively, to improve the solubility of the homo thymine polymer (overall yield: 24%).
After cleavage and deprotection, the oligomer **5** was purified by semipreparative RP-HPLC, and successively characterized by LC-ESIMS (Figure 3.4).

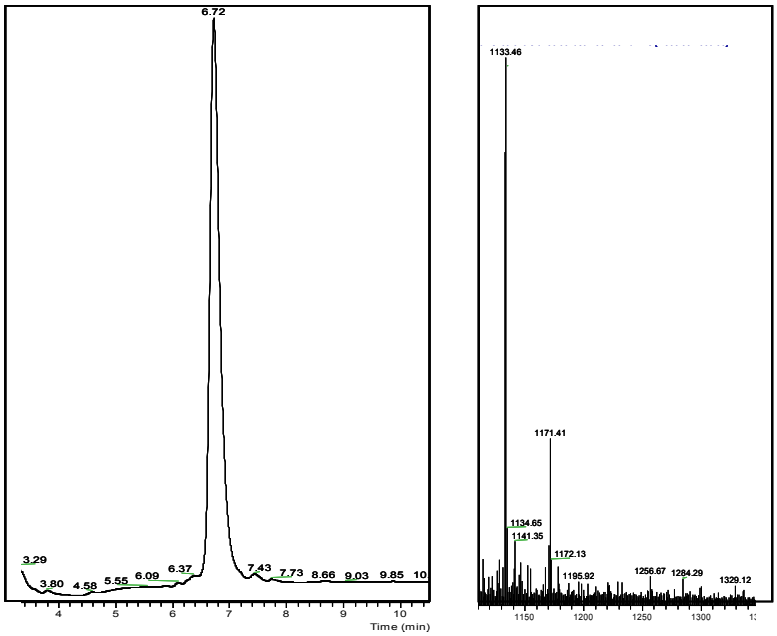


Figure 3.4 : LC-ESI MS of H-G-$(t_{L\text{-}dab})_{12}$K-NH_2 oligomer.

A t_{12} aegPNA (**6**, Table 1) was synthesised as reference oligomer for the binding studies. The synthesis of **6** was realized on the automatic synthesizer showed in Figure 3.5 using standard 2 µmol scale protocol and the commercial PNA monomers (Figure 1.13). Overall yield of **6** was only of 18 % probably for aggregation problems concerning the homothymine sequence and enhanced by the automatic *flow through* synthesis.

Figure 3.5: ABI / Perceptive Biosystems Expedite model 8909

Furthermore, other two dodecamer (**7** and **8**, Table 1), both carrying a single $t_{L\text{-}dab}$ unit at N-terminal and central position of the aegPNA chain, respectively, were synthesized in order to explore the influence of the new DABA-based monomer on the aegPNA binding ability.

These oligomers were assembled on automatic synthesizer using a standard 2 μmol scale protocol for the aegPNA part and with manual single insertion of $t_{L\text{-}dab}$ monomer using the protocol described for **5**. The overall yields of **7** and **8** were 18% and 19%, respectively. After deprotection and detachment from the solid support with TFA, oligomer **6-8** were purified by RP-HPLC and characterized by LC-ESIMS.
All the dodecamers, **5-8**, were quantified by UV spectroscopy, measuring the 260 nm absorbance at 85 °C, due to the tendency of PNA to aggregate at room temperature, and calculating the overall extinction coefficient (ε_{260}) as the sum of the ε_{260} of the PNA thymine monomer.
For each oligomer the CD spectrum was registered. As expected, a solution of the aegPNA **6** didn't show a significant CD spectrum. In contrast, CD spectrum of the oligomer H-G-$(t_{L\text{-}dab})_{12}$K-NH_2, due to its chiral nature, showed CD signal. In particular, a negative band in the 260-290 nm region was detected (Figure 3.5a) analogously to the spectrum reported in literature for the PNA analogue based on the L-ornithine, H-$(t_{L\text{-}orn})_{10}$K-NH_2, that presents a negative band in the same region (Figure 3.5b). The chiral $t_{L\text{-}dab}$ unit in **7** and **8** seemed to be not sufficient to these oligomers to show a significant CD signal.

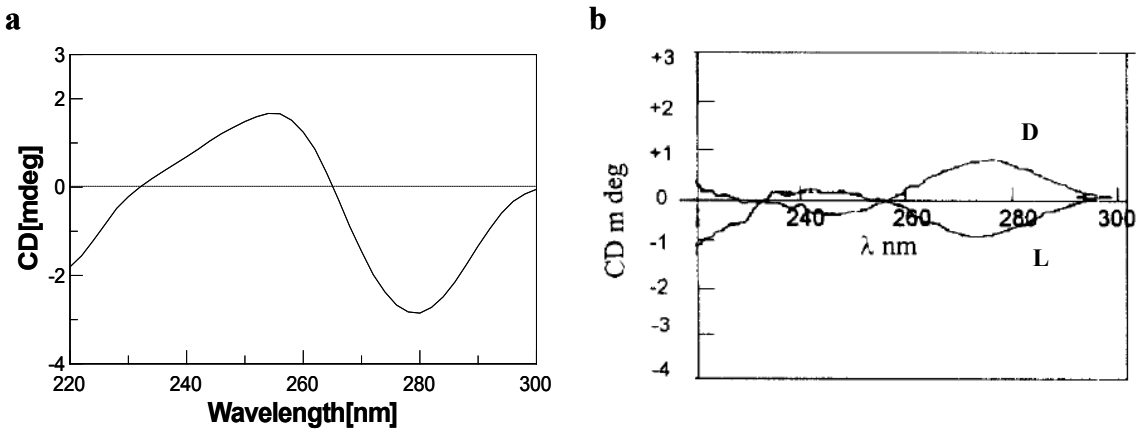


Figura 3.6: (a) CD spectrum of oligomer 5 H-G-$(t_{L\text{-}dab})_{12}$K-NH_2 (4 μM) in 10 mM, phosphate buffer pH 7.5; (b) CD spectra of oligomers H-$(t_{L\text{-}orn})_{10}$K-NH_2 (L, 10 μM) e H-$(t_{D\text{-}orn})_{10}$K-NH_2 (D, 10 μM) in 10 mM phosphate buffer, 100 mM NaCl, 0.1M EDTA, pH 7.

Successively, the ability of the new oligomer $(t_{L\text{-}dab})_{12}$ to bind natural nucleic acids was evaluated by CD experiments, using a Tandem cell, constituted from two separated reservoir communicating just by the upper part of the cell, in which two solutions, initially separated, come in contact only after mixing by turning upside down the cell (Figura 3.6).

Light Path:	2 x 4.375 mm
Height:	46 mm
Width:	12.5 mm
Depth:	12.5 mm
Width:	9.5 mm
Base Thickness:	1.5 mm

Figure 3.6 Hellma Tandem mix cell.

Initially, we considered a DNA strand complementary to the PNA dodecamers (dA_{12}): a solution of dA_{12} in phosphate buffer was kept in one of the Tandem cell reservoir (1 mL), while oligomer **5**, in the same buffer conditions, was in the other one.
CD spectrum from 200 to 320 nm, corresponding to the "sum" spectrum of the separated strands, was registered. Successively the cell was turned upside down to allow the mixing of the two samples and again a CD spectrum was recorded. As shown in Figure 3.7a, since the "sum" and "mix" CD spectra were almost the same, no binding evidence was revealed, even recording CD spectra of the "mix" sample at different time (10 min, 30 min, 1 h, 2 h, 3 h, 24 h), performing a kinetic experiment. Furthermore, also adding one equivalent more of oligomer **5**, in a 2:1 total ratio of $(t_{L\text{-}dab})_{12}/dA_{12}$, in order to verify the possibility to form a triple helix complex, the binding was not detected.As positive reference, the aforementioned CD experiment was also performed between the aegPNA **6** and dA_{12}, obtaining the spectra reported in Figure 3.7b: in this case a clear evidence of the binding was detected.

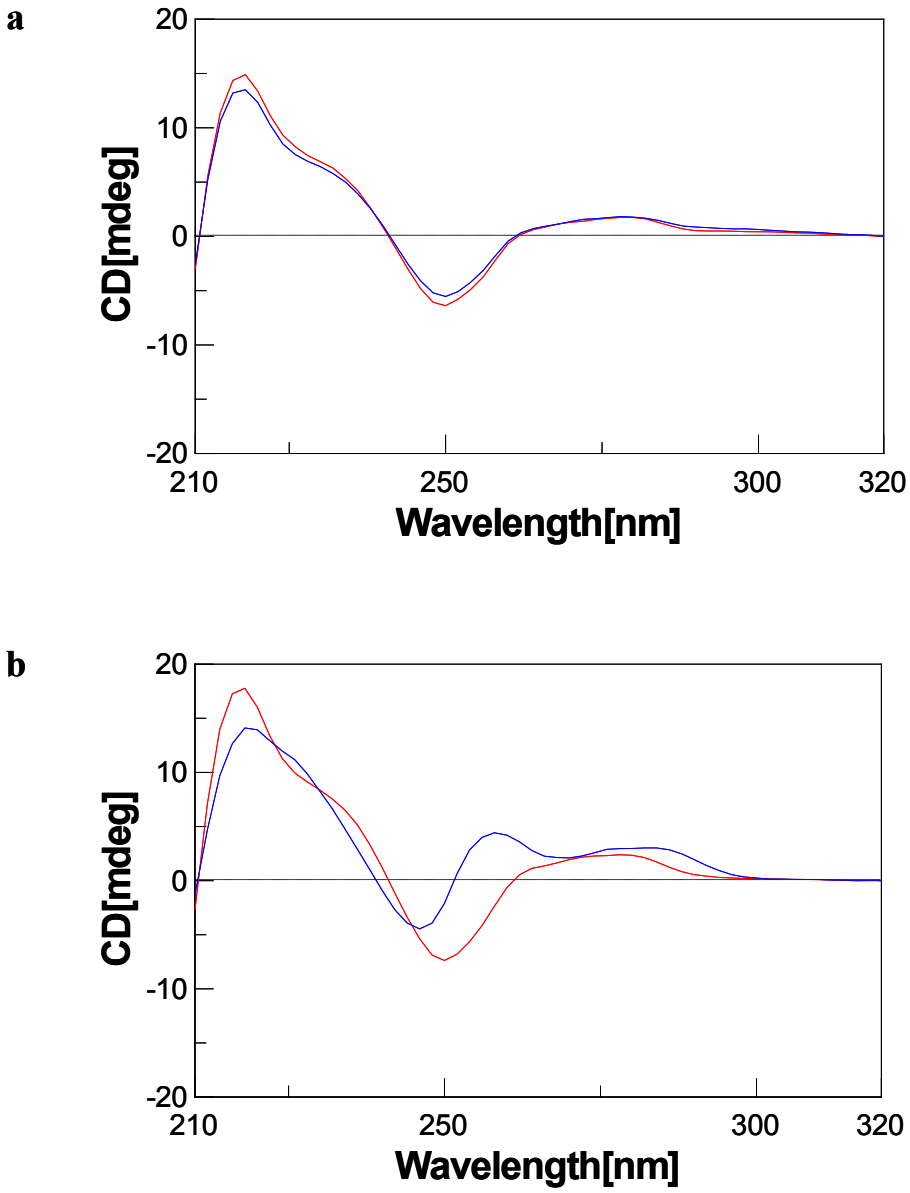


Figura 3.7: Sum (red) and Mix (blue) CD spectra of (a) $(t_{L\text{-}Dab})_{12}$ 6/dA_{12} H-G-$(t_{L\text{-}dab})_{12}$K-NH_2 4 μM and dA_{12} 4μM in 10 mM phosphate buffer of (b) t_{12} PNA 7/dA_{12}.

Furthermore, also from UV melting experiment on the solution of **5** and dA_{12}, correctly annealed, no binding was revealed, as shown in Figure 3.8 the melting curve of H-G-$(t_{L\text{-}dab})_{12}$-K-NH_2/dA_{12} was a drift (red line).
In order to study the effect of the single dabPNA insertion on the ability of aegPNA strands to bind complementary DNA, UV melting experiments on annealed samples containing dA_{12} and, respectively, oligomers **6**, **7** and **8**, were performed. Overlapped melting curves are shown in Figure 3.8 and the corresponding T_m values are summarized in Table 1. From the melting data it was clear that the insertion of $t_{L\text{-}dab}$ monomer in the middle (oligomer **8**) or at N terminus (oligomer **7**) of the homothymine aegPNA chain leads to a decreased binding efficiency to the target DNA in comparison to aegPNA **6**.

Sequences		T_m (°C) ± 0.5
H-G-$(t_{L\text{-}dab})_{12}$-K-NH_2/dA_{12}	**(5)**	-
H-G-t_{12}-K-NH_2/dA_{12}	**(6)**	86.2
H-G-$t_{L\text{-}dab}t_{11}$-K-NH_2/dA_{12}	**(7)**	84.8
H-G-$t_6t_{L\text{-}dab}t_5$-K-NH_2/dA_{12}	**(8)**	72.0

Table 1 Homo thymine sequences synthetized.

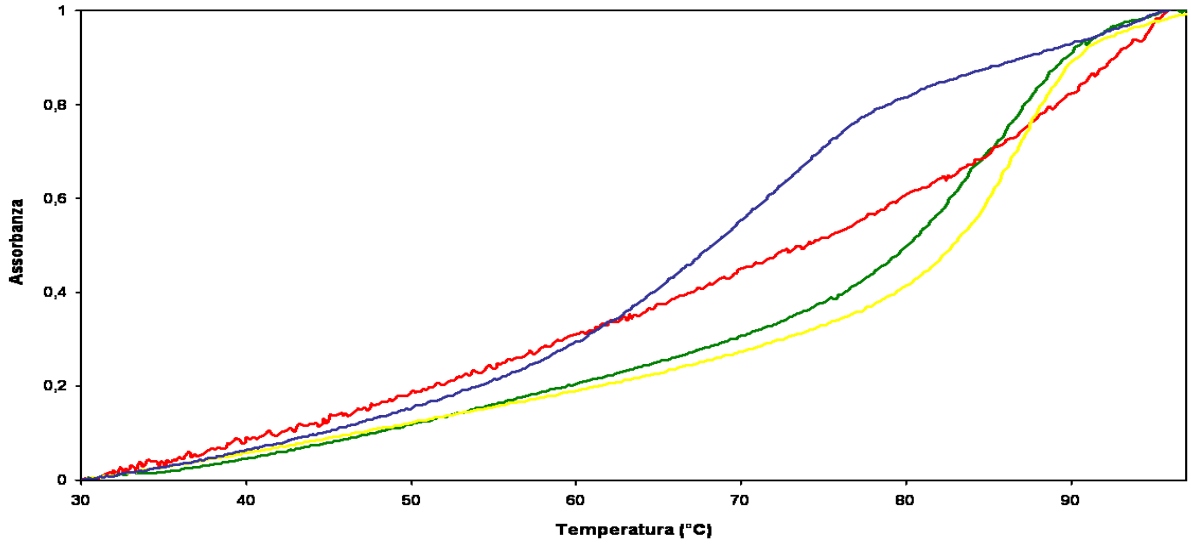


Figure 3.8: Melting curves of (**5**) H-G-$(t_{L\text{-}dab})_{12}$-K-NH_2/dA_{12}, (**6**) H-G-t_{12}-K-NH_2/dA_{12}, (**7**) H-G-t_L-$dabt_{11}$-K-NH_2/dA_{12}, (**8**) H-G-$t_6t_{L\text{-}dab}t_5$-K-NH_2/dA_{12}

In addition, the ability of the H-G-$(t_{L\text{-}dab})_{12}$K-NH_2 oligomer to hybridise a complementary strand of RNA (A_{12}) was explored. Even in this case, from the binding experiment with the tandem cell (Figura 3.9a), as well as from the kinetic CD experiment, no binding evidence was revealed.
As reference, the same experiment on the RNA A_{12} was performed with the aegPNA strand (**6**) and, as expected, in this case the “sum” and “mix CD spectra resulted different, clear evidence of binding (Figura 3.9b).

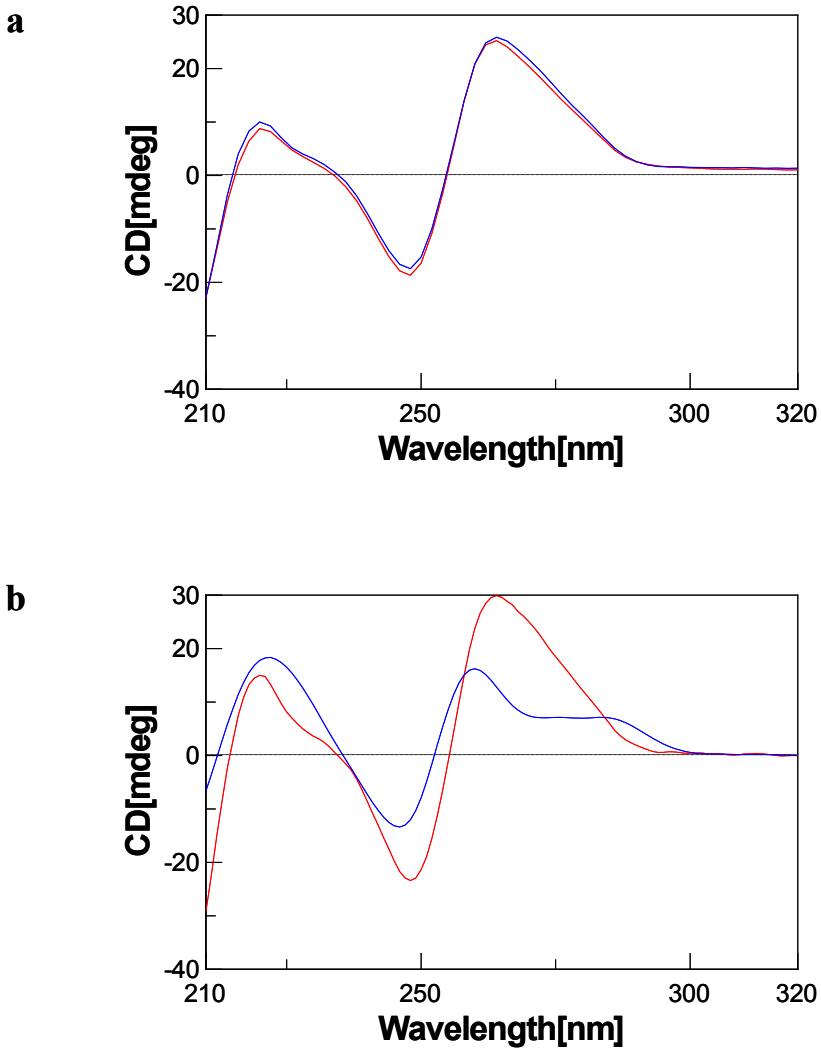


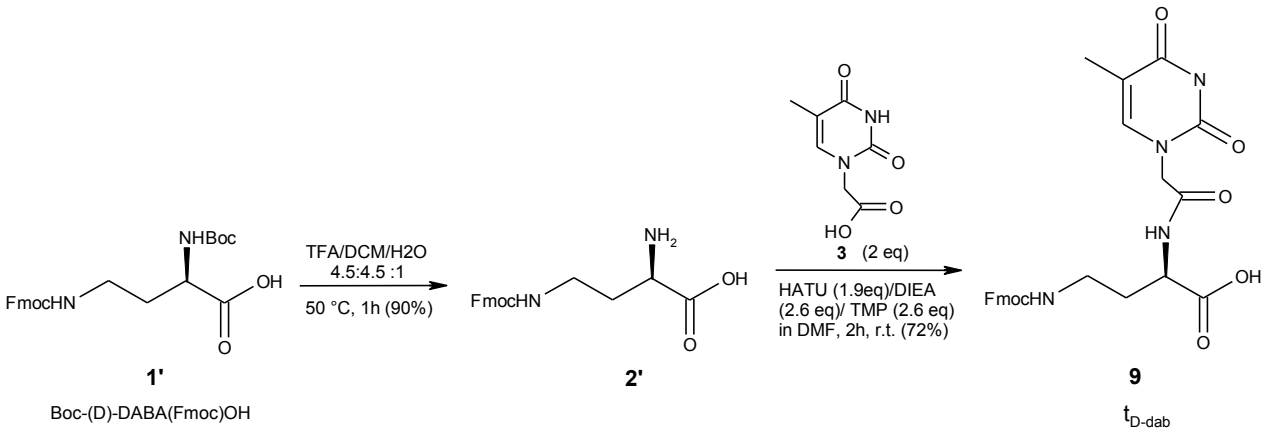
Figure 3.9: sum (red) and Mix (blue) CD spectra of (a) H-G-$(t_{L\text{-}dab})_{12}$K-NH_2 4 μM and A_{12} RNA 4μM in 10 mM phosphate buffer and (b) t_{12} **7**/A_{12}.

On the basis of these findings we concluded that L-*dab*PNAs based oligomers were not able to bind natural nucleic acids.

3.2 Synthesis and oligomerization of $t_{D\text{-}dab}$ monomer, and binding studies on $(t_{D\text{-}dab})_6$ oligomer

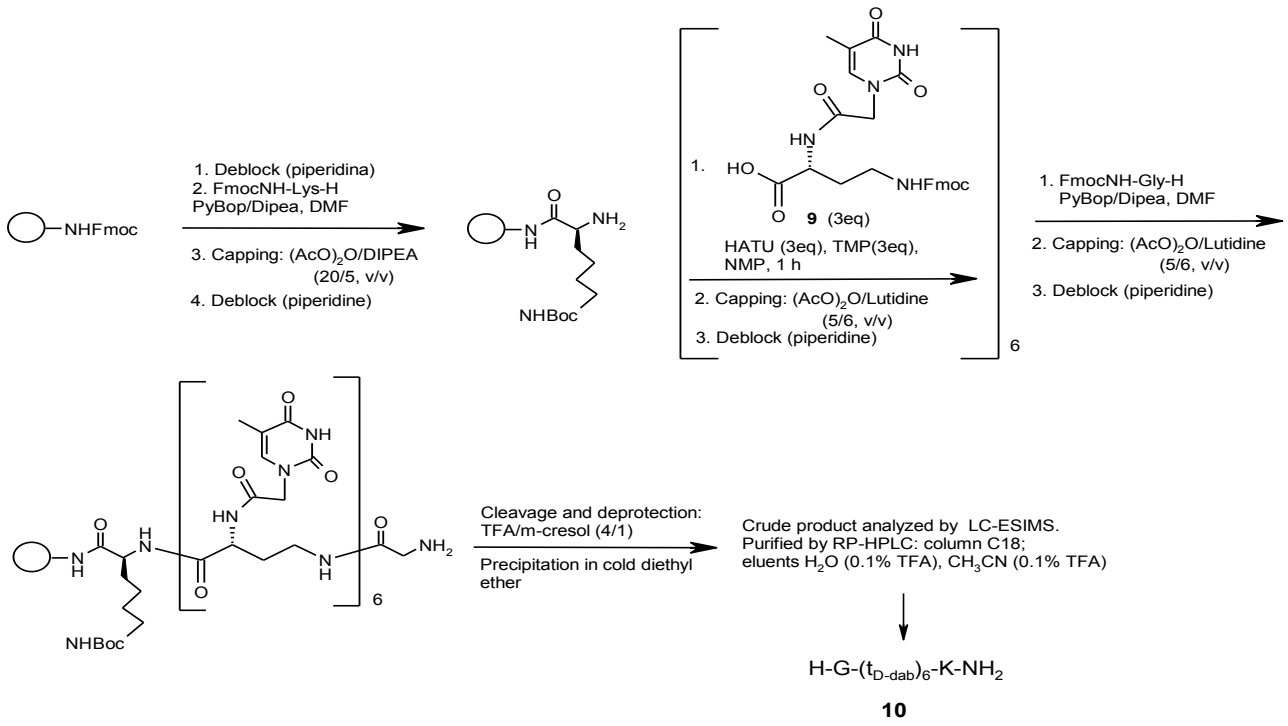
Since the PNA oligomer based on L-DABA don't bind natural nucleic acids and considering that different stereoisomers have different ability to adopt the conformation required to bind DNA or RNA, we thus explore the hybridization of the D-DABA-based nucleopeptides to nucleic acid targets. Nevertheless, in this way we continue our investigation on the hypothized prebiotic role of dabPNAs, after the recovery of both L and D-DABA enantiomers in meteoritic soil (Meierhenrich et al.,

2004). Our interest was successively devoted to the realization of the enantiomeric $t_{D\text{-}dab}$ monomer, starting from the commercial Boc-(D)-DAB(Fmoc)-OH (Scheme 3).



Scheme 3 Synthesis of $t_{D\text{-}dab}$ monomer.

The $t_{D\text{-}dab}$ monomer **9**, purified by RP-HPLC and characterized by NMR and LC-ESI MS, was oligomerized manually on solid phase (Scheme 4) to the corresponding H-G-$(t_{D\text{-}dab})_6$K-NH_2 (**10**) following the same synthetic procedure used for the obtainment of oligomer **5**.



Scheme 4 Synthesis of H-G-$(t_{D\text{-}dab})_6$K-NH_2 oligomer.

After cleavage and deprotection, homopolymer **10** was purified by semipreparative RP-HPLC on a C18 column, and successively characterized by LC-ESIMS (Figure 3.10).

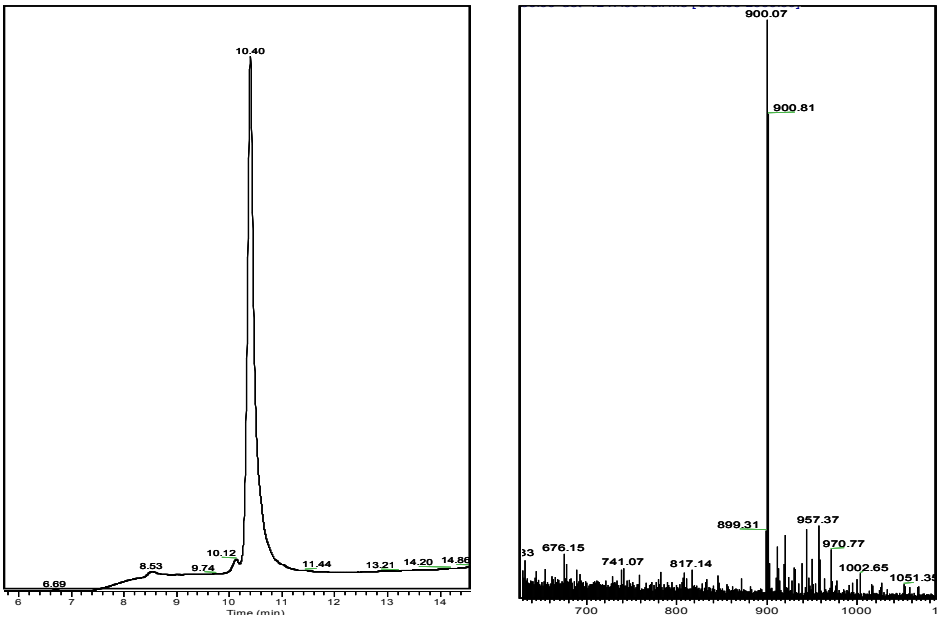
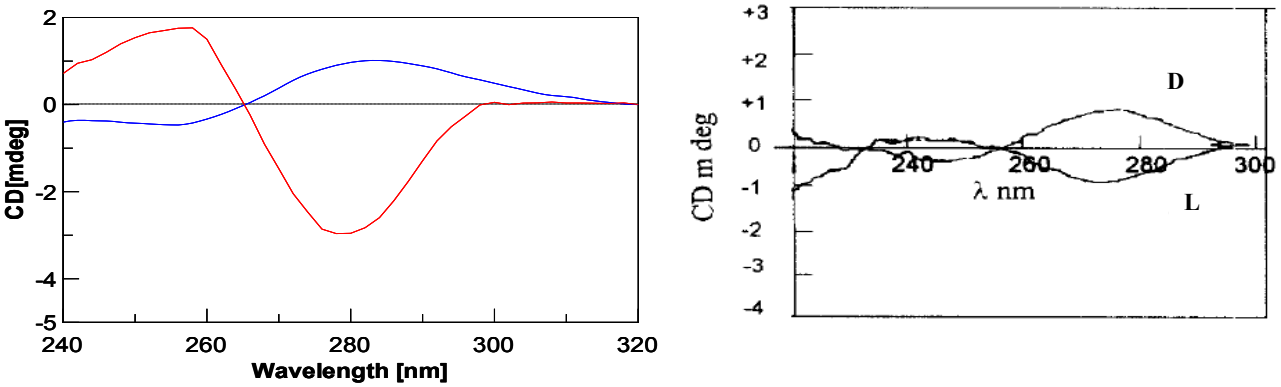


Figure 3.10 LC-ESIMS of H-G-($t_{D\text{-}dab}$)$_6$K-NH_2 homopolymer .

CD spectrum of single strand H-G-($t_{D\text{-}dab}$)$_6$K-NH_2 (**10**), depicted in Figure 3.11a (blu line), showed a mirror image relationship relative to the enantiomer H-G-($t_{L\text{-}dab}$)$_{12}$K-NH_2 **5** (red line, Figure 3.11a), in analogy to the CD spectra reported in literature by Sforza et al.(2002) for the L- and D-ornPNAs H-(t_{orn})$_{10}$K-NH_2, showed in Figure 3.11b.



a **b**

Figure 3.11 a) Mirror-image relationship between H-G-($t_{L\text{-}dab}$)$_{12}$-K-NH_2 (red line) and H-G-($t_{D\text{-}dab}$)$_6$K-NH_2 (blu line), 4 uM, 10 mM Pi, 10 °C; b) H-($t_{L\text{-}orn}$)$_{10}$K-NH_2 (**L**, 10 μM) e H-($t_{D\text{-}orn}$)$_{10}$K-NH_2 (**D**, 10 μM) in 10 mM phosphate, 100 mM NaCl, 0.1M EDTA, pH 7.

Furthermore, we performed CD experiments to verify the ability of H-G-$(t_{D\text{-}dab})_6$K-NH_2 to bind DNA and RNA (dA_{12}/A_{12}) using the "Tandem cell". No difference between the sum CD spectrum, obtained before mixing the two components, and the complex CD spectrum, recorded after the mixing, was revealed for oligomer **10** with both DNA and RNA. The CD spectra remained almost the same also at different times by performing kinetic CD studies, or adding another equivalent of H-G-$(t_{D\text{-}dab})_6$K-NH_2 to the solution obtaining a 2:1 final ratio of $(t_{D\text{-}dab})_6/dA_{12}$ to verify the possibility to form triple helix. In addition UV thermal experiments of the annealed mixture confirmed the absence of any significant H-G-$(t_{D\text{-}dab})_6$K-NH_2 /DNA(RNA) interaction, showing little drift curves.

3.3 Synthesis and oligomerization of $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ monomers, and binding studies

Since dabPNAs based on L- and D-DABA don't bind natural nucleic acids, we explored the possibility that complementary nucleopeptides based on D or L-DABA could bind between themselves.
This property would be interesting in order to develop novel DABA-based dendrimeric systems, as new materials, and also to realize new diagnostic tools, as for example new molecular beacon probe.
This idea is based on some scientific studies reported in the literature concerning alanylPNA (Figure 3.12 a).
In particular, it was recently demonstrated that these analogues of PNA, whose backbone is composed of units of aminoacid L-alanyne that is grafted methylene-nucleobase, while not natural binding target, are able to hybridize with themselves and are therefore been proposed as constituents of the duplex portion of the stem found in molecular beacons (Figure 3.12 b) are useful diagnostic tools for the analysis (detection) of target nucleotide sequences as well as for the detection of mutations (Diederichsen, U. *et al*.,1996).

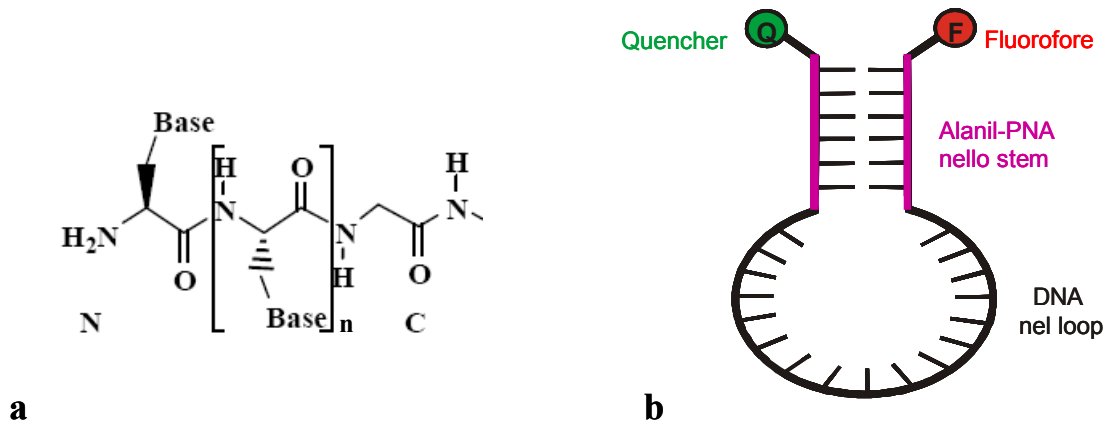


Figure 3.12: : (A) single strand of L-alanylPNA, (b) molecular beacons with stem-based alanylPNA.

Similarly, one might think, if the dabPNA itself to a hybrid system like the one shown in Figure 3.13 for dabPNA

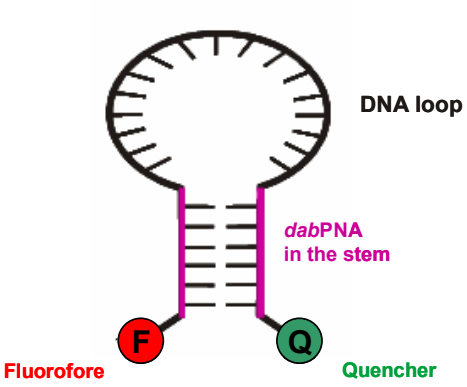
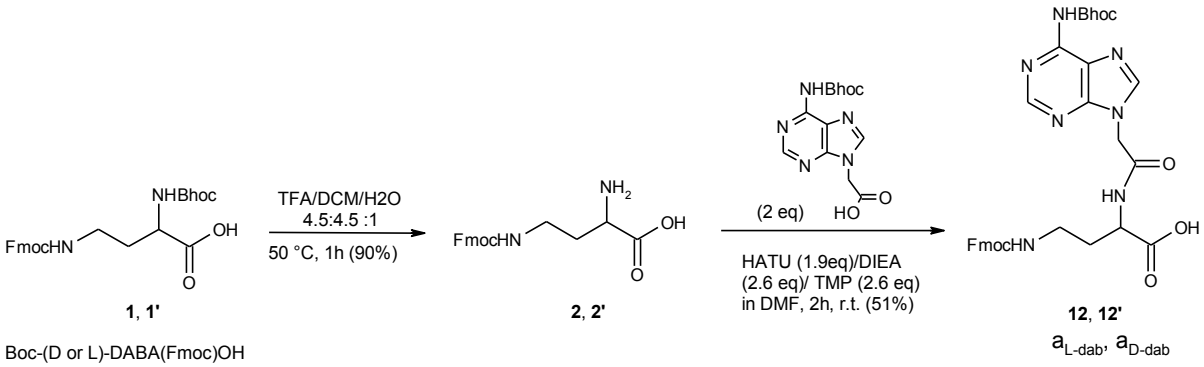


Figure 3.13: molecular beacon with *dab*PNA-based stem.

To verify this interesting possibility, homoadenine and homothymine hexamers based on L- and D-DABA were realized in order to find, if possible, the correct combination of chirality suitable for obtaining the binding.
In particular, we obtained and studied the following systems:

- strands of the same chirality: H-G-($a_{L\text{-}dab}$)$_6$K-NH_2 / H-G-($t_{L\text{-}dab}$)$_6$K-NH_2;
- strands of opposite chirality: H-G-($a_{L\text{-}dab}$)$_6$K-NH_2 / H-G-($t_{D\text{-}dab}$)$_6$K-NH_2;
- an alternating chirality strand and a homochiral strand: H-G-($a_{L\text{-}dab}$)$_6$K-NH_2/H-G-($t_{L\text{-}dab}$-$t_{D\text{-}dab}$)$_3$K-NH_2
- two alternating chirality strands: H-G-($a_{L\text{-}dab}$-$a_{D\text{-}dab}$)$_3$K-NH_2/H-G-($t_{L\text{-}dab}$-$t_{D\text{-}dab}$)$_3$K-NH_2;
- two alternating chirality strands: H-G-($a_{L\text{-}dab}$-$t_{D\text{-}dab}$)$_3$K-NH_2/ H-G-($a_{L\text{-}dab}$-$t_{D\text{-}dab}$)$_3$K-NH_2.

With the previously synthesized $t_{L\text{-}dab}$ and $t_{D\text{-}dab}$ monomers, we initially realized the sequences H-G-($t_{L\text{-}dab}$)$_6$K-NH_2 (**11**) and H-G-($t_{L\text{-}dab}$-$t_{D\text{-}dab}$)$_3$K-NH_2 (**12**), following the same synthetic procedure for **5**.
In order to assemble the poliadenine sequences, the new $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ monomers were synthesized, by reacting L- and D-Boc-DAB(Fmoc)-OH (**1** and **1'**), respectively, with Bhoc-protected carboxy methylated adenine. (Scheme 5).



1, **1'**
Boc-(D or L)-DABA(Fmoc)OH

2, **2'**

12, **12'**
$a_{L\text{-}dab}$, $a_{D\text{-}dab}$

Scheme 5: Synthesis of $a_{L\text{-}dab}$ / $a_{D\text{-}dab}$ monomers.

The Fmoc-protected monomers (**12** and **12'**) synthesized, were purified by RP-HPLC without TFA in the eluents, to avoid the loss of the acid labile Bhoc protecting group. $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ were characterized by ^{1}H /^{13}C-NMR and LC-ESI MS (Figure 3.14).

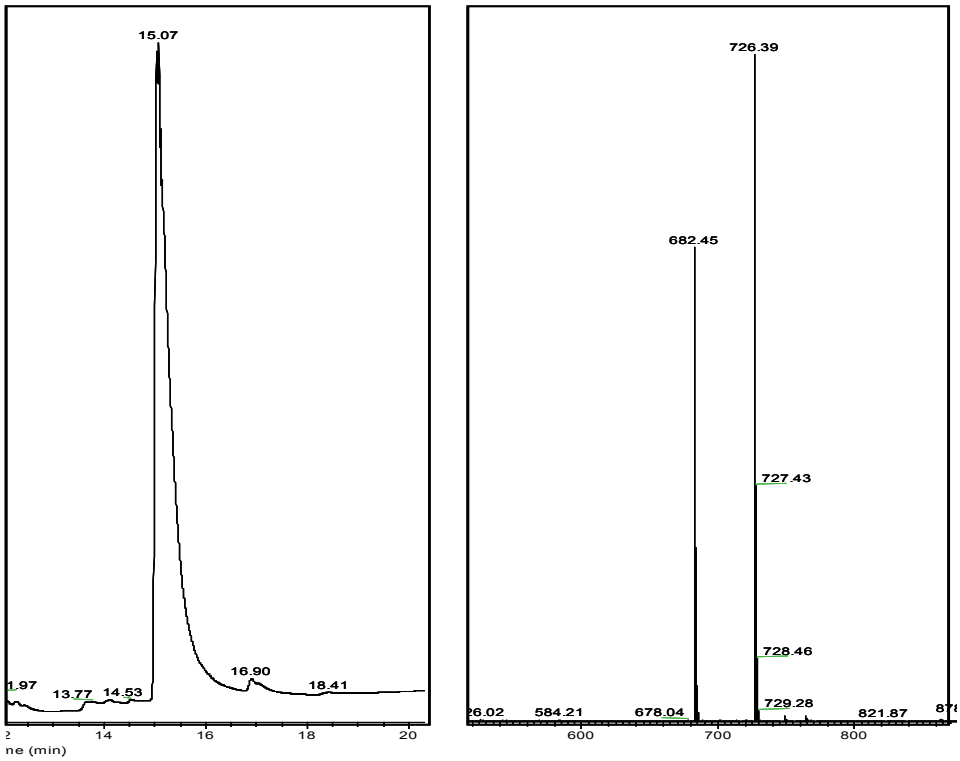
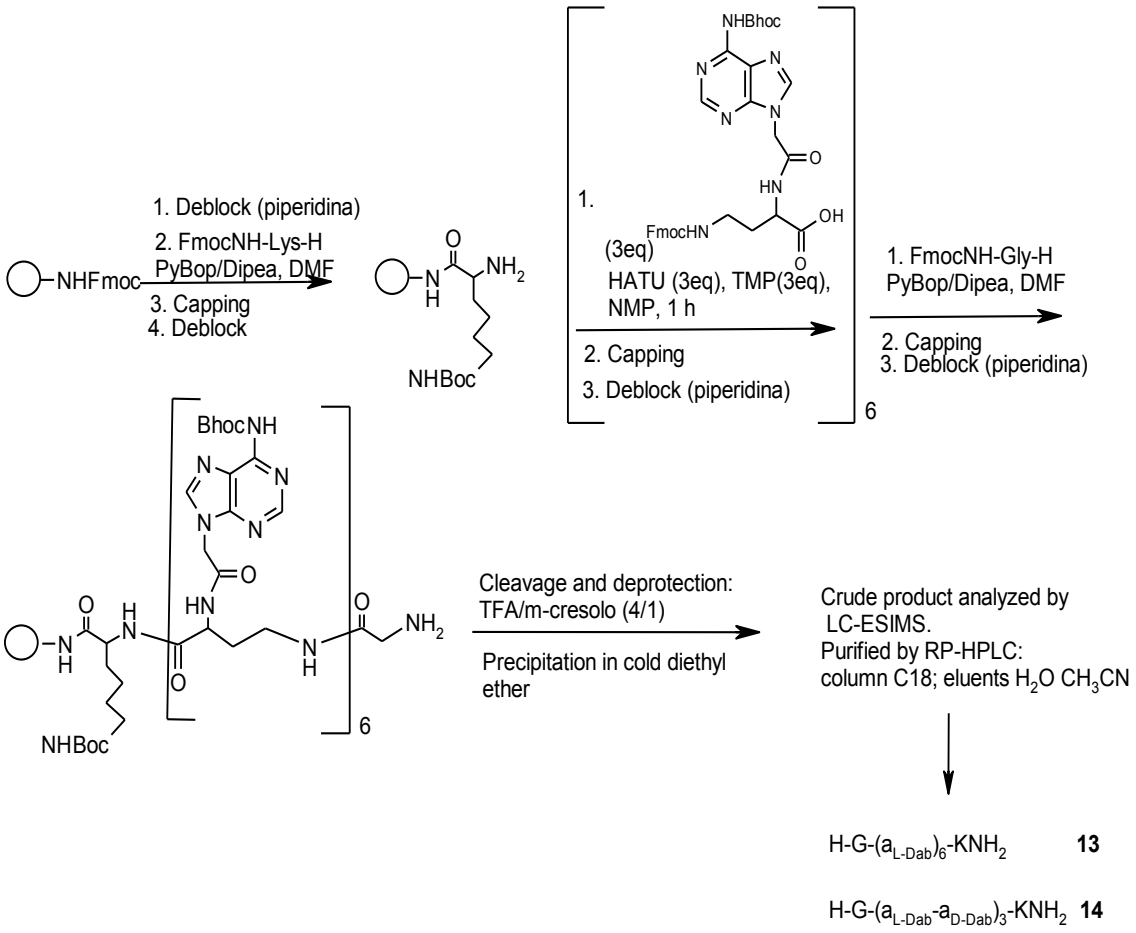


Figure 3.14: LC-ESI MS profile of $a_{L\text{-}dab}$ monomer.

The new chiral monomers $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ were used to synthetize H-G-$(a_{L\text{-}dab})_6$K-NH_2 **13**, and H-G-$(a_{L\text{-}dab}$-$a_{D\text{-}dab})_3$K-NH_2 **14**, with alternate chirality, following the same manual synthetic protocol used for all the previous syntheses to minimize racemization problems.



Scheme 6: Synthesis of polyadenine DABA-based oligomers.

The coupling efficiency for the synthesis of **13** and **14**, checked by UV Fmoc test, was on average around 70% in the first five synthetic steps and decreased rapidly in the last ones, giving an overall yields of 16% (**13**) and 18% (**14**).
After cleavage and deprotection, the oligomers were purified by semipreparative RP-HPLC, and successively characterized by LC-ESIMS, which confirmed the identity of the products. The LC-ESIMS profile of the new oligomer **11** and **13** is reported in Figure 3.15 as an example.

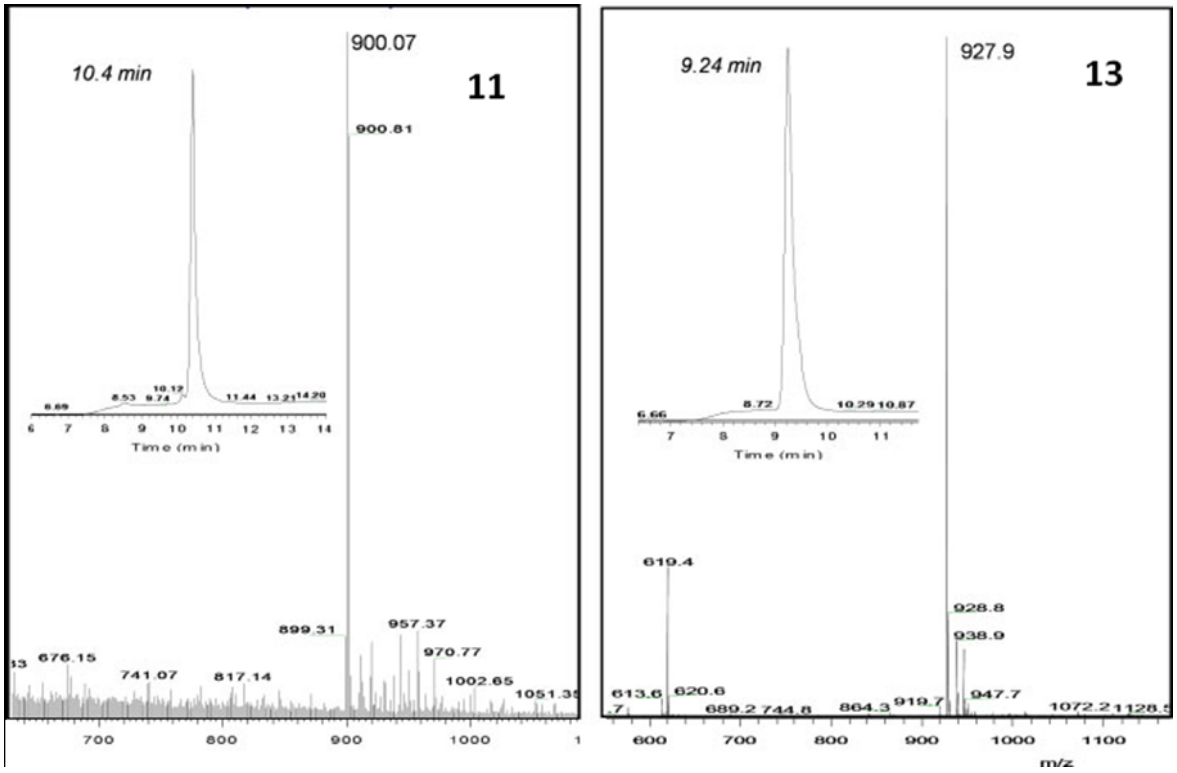
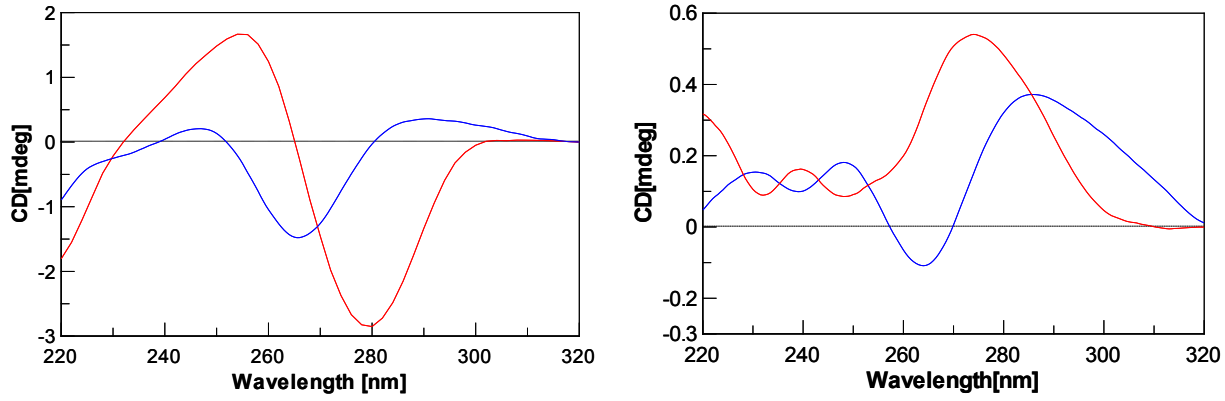


Figure 3.15: LC-ESI MS of H-G-$(t_{L\text{-}dab})_6$K-NH_2 (**11**) and H-G-$(a_{L\text{-}dab})_6$K-NH_2 (**13**)

All the dabPNA hexamers were quantified by UV spectroscopy using the molar extinction coefficients (ε_{260}) of the aegPNA monomers, and measuring the A_{260} at 85 °C.

Oligomer **13**, due to its chiral nature, showed CD signal, as evidenced by its CD spectrum reported in Figure 3.16 (blue line). CD experiments revealed that the spectrum of $(a_{L\text{-}dab})_6$ showed the same trend as that of homo polymer having the same chirality $(t_{L\text{-}dab})_{12}$ (red line, Figure 3.16 b), with a band shift of the minimum between 250-300 nm from 276 nm of $(t_{L\text{-}dab})_{12}$ to 266 nm because of the different chromophores.



a **b**

Figure 3.16 a) Overlapped CD spectra of $(a_{L\text{-}dab})_6$ (blue line)and $(t_{L\text{-}dab})_{12}$ (red line), 8 µM in 10 mM phosphate buffer, pH 7.5; b) Overlapped CD spectra of $(a_{L\text{-}dab}\text{-}a_{D\text{-}dab})_3$ (blue line) and $(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ (red line), 4 µM in 10 mM phosphate buffer, pH 7.5.

In addition, by comparing the CD spectra of the alternate oligomers $(a_{L\text{-}dab}\text{-}\ a_{D\text{-}dab})_3$ and $(t_{L\text{-}dab}\text{-}\ t_{D\text{-}dab})_3$, a positive band between 260-320 nm was revealed, with the maxima centred at 274 nm, in the case of $(t_{L\text{-}dab}\text{-}\ t_{D\text{-}dab})_3$, and at 284 nm, in the case of $(a_{L\text{-}dab}\text{-}a_{D\text{-}dab})_3$ (Figure 3.16b).
The presence of clear CD signals for all oligomers suggested a structural preorganization of the chiral strands, confirmed by the temperature-dependent CD profiles (data not shown).
CD hybridization studies were performed with tandem cell on H-G-$(a_{L\text{-}dab})_6$K-NH_2 /$(t_{L\text{-}dab})_6$: no significant binding evidence was revealed in this case. Indeed only little changes are recorded for the sum and mix spectra relative to this complex (Figure 3.17).

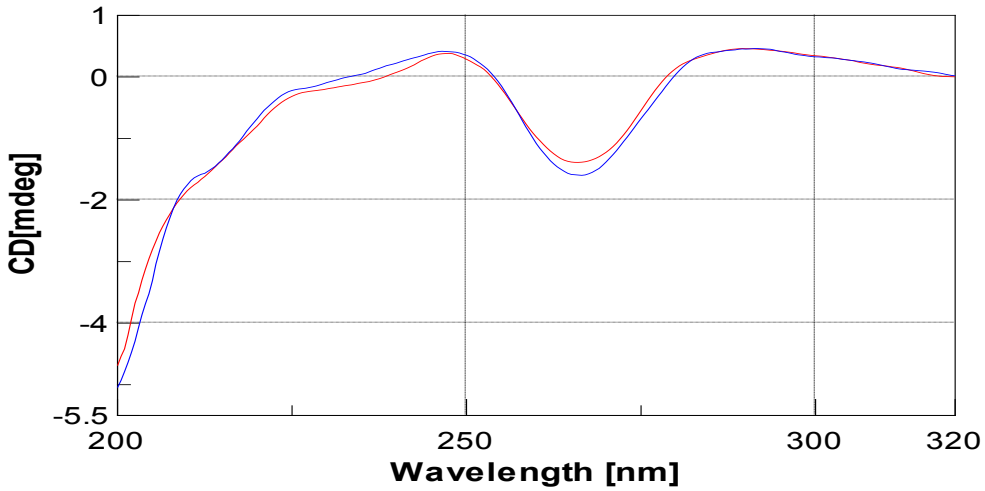


Figure 3.17: Red sum of $(a_{L\text{-}dab})_6$/ $(t_{L\text{-}dab})_6$, Blue mix of $(a_{L\text{-}dab})_6$/ $(t_{L\text{-}dab})_6$ (4 µM each strand in 10 mM phosphate buffer, pH 7.5)

Other CD experiments were carried out on the following systems: $(a_{L\text{-}dab})_6$/ $(t_{D\text{-}dab})_6$, $(a_{L\text{-}dab})_6$/$(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ and $(a_{L\text{-}dab}\text{-}a_{D\text{-}dab})_3$/$(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$. In the first case, no binding evidence was revealed, while in the second one, a little difference between the sum spectra (red line) and the mix (blue line) was observed (Figure 3.18), suggesting a possible hybridization between the strand with alternate chirality and homochiral oligomer. However, this finding was not supported by the UV melting experiment on the annealed strands: drift curve was recorded for this system, suggesting a possible transition at a temperature below 5°C (data not shown).

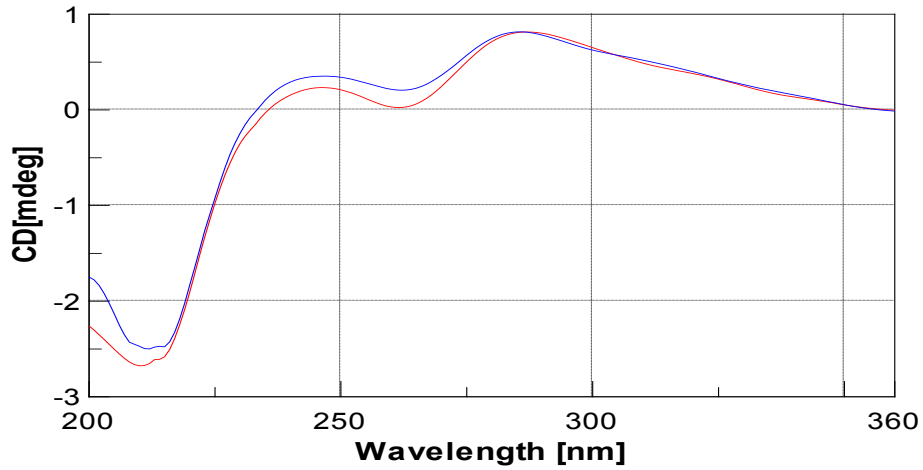


Figure 3.18: Red sum and blue mix $(a_{L\text{-}dab})_6/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$, (4 μM each strand in 10 mM phosphate buffer, pH 7.5).

In the case of $(a_{L\text{-}dab}\text{-}\ a_{D\text{-}dab})_3/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$, CD measurements with tandem cell revealed a difference between the sum and mix spectra (Figure 3.20), which, in this case, was confirmed also by the UV thermal denaturation curve (Figure 3.21): indeed, a transition phase at 11 °C was detected.

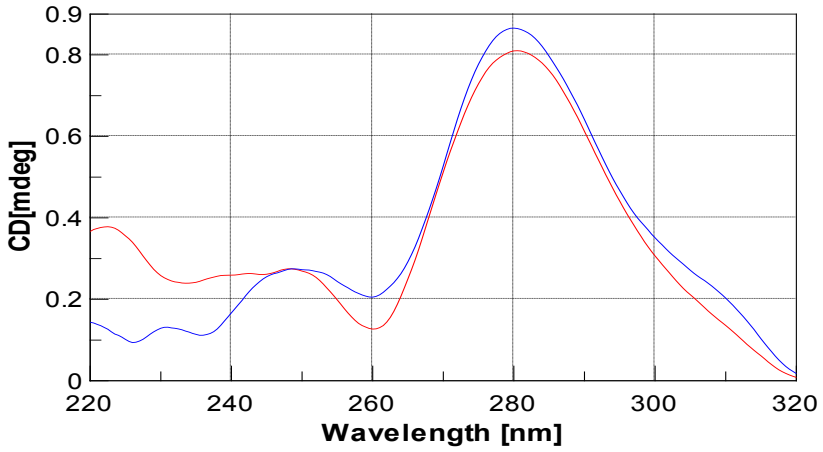


Figure 3.20: Red sum and blue mix of $(a_{L\text{-}dab}\text{-}\ a_{D\text{-}dab})_3/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$,(4μM each strand in 10 mM phosphate buffer,pH 7.5).

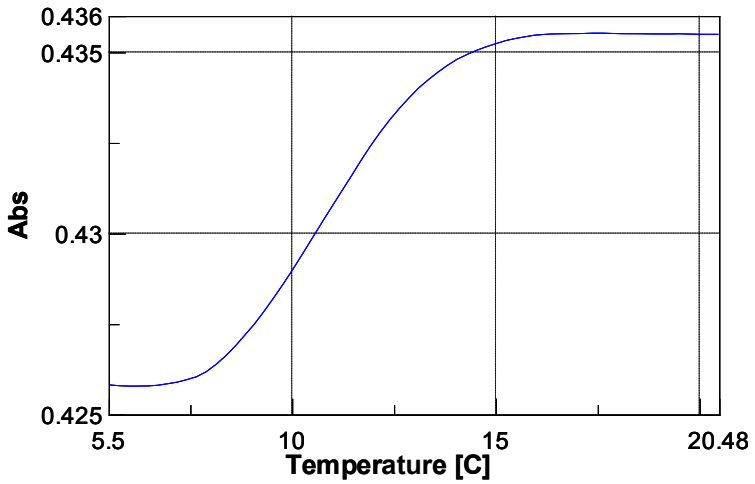


Figure 3.21: UV melting of $(a_{L\text{-}dab}\text{-}a_{D\text{-}dab})_3/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ 2 μM each strand, in 10 mM phosphate buffer, pH 7.5).

Also the self-pairing strand [H-G-$(a_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$-K-$NH_2$](**15**), forming palindromic complex with complementary sequences, which showed the CD spectrum reported in Figure 3.22, revealed a transition phase in the UV-melting curve (Figure 3.23) at 22 °C, confirming the binding between dabPNA strands of alternating chirality.

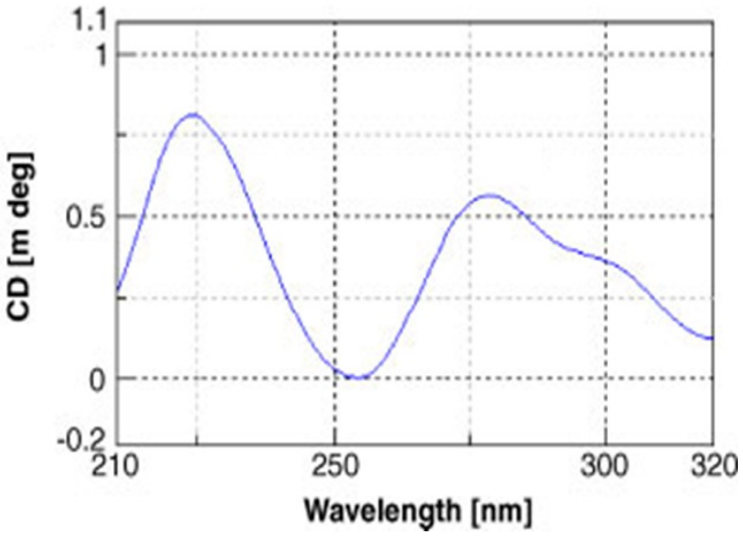


Figure 3.22: CD spectra in 10 mM phosphate buffer (pH 7.5), 10 ◦C, 1-cm path length cell, of 4 μM $(a_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$.

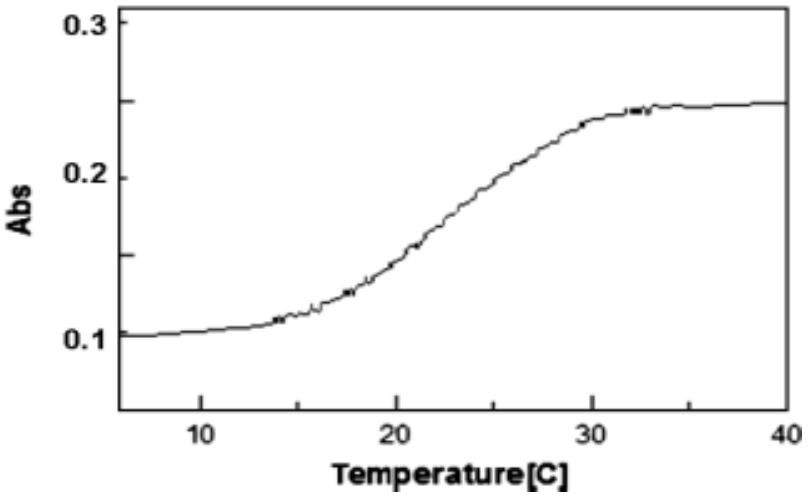


Figure 3.23: UV-melting experiments of the self-pairing strand $(a_L\text{−dab-}t_D\text{−dab})_3$; (2 µM each strand, in 10 mM phosphate buffer, pH 7.5)

The sigmoidal profile of the UV-melting curves suggested the formation of complexes based on cooperative hydrogen bonds and base stacking. The processes were reversible and the pairing was completed in about 9 °C (18min at 0.5°C min^{-1}) for system $(a_{L\text{-}dab}\text{- }a_{D\text{-}dab})_3/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ and 18 °C (36 min at 0.5 °Cmin^{-1}) for system $(a_{L\text{−}dab}\text{-}t_D\text{−}_{dab})_3$. A further analysis of the melting behavior relative to alternating chirality systems($(a_{L\text{-}dab}\text{- }a_{D\text{-}dab})_3/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ and $(a_{L\text{−}dab}\text{-}t_D\text{−}_{dab})_3$) revealed very substantial changes in the T_m as a function of the concentration only for the self-assembling system $(a_L\text{−dab-}t_D\text{−dab})_3$. Thus, for $(a_{L\text{−}dab}\text{-}t_D\text{−}_{dab})_3$ the formation of structures held together by an intricate network of hydrogen bonds based on a stoichiometry of more than 1 : 1 should be considered.
This hypothesis was supported by preliminary experiments of DLS that revealed for nucleopeptide $(a_{L\text{−}dab}\text{-}t_D\text{−}_{dab})_3$ (100 µM in 10 mM phosphate buffer, pH 7.5), the formation of multimeric aggregates with an molecular weight (MW) ranging between 1.6 × 104 and 4.5 × 105 kDa (with hydrodynamic radii, Rh, comprised between 109 and 634 nm at 16 ◦C), and between 1.7 × 103 and 4.5 × 104 kDa at 20 °C (Rh reduced to 34–203 nm) (Figure 3.24).

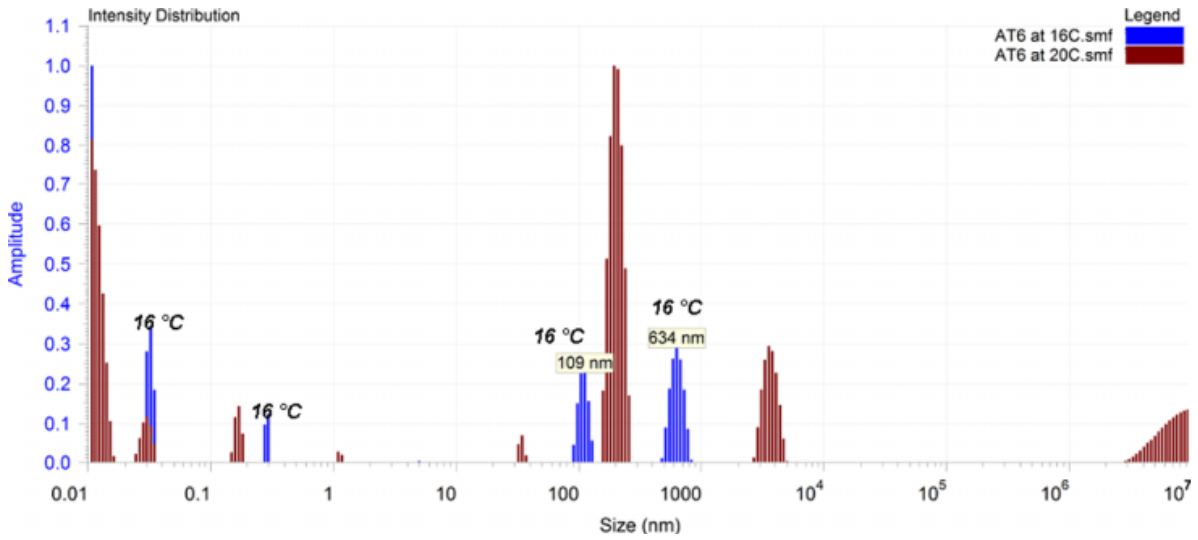


Figure 3.24: Dynamic light-scattering experiments on the self-complementary dabPNA oligomer $(a_L{-}dab\text{-}t_D{-}dab)_3$.

Further investigations on the viscoelastic properties of system $(a_{L-dab}\text{-}t_D{-}_{dab})_3$ are required to elucidate its bent to form hydrogels; moreover, NMR and X-ray experiments on both $(a_{L-dab}\text{-}t_D{-}_{dab})_3$ and $(a_{L\text{-}dab}\text{-}\ a_{D\text{-}dab})_3/(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ complexes are necessary to establish the orientation of the strands (parallel/antiparallel) and whether the Watson–Crick or Hoogsteen pairing is involved. In this frame, large-scale synthesis of oligomers $(a_{L\text{-}dab}\text{-}\ a_{D\text{-}dab})_3$, $(t_{L\text{-}dab}\text{-}t_{D\text{-}dab})_3$ and $(a_{L-dab}\text{-}t_D{-}_{dab})_3$ are in progress by our research group. In addition, we are going to synthesize also the C and G dabPNA monomers to assemble mixed sequence and different length oligomers. The binding properties of dabPNAs could be explained by comparing with the pairing properties of alanylPNAs containing an amino acid backbone with alternating configuration (Diederichsen et al, 1996). In analogy to alanylPNAs, which have a backbone differing from dabPNA for two C atoms, we expected for the γ -peptide backbone of dabPNA an anti-periplanar orientation of the α-amino group and thus of the nucleobases. When L- and D-configuration is alternate in the backbone, the nucleobases should be orientated in syn-periplanar geometry. Finally, serum stability assays, performed by HPLC, showed that dabPNA $(a_{L-dab}\text{-}t_D{-}_{dab})_3$ had a half-life greater than 48 h (Figure 3.25); even if a good stability to enzymatic degradation is a fundamental characteristic in view of potential bioengineering and biomedical applications of dabPNAs, it will be necessary to study the toxicity of these compounds.

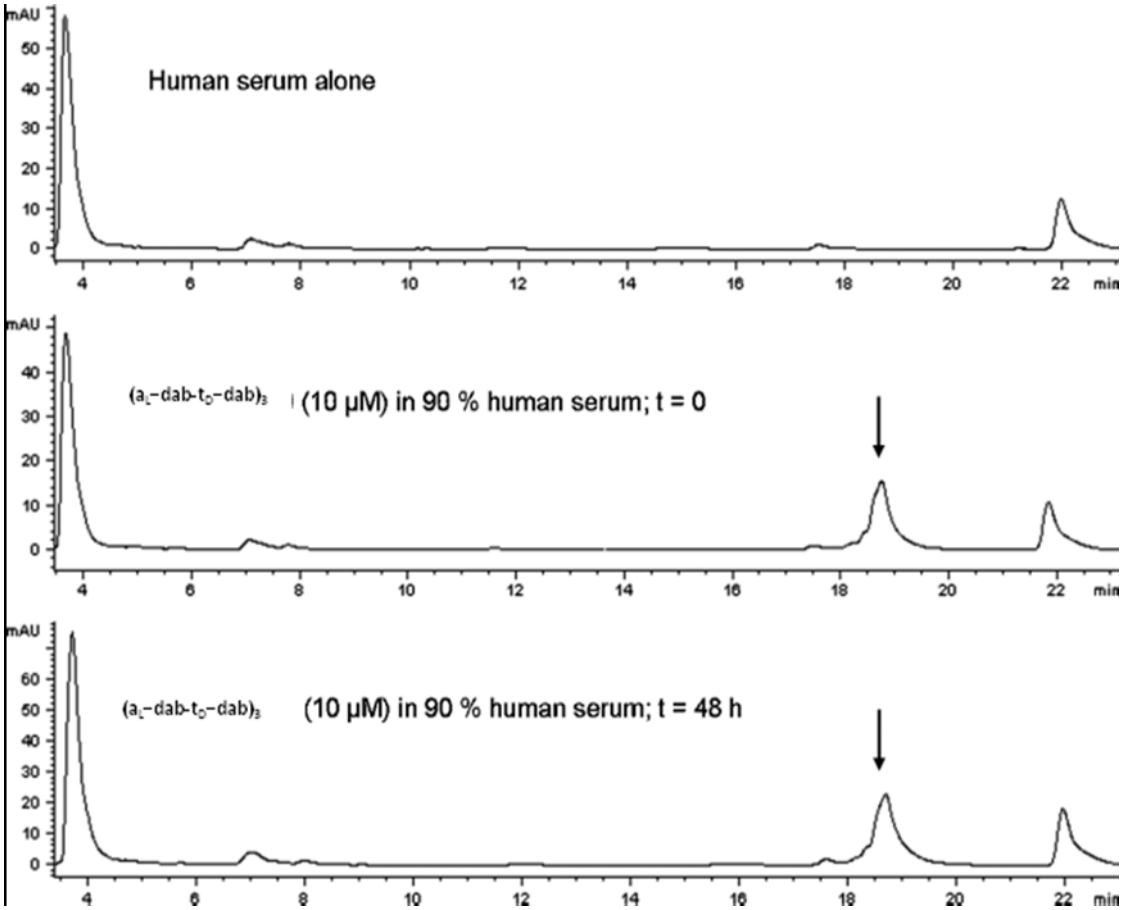


Figure 3.25: Serum stability assay on dabPNA $(a_L{-}dab\text{-}t_D{-}dab)_3$ performed by HPLC.

4. Conclusions

In conclusion, during the period of research done by the candidate in the PhD program, was designed and realized chiral nucleo-γ-peptides, dabPNAs, isomer of the aegPNAs and characterized by a 2,4-diaminobutyric acid (DABA)-based backbone carrying the carboxymethylated nucleobase on its α amino group. Our interest to dabPNAs arises, besides from the obtainment of new molecules for potential therapeutic and diagnostic applications, also from the possibility to explore their involvement in the prebiotic world, as recently proposed by Meierhenrich et al. (2004). This proposal followed the recovery of DABA in the extraterrestrial soil of the Murchison meteorite together with the molecular DNA constituent purine and pyrimidine bases, and other diaminoacids (Meierhenrich et al., 2004).
In order to realize the chiral dabPNA oligomers, the new monomers $t_{L\text{-}dab}$, $t_{D\text{-}dab}$, $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ were synthesized in good yield using suitable protected building blocks and characterized by NMR and ESI-MS techniques.
Initially, the homothymine oligomers $(t_{L\text{-}dab})_{12}$ and $(t_{D\text{-}dab})_6$ were synthesized, using a synthetic strategy that ensured the maintenance of chirality during the coupling steps, and tested for hybridization towards natural nucleic acids. No binding evidence with both DNA (dA_{12}) and RNA (A_{12}) was revealed by CD and UV experiments. Furthermore, the insertion of a single $t_{L\text{-}dab}$ unit in the middle or at N terminus of a homothymine aegPNA chain leads to a decreased binding efficiency to the target DNA in comparison to full aegPNA.
Since dabPNAs based on L- and D-DABA don't bind natural nucleic acids, we explored the possibility that complementary nucleopeptides based on D or L-DABA could bind between themselves. This property would be interesting in order to develop novel DABA-based dendrimeric systems, as new materials, and also to realize new diagnostic tools, as for example new molecular beacon probe. To verify this interesting possibility, the monomers $a_{L\text{-}dab}$ and $a_{D\text{-}dab}$ were oligomerized to the homoadenine hexamers $(a_{L\text{-}dab})_6$ and $(a_{D,L\text{-}dab})_6$.
In order to find the correct combination of chirality suitable for obtaining the binding, also the $(t_{D,L\text{-}dab})_6$ oligomer was realized to perform the hybridization studies. From CD and UV experiments, binding evidence was revealed in the case of the complementary oligomers with alternate chirality, $(a_{D,L\text{-}dab})_6$ and $(t_{D,L\text{-}dab})_6$, even if the stability of the complex formed was not so high (T_m 11°C).
Furthermore, some interesting properties relative to the self-complementary oligomer $(a_{L-dab}\text{-}t_{D-dab})_3$ emerged from preliminary DLS experiments that evidenced the formation of multimeric aggregates for this system.
These results, together with the high serum stability of the DABA-based oligomers, suggest further studies on dabPNAs as new self-recognizing bio-inspired polymers, with the potentiality to develop new nanomaterials or new biotechnological tools in bioengineering and biomedical applications.

5. Experimental section

5.1 Materials

Fmoc-Gly-OH, HATU, Fmoc-Lys(Boc)-OH, and PyBOP were purchased from Novabiochem. Anhydroscan DMF and NMP were from LabScan. Piperidine was from Biosolve. Solvents for HPLC and Ac_2O were from Reidel-de Ha¨en. TFA, Rink-amide resin, and TCH_2COOH were from Fluka. [*N*6-(benzhydryloxycarbonyl)-adenine-9-yl] acetic acid was purchased from ASM Research Chemicals GmbH and Co. Perspective Biosystem PNA kit (Fmoc/Bhoc monomers, HATU activator, Base solution (0.2 M DIEA, 0.3 M lutidine), Wash B (anhydrous DMF), DIEA, Capping Solution (5% Ac2O, 6% lutidine), Deblock solution (20% piperidine in DMF)) was purchased from PRIMM (Milan, Italy). Boc-L-DAB(Fmoc)-OH and Boc-D-DAB(Fmoc)-OH was from Bachem. Dry CH_3CN, DCM (for synthesis), DIEA and TFA (for HPLC) were from Romil. Deuterated solvents (DMSO, methanol) and TMP were from Aldrich. Thin-layer chromatography (TLC) analyses were performed on silica gel Macherey–Nagel G-25 UV254 plates (0.25-mm thick) visualized by UV light and by a ninhydrin staining solution. The reactions were monitored by TLC until all starting material had been consumed. Solvents for TLC analyses, and diethyl ether were from Carlo Erba. dA_{12} and A_{12} were purchased from Biomers (Ulm, Germany).

5.2 Instrumentations

^{1}HNMR and ^{13}C NMR spectra were recorded at 25 °C on Varian unity 300 MHz and Varian Inova 600 MHz spectrometers. Chemical shifts (δ) are given in parts per million (ppm) and all coupling constants (J) in Hz. Proton chemical shifts were referenced to residual CHD_2SOCD_3 (δ = 2.49, quin) and CHD_2OD (δ = 3.30, quin) signals.^{13}C NMR chemical shifts were referenced to the solvent (CD_3SOCD_3: δ = 39.5, sept; CD_3OD: δ = 49.0, sept). *aeg*PNA oligomers were assembled on solid phase with an Applied Biosystems Expedite 8909 oligosynthesizer. Crude samples containing PNA oligomers were centrifuged for 4 min at 4000 rpm (Z 200 A, Hermle). Products were analyzed and characterized by LC-MS, performed on an MSQ mass spectrometer (Thermo Electron, Milan, Italy) equipped with an ESI source operating at 3 kV needle voltage and 320 °C, and with a complete Surveyor HPLC system, comprising an MS pump, an autosampler and a photo diode array (PDA) detector, by using a Phenomenex Jupiter C18 300 A° (5 µm, 4.6 × 150 mm) column. Gradient elution was performed at 40 °C (monitoring at 260 nm) by building up a gradient starting with buffer A (0.05% TFA in water) and applying buffer B (0.05% TFA in acetonitrile) with a flow rate of 0.8 ml/min.
Semipreparative purifications were performed by RP-HPLC on a Shimadzu LC-8A equipped with an SPD-10A VP UV–vis detector, and on a Hewlett Packard/Agilent 1100 series equipped with a diode array detector, by using a Phenomenex Jupiter C18 300 A° (10 µm, 10 × 250 mm) column. Gradient elution was performed at 45 °C (monitoring at 260 nm) by building up a gradient starting with buffer A (0.1% TFA in water) and applying buffer B (0.1% TFA in acetonitrile) with a flow rate of 4 ml/min. Samples containing PNAs (crude or purified) were lyophilized in an FD4 freeze dryer (Heto Lab Equipment) for 16 h. CD spectra were obtained on a Jasco J-810 spectropolarimeter. UV spectra and UV-melting experiments were recorded on a UV–Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC- 505T

temperature controller. UV and CD measurements were performed in Hellma quartz Suprasil cells, with a light path of 1 cm and 2 × 0.4375 cm (Tandem cell). Dynamic light-scattering (DLS) experiments were performed on a Viscotek 802 DLS instrument equipped with a 50 mW internal laser operating at a wavelength of 630 nm.

5.3 Methods

Synthesis of the dabPNA monomers

Commercial Boc-L-DAB(Fmoc)-OH(200 mg, 0.45 mmol) and Boc-D-DAB(Fmoc)-OH (200 mg, 0.45 mmol) were separately reacted with a solution of TFA/DCM/H2O 4.5 : 4.5 : 1 at 50 ◦C for 1h, and subsequently treated as we recently reported (Roviello et al., 2006). The D- and L-products obtained, dissolved in dry DMF (8 ml) and DIEA (0.6 eq, 41.2 µl, 0.24 mmol) and TMP (0.6 eq, 33.3 µl, 0.24 mmol), were separately reacted with both the thymin-1-acetic acid (2 eq, 149 mg, 0.81 mmol) and the Bhoc-protected adenin-9-acetic acid (2 eq, 149 mg, 0.81 mmol), previously preactivated with HATU (1.9 eq, 293 mg, 0.77 mmol), DIEA (2 eq, 137.5 µl, 0.81 mmol), and TMP (2 eq, 107.0 µl, 0.81 mmol) for 2 min. After 2 h the reaction was quenched by removing the solvent under vacuum, suspending in H_2O/CH_3CN (7 : 3) and lyophilizing. The lyophilized crude was resuspended in cold water, sonicated, transferred into a falcon tube, and then centrifuged at 1200 rpm at room temperature. After the supernatant was removed, the white pellet obtained was washed twice with cold water and dried. In this way, products 4 and 9 were obtained as almost pure samples (95% purity), while for 12 and 12' the white pellets were dissolved in H2O/CH3CN 7 : 3 (v/v) and purified by semi-preparative RP-HPLC using increasing amounts of acetonitrile (from 35 to 75%in 25 min) in water at 25 ◦C with a flow rate of 4 $mlmin^{-1}$. Pure samples 3 and 4 were obtained as white powders in 51 and 49% yields, respectively. Compound 4: the LC-ESI-MS *m*/*z*: 507.32 (found), 507.52 (expected for MH^+); *δ*H (600 MHz, DMSO-*d*6) 11.35 (1H, br s, NH thymine), 8.54 (1H, br s, NH amide), 7.98 (2H, d, *J* = 7.3, aromatic CH Fmoc), 7.77 (2H, d, *J* = 7.3, aromatic CH Fmoc), 7.51 (2H, t, *J* = 7.3, aromatic CH Fmoc), 7.49–7.51 (1H, m, Fmoc-NH), 7.42 (2H, t, *J* = 7.3, aromatic CH Fmoc), 7.41 (1H, s, CH thymine), 4.43 (2H, s, CH_2 acetyl linker), 4.05–4.50 (4H, m, FmocCH-CH_2 and CH_α), 3.14 (2H, m, C*H*2NH), 2.00 (1H, m, part of an AB system centred at 1.91, C*H*2CH*α*), 1.83 (1H, m, part of an AB system centred at 1.91, CH_2CH_α), 1.82 (3H, s, CH_3 thymine); *δ*H (600 MHz, CD_3OD) 7.78 (2H, d, *J* = 7.3, aromatic CH Fmoc), 7.64 (2H, d, *J* = 7.3, aromatic CH Fmoc), 7.38 (2H, t, *J* = 7.3, aromatic CH Fmoc), 7.36 (1H, s, CH thymine), 7.30 (2H, t, *J* = 7.3, aromatic CH Fmoc), 4.47 (2H, s, CH_2 acetyl linker), 4.19–4.50 (4H, m, FmocCH-CH_2 and CH_α), 3.19 (1H, m, part of an AB system centred at 3.22, CH_2NH), 3.25 (1H, m, part of an AB system centred at 3.22, CH_2NH), 2.11 (1H, m, part of an AB system centred at 1.98, CH_2CH_α), 1.85 (1H, m, part of an AB system centred at 1.98, CH_2CH_α), 1.84 (3H, s, CH_3 thymine); *δ*C (150 MHz, DMSO-*d*6) 170.98 (COOH), 168.48 (CH_2*C*ONH), 160.10 (thymine C-4), 155.00 (OCONH), 147.96 (aromatic Fmoc 2C), 147.89 (thymine C-2), 146.36 (aromatic Fmoc 2C), 144.73 (thymine C-6), 131.65 (aromatic Fmoc 2CH), 131.11 (aromatic Fmoc 2CH), 129.18 (aromatic Fmoc 2CH), 124.15 (aromatic Fmoc 2CH), 111.96 (thymine C-5), 69.40 (Fmoc CH_2), 53.02 (CH_2 acetyl linker), 50.73 (CH_α), 44.1 (Fmoc CH), 41.30 (CH_2NH), 22.56 *(*CH_2CH_α*)*, 15.91 (thymine CH_3). Compound 9: the LC-ESI-MS were almost identical to the enantiomer 4; compound 12: LC-ESI-MS *m*/*z*: 726.36 (found), 726.00 (expected for

MH+); *δ*H (600 MHz, DMSO-*d*6) 8.43 (1H, s, H-8 adenine), 8.22 (1H, s, H-2 adenine), 7.13–7.75 (18H, aromatic CH Fmoc and Bhoc, and 1H Fmoc-N*H*), 4.47 (2H, s, CH_2 acetyl linker), 4.19–4.50 (4H, m, FmocC*H*-CH_2 and CH_α), 3.19 (1H, m, part of an AB system centered at 3.22, CH_2NH), 3.25 (1H,m, part of an AB system centered at 3.22, CH_2NH), 2.11 (1H, m, CH_2CH_α), 1.85 (1H, m, CH_2CH_α); Compound 12': the LC-ESI-MS were almost identical to the enantiomer 12.

Solid-phase Synthesis of Oligomers 5–8 (Table 1)

Solid support functionalization and manual solid-phase oligomerizations were carried out in short PP columns (4 ml) equipped with a PTFE filter, a stopcock and a cap. Solid support functionalization: Rink-amide resin (0.50 mmol NH_2/g, 128 mg) was functionalized with a lysine (Fmoc- Lys(Boc)-OH, 0.5 equiv., 14.8 mg, 32 μmol) using PyBOP (0.5 equiv., 16.8 mg, 32 μmol) as activating agent and DIEA (1 equiv., 12 μl, 64 μmol) as base for 30 min at room temperature. Capping of the unreacted amino groups was performed with Ac_2O (20%)/DIEA (5%) in DMF. Loading of the resin was checked by measuring the absorbance of the released Fmoc group (ε_{301} = 7800, quantitative yield) after treatment with a solution of piperidine (30%) in DMF (UV Fmoc test) and the resultant reduced to 0.25 mmol/g with respect to the initial functionalization. Automatic solid-phase assembly of the aegPNAs was performed on an Expedite 8909 nucleic acid synthesis system using a standard 2-μmol-scale protocol and Fmoc chemistry leaving the final Fmoc on, in order to evaluate SPS yields by the UV Fmoc test. Manual couplings of dabPNA monomers were monitored by the UV Fmoc test in order to evaluate the incorporation yields of the new monomer. A glycine residue (3 equiv.) was attached to the *N*-terminus of all the oligomers by using PyBOP (3 equiv.)/DIEA (6 equiv.) in DMF as the activating system. After removal and quantification of the Fmoc group, all oligomers were cleaved from the resin and deprotected under acidic conditions (TFA/*m*-cresol 4 : 1 v/v). The oligomers were isolated by precipitation with cold diethyl ether, centrifugation and lyophilization. Purified oligomers (semipreparative HPLC) were quantified and characterized by LC-ESI-MS.

H-Gly-($t_{L\text{-}dab}$)$_{12}$-Lys-NH_2 5. (t_L-dab)$_{12}$ was assembled manually on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) using the following protocol: 0.2 M Fmoc-tdab-OH monomer in NMP (3 equiv., 120 μl, 24 μmol), 0.2 M PyBOP in DMF (3 equiv., 120 μl, 24 μmol) and 0.2 M NMM in DMF (6 equiv., 240 μl, 48 μmol) were added in sequence to the resin and the coupling was left for 30 min with shaking at room temperature. Successively, the unreacted amino groups were capped with PNA Cap solution for 5 min. Fmoc removal was accomplished with PNA Deblock solution (5 min) and was monitored at every step by UV measurements: average yield for the first six steps was 96%, while for the last six steps a lowering of the yields from 96 to 70% was observed probably because of the enhanced aggregation tendency of the increasing homothymine chain (overall yield: 25%). Yield of the last coupling with glycine was 97%. After cleavage and deprotection, the oligomer 5 was purified by semipreparative RP-HPLC using a linear gradient of 8% (for 5 min) to 25% B in A over 30 min: t_R = 31.0 min; UV quantification of the purified product gave 100 nmol of 6; ESI-MS (Figure 3) *m*/*z*: 1134.8 (found), 1133.4 (expected for [M + 3H]3+).

H-Gly-t_{12}-Lys-NH2 6. Standard t_{12} aegPNA was assembled on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) on an automatic synthesizer. After the synthesis was complete, the final Fmoc was left on and removed manually to perform the Fmoc UV

test. The overall yield of t_{12} was only 18% probably because the *flow through* synthesis of the automatic synthesizer enhances the aggregation problems of the homothymine sequence. Glycine was incorporated manually as the last residue with high efficiency (98% yield). The cleaved and deprotected oligomer was purified by semipreparative RP-HPLC using a linear gradient of 8% (for 5 min) to 20% B in A over 30 min: t_R = 27.9 min; UV quantification of the purified product gave 60 nmol of **8**; ESIMS *m*/*z*: 1132.0 (found), 1697.1 (found), 1133.4 (expected for [M + 3H]3+), 1699.7 (expected for [M+ 2H]2+).

H-Gly-(t_{dab})t11-Lys-NH2 7. The *aeg*PNA part of 7 was assembled on Rink-amide-Lys-NH2 resin (0.25 mmol/g, 32 mg) in an automatic synthesizer leaving the final Fmoc on (yield: 20%), while the *dab*PNA monomer was attached manually as the last residue following the protocol described for 5 (yield: 95%). After the last coupling with glycine, an overall yield of 18% was obtained. After cleavage and deprotection, the oligomer was purified by semipreparative RP-HPLC using a linear gradient of 8% (for 5 min) to 17% B in A over 35 min: t_R = 29.8 min; UV quantification of the purified product gave 70 nmol of 7; ESI-MS *m*/*z*: 1133.0 (found), 1699.8 (found), 1133.4 (expectedfor $[M+ 3H]^{3+}$), 1699.7 (expected for $[M+ 2H]^{2+}$).

H-G-$t_6t_{L\text{-}dab}t_5$-K-NH_2 8. The synthesis of the aegPNA part of **8** was performed on Rink-amide-Lys-NH_2 resin (0.25 mmol/g, 32 mg) by the automatic synthesizer except for the dabPNA monomer, which was attached manually in the middle of the aegPNA sequence (overall yield: 20%). Glycine was incorporated manually as the last residue with 95% yield. The cleaved and deprotected oligomer was purified by semipreparative RP-HPLC using a linear gradient of 7% (for 5 min) to 19% B in A over 40 min: t_R = 30.1 min; UV quantification of the purified product gave 80 nmol of **8**; ESIMS *m*/*z*: 1132.8 (found), 1698.5 (found), 1133.4 (expected for $[M + 3H]^{3+}$), 1699.7 (expected for $[M+ 2H]^{2+}$).

Solid Phase Syntheses of dabPNA oligomers (10-15)

Solid phase oligomerizations were carried out in short PP columns (4 ml) equipped with a polytetrafluoroethylene (PTFE) filter, a stopcock and a cap. Solid support functionalization: Rink-amide resin (0.50 mmol NH_2/g, 128 mg) was functionalized with a lysine (Fmoc-Lys(Boc)-OH, 0.5 eq, 14.8 mg, 32 µmol) using PyBOP (0.5 eq, 16.8 mg, 32 µmol) as activating agent and DIEA (1 eq, 12 µl, 64 µmol) as base for 30 min at room temperature. Capping of the unreacted amino groups was performed with Ac_2O (20%)/DIEA (5%) in DMF. Loading of the resin was checked by measuring the absorbance of the released Fmoc group (ε_{301} = 7800, quantitative yield) after treatment with a solution of piperidine (30%) in DMF (UV Fmoc test)andresulted reduced to 0.25 $mmolg^{-1}$ with respect to the initial functionalization. DabPNA monomers were coupled manually to the solid support using the following protocol: a mixture of the monomer (75 µl of a 0.2 M solution in DMF, 15 µmol, 3 eq), HATU (75 µl of a 0.2 M solution in DMF, 15 µmol, 3 eq), and DMF (100 µl) was introduced into the reactor containing the Rink-amide-Lys-NH2 resin (0.25 mmol g−1, 20mg,5 µmol). Successively, 75 µl (15 µmol, 3 eq) of the TMP solution (0.2 M in NMP) was added to

the stirred reaction in four portions over 1 h. After the coupling step, unreacted amino groups were capped with PNA capping solution for 5 min. Fmoc removal was accomplished with PNA deblocking solution for 10 min, and was monitored by UV Fmoc test to evaluate the incorporation yields of the monomers. A glycine residue (3 eq) was attached to the N-terminus of all the oligomers by using PyBOP (3 eq)/DIEA (6 eq) as activating system in DMF. After removal and quantification of the final Fmoc group, all oligomers were cleaved from the resin and deprotected under acidic conditions (TFA/*m*-cresol 4 : 1 v/v). After precipitation with cold diethyl ether, centrifugation and lyophilization, crude oligomers, were purified by semi-preparative RP-HPLC at 45 °C using a linear gradient of 5% (for 5 min) to 22% acetonitrile (0.1% TFA) in water (0.1% TFA) over 25 min. Purified oligomers were quantified by UV and characterized by LC-ESI-MS. UV quantification was performed by dissolving oligomers in a known amount of milliQ water and measuring the A_{260} at 85 °C, using as extinction coefficients those of the aegPNA monomers (ε_{260} = 8600 cm^{-1} M^{-1} for thymine monomer, ε_{260} = 13 700 cm^{-1} M^{-1} for adenine monomer). H-G-$(t_{L-dab})_6$-K-NH_2 : HPLC t_R = 25 min; UV quantification (ε_{260} = 51 600 cm^{-1} M^{-1}) of the purified product gave 110 nmol (2.2%yield); ESI-MS *m*/*z*: 900.07 (found), 900.8 (expected for $[M + 2H]^{2+}$). H-G-$(t_{D-dab})_6$-K-NH_2 : HPLC t_R = 25 min; UV quantification of the purified product gave 120 nmol (2.4% yield); ESI-MS *m*/*z*: 900.17 (found), 900.8 (expected for $[M + 2H]^{2+}$). H-G-$(a_{L-dab})_6$-K-NH_2 : HPLC t_R = 20 min; UV quantification (ε_{260} = 82 200 cm^{-1} M^{-1}) of the purified product gave 204 nmol (4.1%yield); ESI-MS *m*/*z*: 927.9 (found), 927.08 (expected for $[M + 2H]^{2+}$). H-G-$(t_{L-dab}$-$t_{D-dab})_3$-K-NH_2 : HPLC t_R = 23 min; UV quantification of the purified product gave 95 nmol (1.9% yield); ESI-MS *m*/*z*: 900.30 (found), 900.8 (expected for $[M + 2H]^{2+}$). H-G-$(a_{L-dab}$-$a_{D-dab})_3$-K-NH_2 : HPLC t_R = 22 min; UV quantification of the purified product gave 130 nmol (2.6% yield); ESI-MS *m*/*z*: 928.0 (found), 927.08 (expected for $[M + 2H]^{2+}$). H-G-$(a_{L-dab}$-$t_{D-dab})_3$-K-NH_2 : HPLC t_R = 22 min; UV quantification (ε260 = 66 900 cm^{-1} M^{-1}) of the purified product gave 140 nmol (2.8% yield); ESI-MS *m*/*z*: 915.3 (found), 914.8 (expected for $[M + 2H]^{2+}$).

UV and CD Studies

Annealing of all PNA oligomers with complementary dA_{12} was performed by mixing equimolar amounts of the two strands in 10 mM phosphate buffer at pH 7.5 to achieve a duplex concentration of 4 μM. The solution was heated at 85 °C (5 min) and then allowed to cool slowly to room temperature. Thermal melting curves were obtained by recording the UV absorbance at 260 nm while the temperature was ramped from 20 to 97 °C at a rate of 0.5°C/min. *T*m values, calculated by the first derivative method. CD spectrum was recorded from 320 to 200 nm at 20 °C under the following conditions: scan speed 50 nm/min; data pitch 2 nm; bandwidth 2 nm; response 4 s; accumulations 3.

Annealing of complementary dabPNA oligomers was performed by mixing equimolar amounts of the two strands in 10 mM phosphate buffer (pH 7.5). The solution was heated at 85 ◦C (5 min), allowed to cool slowly to room temperature and then kept at 4 ◦C for 2 h. Thermal-melting curves were obtained by recording the UV absorbance at 260 nm, while the temperature was increased at a rate of 0.5 ◦Cmin^{-1}. *T*m values were calculated by the first derivative plot. CD spectra were recorded from 360 to 200 nm: scan speed 50 nm min^{-1}, data pitch 2 nm, band width 2 nm, response 4 s, ten accumulations. CD-binding experiments were performed with a tandem cell which

allows to record the ‘sum’ spectrum of two separated sample solutions kept each in one of the two reservoirs of the cell, and the ‘mix’ spectrum obtained after the cell was turned upside down to allow the mixing of the two samples.

Dynamic Light-Scattering Experiments

Hydrodynamic radii of the nucleopeptide complexes were measured via scattered light recorded at 90◦ angle to incident radiation at a wavelength of 630 nm. From standard autocorrelation functions, measured diffusion coefficients were related to particle hydrodynamic radius via the Stokes–Einstein equation.

Serum Stability Assay

Nucleopeptide $(a_L{-}dab\text{-}t_D{-}dab)_3$ (10 µl, 200 µM) was added to 90 µl of 100% fresh human serum ($(a_L{-}dab\text{-}t_D{-}dab)_3$ was 10 µM in 90% serum) in a microvial and the mixture was incubated at 37 ◦C. Aliquots of 10 µl each were taken at times = 0, 1, 2, 24, and 48 h, quenched with 10 µl of 7 M urea solution, kept at 95 ◦C for 3 min, and then stored at −20 ◦C until subsequent analysis. The withdrawn samples were analyzed by RP-HPLC on a Phenomenex Juppiter C18 300 Å (5 µm, 4.6 × 250 mm) column using a linear gradient of 8% (for 5 min) to 20% acetonitrile (0.1% TFA) in water (0.1% TFA) over 30 min.

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Abbreviations

A	Adenine
ACN	Acetonitrile
Aeg	*N*-(2-aminoethyl) glycine
Bhoc	Benzhydryloxycarbonyl
Boc	*tert*-Butyloxycarbonyl
C	Cytosine
Cbz	Benzyloxycarbonyl
CD	Circular Dichroism
DABA	Acido 2,4-diamminobutanoico
dabPNA	diaminobutyrylPNA
DCM	Dichloromethane
DIPEA/DIEA	Diisopropylethylamine
DMF	N,N-dimethylformamide
DMSO	Dimethylsulphoxide
ESI	Electrospray Ionization
FDA	Food and Drug Administration
FISH	Fluorescent *In Situ* Hybridization
Fmoc	9-Fluorenylmethoxycarbonyl
G	Guanine
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
LC	Liquid Chromatography
LNA	Locked Nucleic Acid
MP	Morpholino phosphoramidite
MS	Mass Spectrometry
NMP	1-Methyl-2-pyrrolidinone
NMR	Nuclear Magnetic Resonance
ODN	Oligodeoxyribonucleotide
PCR	Polymerase Chain Reaction
PDBA	Acido poli(2,4-diaminobutanoico)
PNA	Peptide (polyamide) Nucleic Acid
PP	Polipropilene
PTFE	Politetrafluorethylene
PyBop	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
SNP	Single Nucleotide Polymorphism
T	Thymine
TEM	Transmission Electron Microscope
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
TMP	2,4,6, trimetilpiridine
UV	Ultraviolet

Publications

1. Roviello GN, Musumeci D, Moccia M, Castiglione M, Cesarani A, Bucci EM, Saviano M, Pedone C, Benedetti E. "**Evidences of complex formation between DABA-based nucleo-gamma-peptides with alternate configuration backbone.**" J Pept Sci. 2009 Mar;15(3):147-54.

2. Roviello GN, Musumeci D, Bucci EM, Castiglione M, Cesarani A, Pedone C, Piccialli G."**Evidences for complex formation between L-dabPNA and aegPNA."** Bioorg Med Chem Lett. 2008 ;18(17):4757-60.

3. Roviello GN, Musumeci D, Moccia M, Castiglione M, Sapio R, Valente M, Bucci EM, Perretta G, Pedone C. "**dabPNA: design, synthesis, and DNA binding studies.**" Nucleosides Nucleotides Nucleic Acids. 2008;26(10-12):1307-10.

Book Chapters:

4. G. N. Roviello, D. Musumeci, E. M. Bucci, M. Castiglione, C. Pedone, E. Benedetti, R. Sapio, M. Valente, "**Further studies on nucleopeptides with DABA-based backbone**"; Chemical Engineering Transaction 2008, Volume 14, 2008: 393-400

Congress Comunications

1. Castiglione Mariangela, Roviello Giovanni, Musumeci Domenica, Cesarani Annalisa, Benedetti Ettore, Bucci Enrico and Pedone Carlo **"New insights on DABA-based nucleopeptides**." 11th Naples Workshop on Bioactive Peptides", Napoli 24-27 Maggio 2008.

2. Roviello Giovanni, Musumeci Domenica, Castiglione Mariangela, De Cristofaro Andrea, D'Alessandro Cristian, Pedone Carlo, Bucci Enrico and Benedetti Ettore **"Chiral nucleopeptides for biotechnological applications: synthesis and hybridization studies**." 11th Naples Workshop on Bioactive Peptides", Napoli 24-27 Maggio 2008.

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