Università degli studi di Parma

Dottorato di Ricerca in Scienze Chimiche

XXII ciclo
Triennio 2007-2009

Tuning PNA Properties by Chemical Modifications: Design Synthesis and Applications

Tutors:
Prof. Rosangela Marchelli
Prof. Stefano Sforza

Dottorando:
Alessandro Calabretta

2010

Contents

Preface

The outstanding advances of disciplines related to life sciences during the last decades led to the definition of a new type of scientist, quite different from the traditional figures of the last century. Today, the borderline between chemistry, biology, material science and other disciplines has become less defined. A modern scientist is no longer a person with a high experience in a very well delimited area, but he should rather have the ability to combine his defined background with a wide knowledge in many different fields, being able to deal at the same time with chemistry, biology, physics, medicine, and so on, in order to get a more complete understanding of the processes studied.

In the work reported in this Ph.D. Thesis we tackled several aspects generally related to life sciences starting from a hard core of organic chemistry and trying to combine it with different disciplines, in order to have a deeper understanding concerning the properties of the synthesized molecules. The leading actors in this work are Peptide Nucleic Acids (PNAs), a class of oligonucleotide analogues able to recognize complementary nucleic acid sequences with improved selectivity and sensitivity, if compared to standard DNA oligonucleotides. This property has already made them very attractive tools for applications in many fields. In biology, they are extremely useful for genetic studies; in medicine, they are studied as tool in antisense and antigene therapy, and for the realization of diagnostic devices for genetic diseases; in food science, they can be used for specifically recognizing the ingredients in a food product; in material science, their assembly on surface can be manipulated in order to obtain desired patterns; in chemistry, synthetic approaches and chemical modifications are often investigated as well as their supramolecular binding properties.

In this thesis, studies on modified PNAs will be reported. In particular, new chemical modifications will be introduced in the PNA backbone in order to *ad hoc* tune their properties for different applications. In particular, these new probes will be exploited for the development of new diagnostic methods and in nuclear uptake experiments.

In Chapter 1 PNAs will be introduced, starting from the origins, reporting the various synthetic approaches used to synthesize these molecules, and showing the potential modifications which can be introduced in backbone and nucleobase, together with a summary of their main applications, both in biomedicine and diagnostics.

In Chapter 2 a particular type of modified PNA probe will be presented, PNA molecular beacons. This type of molecule can recognize DNA without the need to label it in a pre-assay step, because of their ability to selectively recognize complementary sequences and to emit a fluorescence signal when hybridization takes place. PNA beacons will be applied in AE-HPLC to develop a system able to detect with good selectivity a single nucleotide polymorphism diagnostic of an olive cultivar.

In Chapter 3 the synthesis of chiral PNAs based on the structure of arginine will be described. These PNAs are designed in analogy to those based on lysine, which already demonstrated enhanced recognition performances compared to standard PNAs, especially in point mutation recognition. The replacement of lysine with arginine is necessary to develop chiral PNAs to be used on surfaces, for example for the fabrication of PNA-microarrays. In the chapter, the synthesis of the modified monomers will be reported and different designs will be tested. Finally the recognition properties of these probes will be compared with those of lysine-PNAs.

Chapter 4 will show their application for the fabrication of microarrays to be used for the recognition of two SNPs into the ApoE gene, which are involved in Alzheimer disease early onset. The design and synthesis of these PNA probes will be described together with the optimization of the method for the realization of the microarray system. Finally, the use of the device for the recognition of the different genotypes which can be given by the combination of possible SNPs will be reported.

The possibility to upgrade the fabrication of the devices presented in Chapter 4 will be discussed in Chapter 5. Here, the use of microcontact printing for the derivatization of surfaces with chiral PNAs will be demonstrated. In the first part the optimization of the method will be described, since this is the first reported study on the use of microcontact printing involving PNAs. The potential applications of these simple devices will be demonstrated, studying the probe performances when linked to the surface, and testing the selectivity of the devices. The fabrication of microarrays performed by coupling a commercial array spotter with microcontact printing will be demonstrated, showing the improvements in terms of time required, reproducibility and cost of the device.

Chapter 6 will explore a new potentiality of chiral PNAs, i.e. the ability to behave as a protein analogue, rather than as an oligonucleotide analogue. The design and synthesis of a model chiral PNA mimicking a Nuclear Localization Signal, a peptide able to be actively internalized in the nucleus by a receptor-mediated system, will be shown. The potential interaction between the modified PNA and the receptor will be shown through nuclear uptake

experiments. The modified PNA labeled with a fluorophore will be demonstrated to behave exactly like the NLS peptide, entering the nucleus, whereas a standard achiral PNA did not show this ability.

The results presented in this Ph.D. Thesis are an important contribution to the field of Peptide Nucleic Acids and the advancements here presented pave the way for new applications.

Chapter 1

PNAs: From birth to adulthood

This chapter presents a general overview about Peptide Nucleic Acid synthesis, development and applications. The section includes the various synthetic approaches to this class of molecules, including standard and modified synthetic strategies. The insertion of many interesting features within the backbone or into the nucleobases are described, together with their influence on the hybridization properties, with particular focus on chiral PNAs, a class of molecules characterized by the insertion of amino acid side chains as substituents in the backbone. Finally, some of the most promising applications in medicine and analytical biochemistry are reported, showing some results obtained in the recognition of nucleic acids so far.

1.1 Introduction

One of the most challenging target for the scientists in the last years is to understand what takes place in the cells and the ability to specifically control cell behavior, directing it towards desired functions. In particular, one of the most important and studied process is the storage, replication and use of all the information encoded in DNA because of its involvement, together with other factors, in the processes that regulate the whole cell life. How the genetic information is stored in this molecule, how it is transferred to RNA and how it is used in all aspects of cell life have been the subject of extensive studies. Nowadays many of these processes have been understood, but still some are unknown. What is clear is that the ability to control and manipulate nucleic acids in a cell might open the possibility to control the cell itself. Targeting a particular gene directly in the nucleus or the messenger RNA produced in the cytoplasm can selectively suppress or enhance the presence of a certain protein in a cell, changing its fate in terms of development, replicative ability, toxicity, or killing it. This ability could be exploited in many fields, but one of the most important is undoubtedly medicine, where selective killing or selective growth of particular cells can make the difference between health or illness. Anyway, gene regulation is only part of the goals that can be achieved by techniques aimed at specific gene targeting, since also diagnostic applications are of paramount importance. A specific and selective detection of particular sequences may be useful in order to better understand cellular processes in which those sequences are involved. Alternatively, when it is already known that a particular sequence is involved in the onset of a disease, its fast recognition can be extremely useful in the early diagnosis. Finally, the recognition of DNA sequences typical of bacteria or contaminant organisms, or RNA sequences typical of viruses, can give very important information about their presence or absence in air, water or food.

1.2 Synthetic DNA Analogues

It is clear that nucleic acid recognition is a very hot topic. In order to achieve this task, highly performing molecular tools, able to form stable complexes only with the desired sequences are needed. As a matter of fact, given a particular DNA sequence in a given genome, there always is a huge number of other sequences, only differing for few bases or even for only

one, that can also be recognized by the same probe due to the similarity with the target sequence. For this reason, the molecular probe used to target a given sequence has to be very selective and, at the same time, it has to form very stable complexes, given the low amount of DNA to be recognized usually present in the biological matrices.

Synthetic DNA oligonucleotides complementary to the target sequence are among the most common probes used for this purpose. Nowadays, DNA oligonucleotides are commercially available and quite cheap, but several drawbacks are associated with them. Synthetic nucleotides identical to natural ones can obviously be recognized and cleaved by DNAse enzymes, a limitation for in vivo applications, and a potential problem also for in vitro applications, when contaminations occur. Moreover, in order to obtain stable complexes, long oligonucleotides are often required (20 nuclobases or more), and this can lead to a decreased selectivity that may disturb target recognition in diagnostic applications or affect unselectively many different cells during in vivo applications.[1]

A further problem is given by the high ionic strength required for overcoming the electrostatic repulsion between the two negatively charged strands (the target DNA and the oligonucleotide probe), limiting diagnostic applications in a low ionic strength environment. Finally, in vivo applications of DNA oligonucleotides are usually limited by the low cellular uptake, since simple oligonucleotides are not able to enter the cells[2]. The answers to these problems might come from the synthesis of new molecules obtained by suitable modifications of the DNA structure[3].

Many modifications have been introduced, and among those which gave good results, some presented only minor changes (for example the bonds between each DNA monomer), others showed major structural changes within the backbone (Figure 1-1).

Phosphorothioate oligonucleotides (PS-DNA) can be obtained by substitution of one non linking oxygen atom from the phosphate group with a sulfur[4]. These molecules are more chemically and enzimatically resistant, and they have been used as antisense drugs, and are usually classified as first generation antisense oligonucleotides. However, they still presented some problems, such as a lower binding efficiency towards RNA, aspecific interactions with some proteins, and some toxicity when used in living organisms. N3'-P5' phosphoroamidates (NPs)[5], are another structure obtained by the substitution of an oxygen atom from the phosphodiester group with a nitrogen atom. These analogues were synthesized and tested in binding assays, showing increased binding towards complementary sequences if compared to DNA oligonucleotides as well as enhanced enzyme resistance. Evolution towards better modifications led to 2'-O-methyl (OMe) and 2'-O-methoxy-ethyl RNA (MOE), usually

classified as second generation antisense oligonucleotides. In these classes of molecules, substituents have been introduced in position 2' of the ribose ring, improving performances in terms of RNA affinity and lowering the in vivo toxicity. These oligonucleotides have been applied as antisense agents thanks to their ability to bind and block m-RNA.

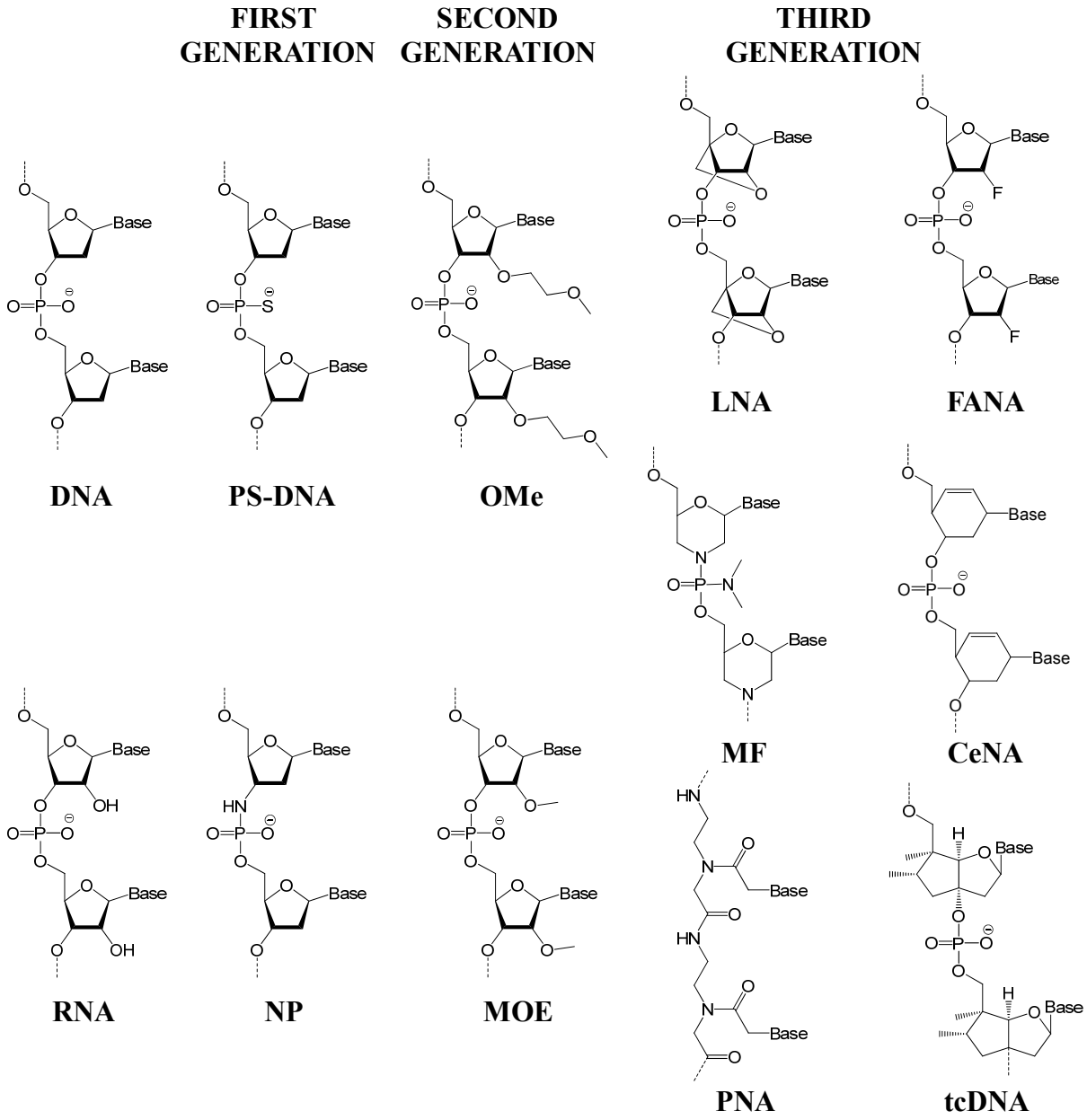


Figure 1-1: Chemical structure of the main oligonucleotide analogues of first, second and third generation

These features have been further improved with the so-called third generation oligonucleotides, where more substantial modifications have been inserted. Locked Nucleic Acids (LNAs) are among the most popular and are obtained by inserting a methylene bridge

between the 2'oxygen and the 4' carbon of the ribose ring[6]. Pure LNA oligonucleotides can be used, as well as mixed oligonucleotides with standard DNA monomers and LNA monomers, obtaining an increased affinity for DNA and RNA, and resistance to enzymes. The good cellular uptake also allowed applications in gene therapy with good results[7]. In morpholino oligonucleotides (MF), the ribose ring is replaced by a morpholino moiety and phosphoroamidate linkages are used instead of phosphodiester bonds[8]. The affinity for complementary sequences is similar to that of DNA oligonucleotides, the enzymatic stability is high and unspecific interactions with proteins are not observed, allowing for their use for in-vivo applications. Cyclohexene nucleic acids (CeNA)[9], tricyclo-DNA (tcDNA)[10], 2'-deoxy-2'-fluoro-β-D-arabino nucleic acid (FANA)[11] are other types of DNA analogues which show an increased affinity towards DNA and RNA, if compared to standard oligonucleotides. The affinity usually increases because of the absence of negative charges in the backbone, avoiding electrostatic repulsions with the target DNA strand, or for the introduction of more rigid groups, able to maintain the correct nucleobase orientation to form Watson-Crick interactions. The increase of in vivo stability usually observed is due to the inability of degrading enzymes to recognize the modified structures[12].

1.3 PNA

The quest for the development of a high performance DNA analogue led in 1991 to the synthesis of another type of modified nucleic acids, called Peptide Nucleic Acids (PNAs)[13].

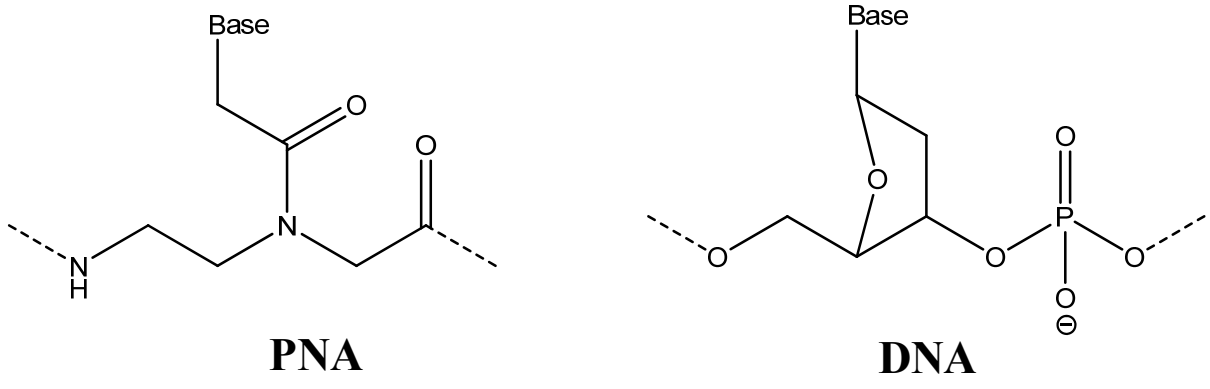


Figure 1-2: Comparison of PNA and DNA chemical structures (P. E. Nielsen et al., *Science*, 1991, 254, 1497)

This class of nucleic acid analogues has been obtained by completely changing the DNA backbone chemistry. The classical sugar-phosphate backbone, in fact, has been replaced with

a psudopeptide one based on the repetition of achiral N-(2-aminoethyl)glycine monomer units linked by amide bonds (Figure 1-2). Nucleobases are derivatised with a carboxymethyl unit that is linked to the nitrogen in position 3 of the backbone by another amide bond. Although this class of molecules appears very different from standard DNA molecules, it preserves the correct features to form Watson-Crick bonds, since the distances between the nucleobases, and their distances from the backbone, are the same as in standard DNA. Although they preserve the ability to form the same interactions as the DNA oligonucleotides, the different chemical structure of PNAs causes some modifications of their chemical and physical properties. The most evident difference is the lack of charge in the PNA backbone, due to the replacement of the phosphate bond with an amide one. This feature avoids charge repulsion when the molecule forms duplexes with DNA or RNA, increasing duplex stability. Unfortunately, the very same feature introduces some problems in the use of the PNAs, since the lack of charges actually reduces probe solubility in water, making solubility highly sequence-dependent[14]. For the same reason, self aggregation in solution often occurs, as well as aspecific adsorption on polystyrene vials, which may be a problem since it reduces the concentration of active molecules in solution.

An important feature for a probe that has to recognize biomolecules is its chemical stability as well as the resistance towards enzymes. From this point of view, PNAs can be considered optimal, because their unnatural structure prevents recognition both by DNAses (because the backbone has a peptide-like structure) and by proteases (because of the pseudopeptide backbone). Moreover, the typical stability of the amide bonds allows for a long storage of the molecules and of the devices built with them[15]. The pseudopeptide structure, finally, allows to obtain these molecules by using solid phase peptide synthesis, rather than oligonucleotide one, making their production easier and less sensitive to synthetic impurities such as moisture.

1.4 Hybridization properties of PNAs

The study of PNA hybridization properties is crucial in order to understand their ability to bind complementary sequences; for this reason, it is important to evaluate the energy necessary to denaturate a PNA-DNA or a PNA-RNA duplex. Melting temperature measurement is one of the simplest method to evaluate the affinity of PNAs for complementary sequences; it consists in warming up a solution containing the duplex and measuring the temperature where the complex is half denaturated. This value can be obtained

exploiting the well nucleobase hypochromicity at 260 nm upon duplex formation. UV melting curves can be registered by measuring absorbance at 260 nm during the temperature increase: a sigmoidal increasing curve is obtained as the duplex dissociates and the flex point of this curve indicates exactly the temperature where the duplex is half denaturated: this value is called melting temperature of the duplex. The same kind of measurement can be done by other spectroscopic techniques relying on the change of spectroscopic properties of the nucleobases when are or not involved in duplex formation. For example, circular dicroism can be exploited: since PNA-DNA and PNA-RNA hybrids are chiral object, whereas standard PNAs are not chiral, a Cotton effect is observed on PNA nucleobases when involved in PNA-DNA or PNA-RNA duplexes, but their denaturation induces a loss of the chiral induction which can be followed by CD. Duplex stability can be studied also by isothermal titration calorimetry (ITC) where the ΔH of the complex formation is measured[16].

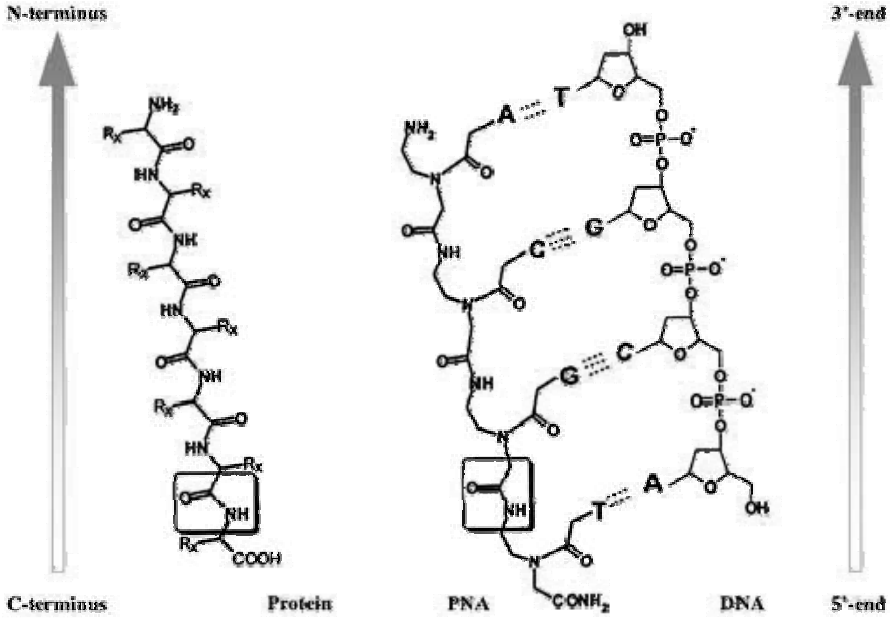


Figure 1-3: Structure of an antiparallel PNA-DNA and comparison with a peptide backbone (F. Pellestor, P. Paulasova, *European Journal of Human Genetics*, 2004, 12, 694)

Due to their chemical features, PNAs show unusually good hybridization properties, better than those shown by natural nucleic acids. As described before, PNA chemical structure allows for the recognition of complementary nucleic acid sequences by Watson-Crick interactions[17], with a stronger binding energy due to the absence of electrostatic repulsion. Moreover, this feature makes the stability of the duplex independent from the ionic strength[18], since there is no need of counterions to mask the repulsive force. Actually, it has been shown that, while DNA-DNA stability increases with salt concentration, PNA-DNA stability shows only a small decrease[22], due to the possibility of DNA to eventually form secondary structures

that may compete with the formation of the duplex. PNAs can also form stable PNA-PNA duplexes, still based on standard Watson-Crick hydrogen bonds. Recognition experiments demonstrated that PNA-PNA duplexes are more stable than PNA-RNA and PNA-DNA complexes.

Although natural nucleic acids form duplexes only in the antiparallel form, PNAs have the peculiar property to bind complementary sequences either in antiparallel and parallel orientation, albeit antiparallel binding is usually strongly preferred, giving more stable duplexes. The antiparallel is conventionally defined as the one in which the amino terminus of the PNA faces the 3' end of the DNA[19] (and obviously parallel complexation is defined in the other way round) (Figure 1-3)

One of the most important features of PNAs is their ability to selectively recognize a complementary sequence and to discriminate it from other sequences only differing for one nucleobase[20]. Recognition experiments done on PNA-DNA duplexes showed a selectivity, expressed as difference in Tm between fullmatch and mismatch duplexes, which is more than 5°C higher than that measured for the corresponding DNA-DNA duplexes. This binding specificity makes PNAs suitable for all the diagnostic and therapeutic applications where mismatch recognition is required.

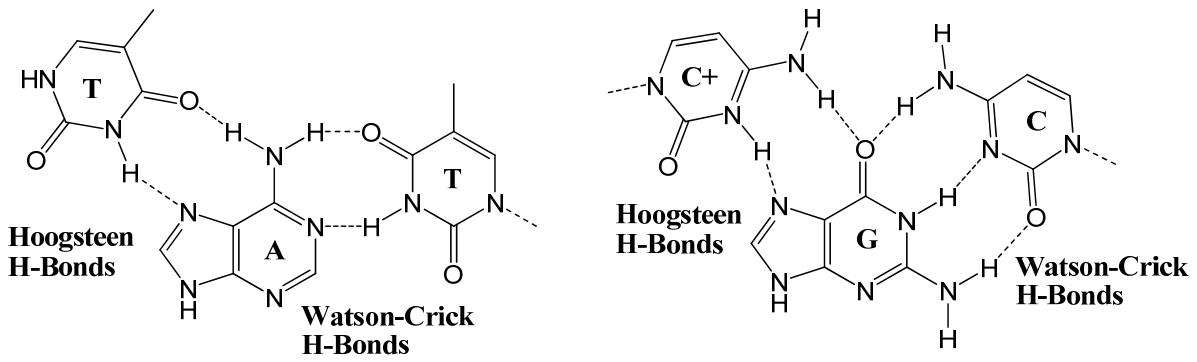


Figure 1-4: Watson-Crick and Hoogstein interaction in triplex formation.

Another interesting feature of PNAs is their ability to form complexes other than duplexes. Homo-pyrimidine PNAs bind to complementary DNA sequences to form $(PNA)_2$-DNA triplexes exploiting Watson-Crick interaction for one PNA strand and Hoogstein for the binding of the second strand. Since a protonated cytosine is involved in the base triplet formation, this is most stable in the pH range of 5.0 –5.5[21] (Figure 1-4). These complexes are characterized by a very high stability: a poly-T PNA decamer hybridized with a poly-A DNA decamer showed a melting temperature of 72°C, while only 23°C was observed for the DNA-DNA complex. The formation of analogous $(PNA)_2$-RNA complexes has been investigated as

well, and their stability has been found to be similar to that of the triplexes formed with DNA. Modified nucleobases, such as isocytosine, which is permanently protonated in one Hoogsteen hydrogen bond donor site, independently from the pH, make the triplex formation easier[22]. The PNA ability to form very stable triplexes can be exploited when the probes are targeted to double stranded DNA: when the target sequence is homopurinic, homopyrimidinic PNAs show the ability to open and invade the double helix, performing strand invasion, to form $(PNA)_2$-DNA triplex (triplex invasion)[23]. Moreover, purine-rich PNAs, are also able to invade DNA-DNA helices by forming PNA-DNA duplexes (duplex invasion)[24], while C rich PNAs triplex forming ability can be exploited in double stranded DNA to form PNA-$(DNA)_2$ triplex complexes[22] (Figure 1-5).

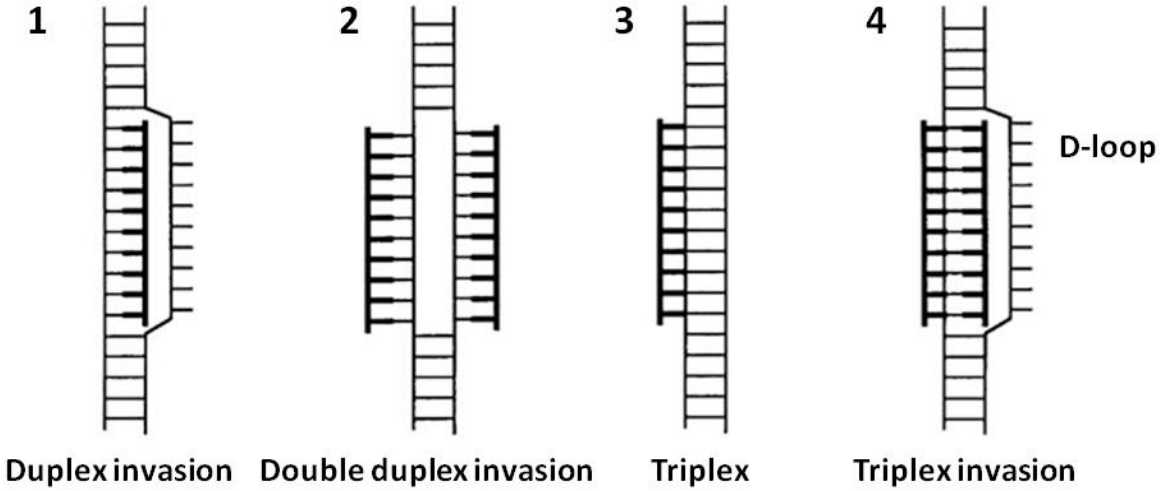


Figure 1-5: Scheme of the complexes formed by PNA and double-stranded DNA. PNA oligomers are drawn in bold. (1) Standard duplex invasion complex formed with some homopurine PNAs. (2) Double-duplex invasion complex, possible with PNAs containing modified nucleobases. (3) Conventional triplex formed with cytosine-rich homopyrimidine PNAs (4) Triplex invasion complex, leading to the displacement of the second DNA strand into a 'D-loop'. (F. Pellestor, P. Paulasova, *European Journal of Human Genetics*, 2004, 12, 694)

This ability can be exploited *in vivo*, because it allows to easily target the desired nucleic acid sequence. Unfortunately, the necessity to have homo-pyrimidine or homo-purine sequences for efficient DNA invasion is a strong limitation, because it limits the choice of the target sequence.

In order to overcome this problem, new methods have been developed to perform strand invasion. One of the most efficient strategy is the so-called double duplex strand invasion, which is possible to achieve by using two PNA sequences targeted to complementary sequences of the double strand DNA; in order to avoid the formation of a PNA-PNA duplexes (the two PNAs are obviously complementary to each other), PNAs should contain

pseudocomplementary nucleobases, such as diaminopurine (as an adenine substitute) and thiouracil (as a thymine substitute), that bind complementary nucleobases (thymine and adenine respectively), but do not bind to each other[25]. Another strategy is PNA tail-clamp, which takes place by coupling the probe complementary to the target sequence with a homopurine sequence (the "tail"). This tail opens the DNA and binds to the double helix, allowing the insertion of the second real targeting sequence[26].

Recently the use of chiral γ-PNA containing an alanine residue with a precise stereochemistry allowed the formation of PNA-DNA Watson-Crick complexes with double stranded DNA. The preorganization given to the probe by the insertion of these substituents increased the stability of the complexes obtaining probes able to perform strand invasion quite easily, simplifying the design of the probe[27]. Chiral PNAs have been also tested in strand invasion tests, in particular PNAs bearing 2D-Lysine monomers have been used in double strand invasion experiments. It was found that when a lysine chain is introduced in the monomers bearing the pseudo complementary nucleobases, double strand invasion efficiency is highly incremented also in difficult sequences where it was never performed quantitatively with other methods[28].

The possibility to avoid the use of pseudo complementary nucleobases is offered by the use of single stranded DNA binding proteins which, trapping the single-stranded portion formed by the invasion, promote the whole process[29]. If this strategy is coupled with the use of 2D-Lysine modified PNAs it can be further improved since shorter and more stable probes can be used[30].

1.5 PNA synthesis

PNAs can be synthesized on account of their pseudopeptide structure by using Solid Phase Peptide Synthesis procedures, by manual synthesis or automatic synthesizers, which also allow parallel syntheses when the screening of multiple sequences is necessary. The generic synthetic procedure can be summarized as in figure 1-6.

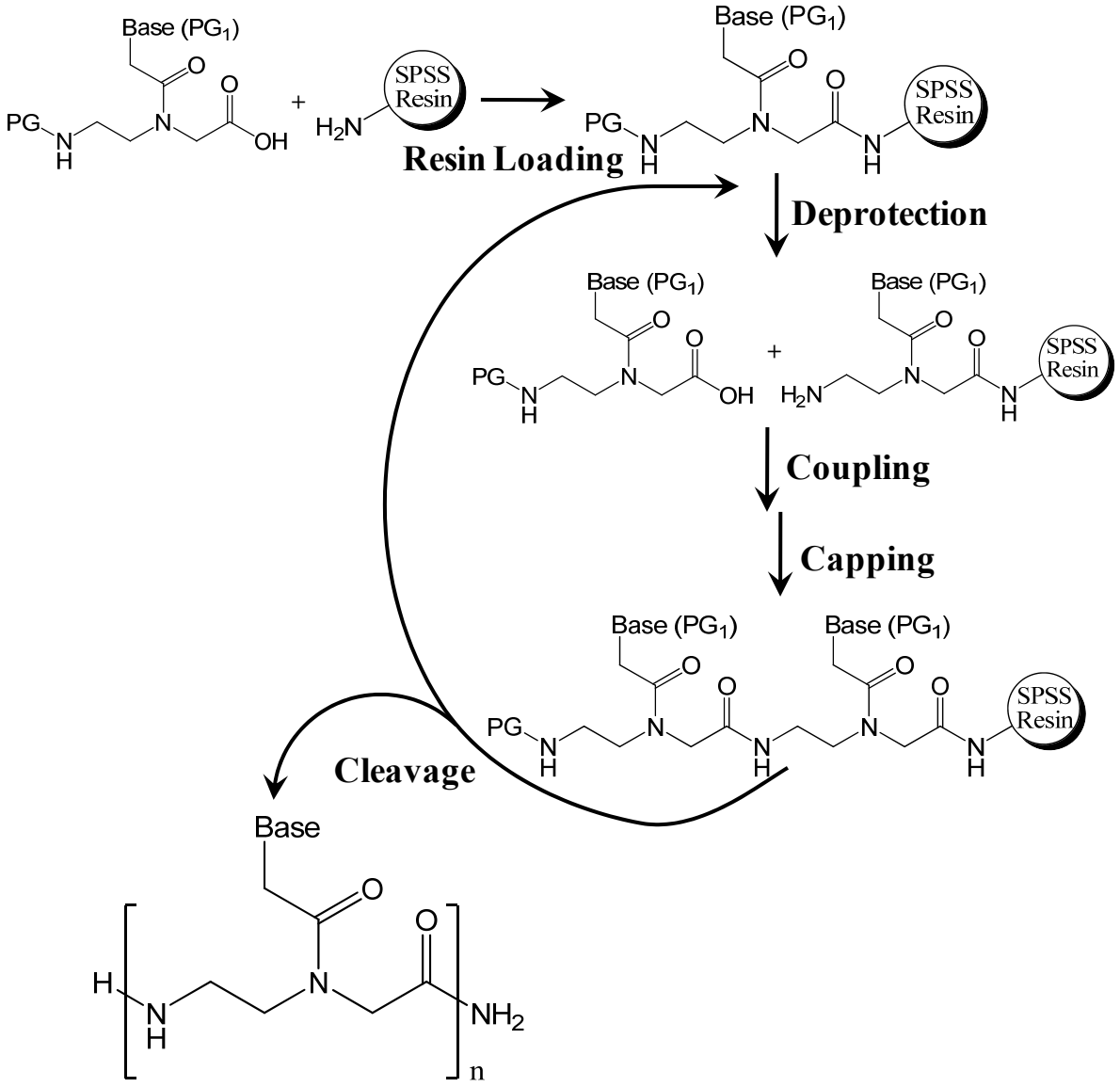


Figure 1-6 Schematic representation of the synthesis of PNAs by Solid Phase Peptide Synthesis (SPSS)

Different strategies can be used, depending on the group used to temporarily protect the amino terminal moiety. In a perfect analogy with standard peptides, the first strategy used for PNA synthesis was the Boc strategy[31], but later also the Fmoc approach has been extensively used[32]; nowadays, these two are the most used strategies for PNA synthesis and also the most affordable, since Boc and Fmoc protected PNA monomers (Figure 1-7) are today commercially available. The synthesis of PNAs always starts with the loading of the resin used with the first monomer of the sequence. The loading of the resin is important, because it sets the amount of molecules growing for each solid support: the high resin loading usually adopted in standard peptide synthesis (0.5-1 mmol/g in active sites) may cause problems during the synthesis, due to the steric hindrance among the growing PNA chains; thus usually

a loading of 0.2 mmol/g is used, and sometimes even lower loadings are required for long or hindered sequences. Unreacted amino groups after the downloading are usually blocked with a mixture of acetic anhydride and pyridine in N-Methylpyrrolidone (NMP). The synthesis continues with the deprotection of the primary amino group of the monomer loaded on the resin. The conditions of this reaction depends on the strategy adopted: Fmoc groups, in fact, are removable by treating the molecule with a solution of a nucleophilic base, such as piperidine, while Boc groups are removed under acidic conditions, usually with neat trifluoroacetic acid. Obviously the reactivity difference between the two strategies implies a difference in the group used for protecting the exociclic amino groups of the nucleobases, which should be stable during all the step of the synthesis. For this reason Bhoc groups are commonly used for Fmoc chemistry, and Z groups for Boc strategy. In the subsequent step, the deprotected amino groups are then linked to the carboxyl group of the next monomer. The coupling reaction is usually carried out with standard techniques derived from peptide synthesis: the carboxylic group is transformed in an active ester that can react with the amines of the growing chains. Many activators can be used, among which uronium reagents such as 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) are the most used, albeit other activators have been employed, like 2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TDBTU) and 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-on (DEPBT), especially when chiral centres are introduced in the molecule and their optical purity has to be preserved[33].

When the synthesis is complete, the molecule has to be removed from the resin and fully-deprotected at the same time. The cleavage of the molecule from the resin is done by exploiting the active linkage of the molecule to the solid support. Depending on the strategy used, different polystyrene based supports can be used: Fmoc chemistry is usually done on 4-Methylbenzhydrylamine (MBHA) Rink-Amide or Wang resin, that can be cleaved in TFA:m-cresol mixture, while Boc chemistry is usually done on MBHA resin, that is cleaved in stronger acidic conditions (TFA:TFMSA mixtures). The presence of scavengers during the cleavage, such as m-cresol and thioanisol, is often necessary in order to block the reactive cations produced during the deprotection of all groups. The final product is the fully-deprotected PNA having a free carboxylic group or a carboxamide at the C-terminus, depending on the linker used: MBHA-based resins produce PNA amides, while Wang resins may allow, when necessary, the production of the PNAs as free carboxylic acids. Due to the

repetitive and easy protocols used, the synthesis can be implemented on an automated synthesizer for peptide chemistry, making these molecules more affordable[34].

Many efforts have been done for finding alternatives to the standard synthetic strategies, in order to make PNA synthesis cheaper, or to use milder conditions for the linking of acid-sensitive groups. This led to the development of other protecting groups: dithiasuccinoyl (Dts) (Figure 1-7) has been used because this group can be removed under mild deprotecting conditions by treatment with a thiol in slightly acidic conditions. The deprotection of Dts can be carried out by using dithiothreitol (DTT) in acetic acid-CH_2Cl_2, followed by neutralization with diisopropylethylamine (DIEA)-CH_2Cl_2 and subsequent coupling of Dts-PNA-monomer by using HBTU/DIEA in NMP[35]. The monomethoxytrityl (Mmt) protecting group (Figure 1-7) strategy has been developed for the same reason, in order to use milder conditions during the synthesis[36], since the Mmt group can be removed by treatment with 3% trichloroacetic acid in CH_2Cl_2. In order to ensure mild synthetic conditions, a new solid phase linker based on an aminohexyl spacer and a base cleavable succinyl linker have also been developed, which allow to perform the cleavage by treatment with an ammonia solution, with the simultaneous deprotection of the acyl-protected nucleobases. The four monomers have been synthesized and a PNA probe was produced by using this technique, obtaining a molecule of purity comparable with those produced by standard technique procedures.

Other examples of different synthetic approaches can be found in the literature, such as the use of the azide groups (Figure 1-7) in order to mask primary amines; these groups protect the amine during the synthesis and can be reduced by phosphine after the coupling[37]. The synthesis of PNAs using 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl (Dde) protected monomers has been demonstrated as well[38] (Figure 1-7). This group represents an alternative to Fmoc group, since it can be removed under mild slightly basic conditions, using a hydrazine solution, that removes also the Fmoc group; lately a deprotection step has been optimized, using hydroxylamine (mixture of $NH_2OH{\cdot}HCl$ and imidazole) under slightly acidic conditions which is fully orthogonal to the Fmoc group.

Another strategy is the so called “fully protected backbone approach”, in which a backbone containing protecting groups in place of the nucleobase is synthesized. The selective deprotection of the protecting groups and coupling with the correspondent nucleobase gives the complete PNA[39]. PNAs were synthesized by using this technique, in order to verify the quality of the technique, only containing cytosine and thymine; more complex sequences still have to be synthesized. A challenging but charming approach is the template synthesis[40], which implies the synthesis of a PNA backbone without nucleobases, and the use of a

complementary PNA sequence to address the nucleobases in the desired position, by exploiting the Watson-Crick complementarity. The nucleobases are linked to the amino group of the backbone by an N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) mediated coupling or deoxyPNA can be obtained when the reaction is a reductive amination. Recently another strategy has been developed in order to make the synthesis faster and cheaper[41]. The key step of this new strategy is the protection of the primary amino group by benzothiazole-2-sulfonyl (Bts), which acts not only as a protecting group for the primary amino group, but also as activating group for the carboxyl moiety of the incoming monomer. The monomers are actually synthesisized as cyclic piperazinones (Figure 1-7), which can be stored for a long time in DMF and are reactive with an amino group. Nowadays this technique is exploited to synthesize commercially available PNAs.

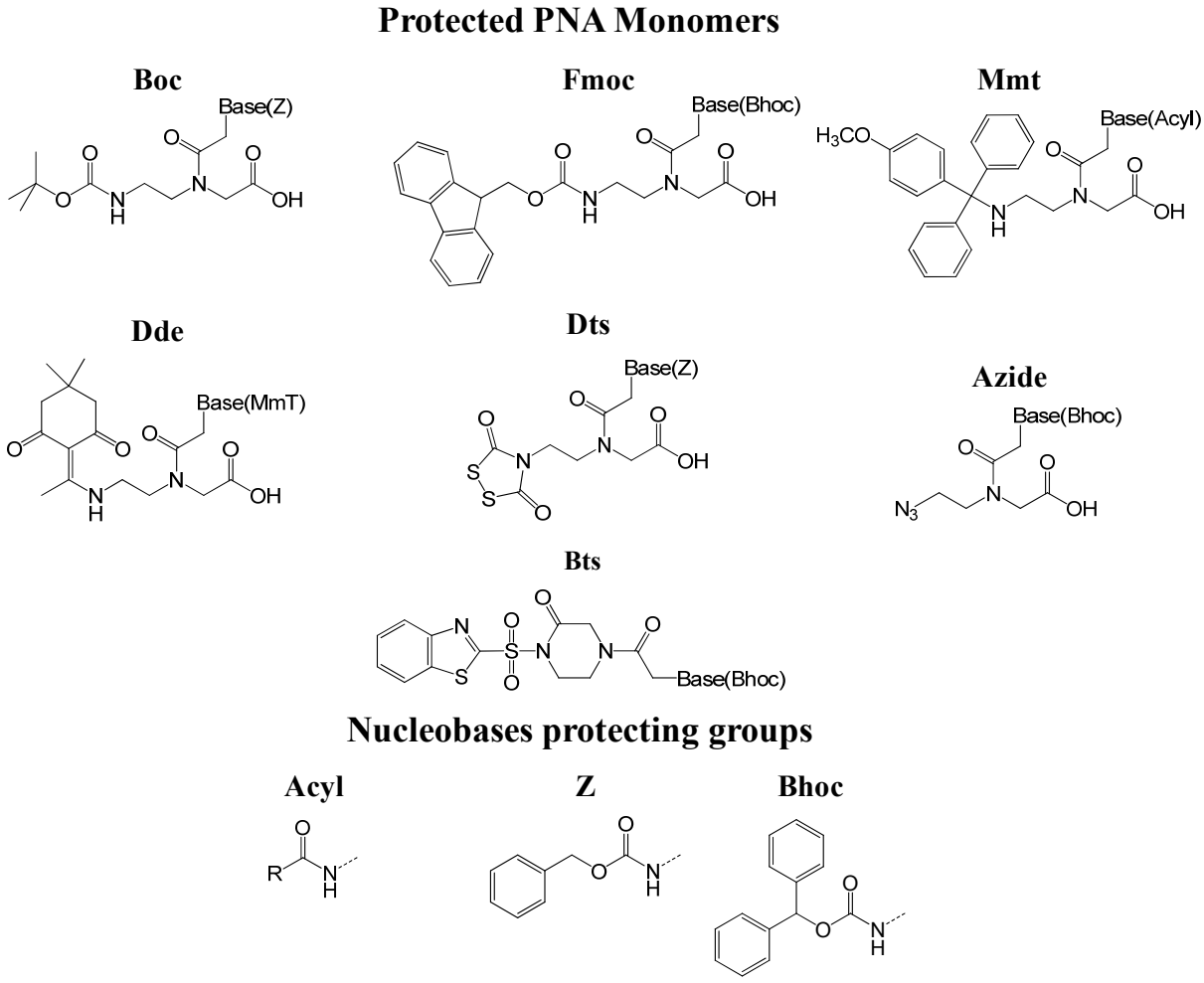


Figure 1-7: Structure of common protecting groups used in PNA synthesis

1.6 Modified PNAs

The advantages of using PNAs in DNA recognition have been extensively discussed in the previous sections but, albeit their interesting properties, continuous improvements are being extensively pursued. These attempts led in the last years to the synthesis of several modified PNAs. PNAs can be modified by covalently linking to them other structures, such as oligonucleotides (PNA-DNA chimeras), peptides or other molecules, or modifying the PNA backbone or the nucleobases. One of the first modifications introduced in a PNA probe, adopted for its simplicity since the very first PNAs synthesized, was obtained by linking a positively charged amino acid (a lysine) at the C-terminus, in order to improve the molecule solubility. The conjugation with more complex peptides has also been subsequently exploited in order to tune the PNA properties, such as cell penetration, DNA affinity and solubility[42]. The linking of amino acids or peptide sequences to PNA is actually very straightforward, since the synthetic approach used for PNA can be readily extended to peptides, making the synthesis of PNA-peptide conjugates a simple task. A more complex chemistry has to be developed for the synthesis of PNA-DNA chimeras, due to the different synthetic strategies and conditions used for the peptide and for the oligonucleotide solid phase synthesis. PNA-DNA chimeras are characterized by an improved solubility, due to the introduction of negative charges, and the possibility to be recognized by enzymes that usually do not recognize standard PNAs, such as DNA polymerases, although the non-standard structure also gives resistance towards nucleases[43].

The evolution of PNAs led to the development of probes containing one or more modified monomers. Most of the modifications have been inserted in order to increase the affinity and the specificity towards the complementary DNA sequences. The modifications can be applied to the nucleobases, in order to extend the hydrogen bond network with the complementary base, or to the backbone, in order to tune the rigidity and induce a preorganization more suited for the hybridization with the complementary DNA sequence[44]. The insertion of such modifications within the backbone can be done by changing the length of the backbone, by introducing substituents on the backbone itself, usually in position 2 or 5, by cyclizing the backbone, by modifying the linker with the nucleobase, or by changing the chemistry of the bonds between the monomers.

The effects of the backbone length on PNA hybridization properties have been studied by synthesizing PNA monomers containing one more methylene group in the glycine or the

aminoehtyl moieties, or in the linker unit to the base. PNAs containing such monomers have been synthesized either as completely modified oligomers, either as partially modified. The results showed that the standard monomer length is the optimal for DNA recognition in terms of duplex stability and selectivity[45]. The distance of the nucleobase from the backbone or between the nucleobases is the most influent factor, while not much difference was found when the backbone was lengthened on either sides.

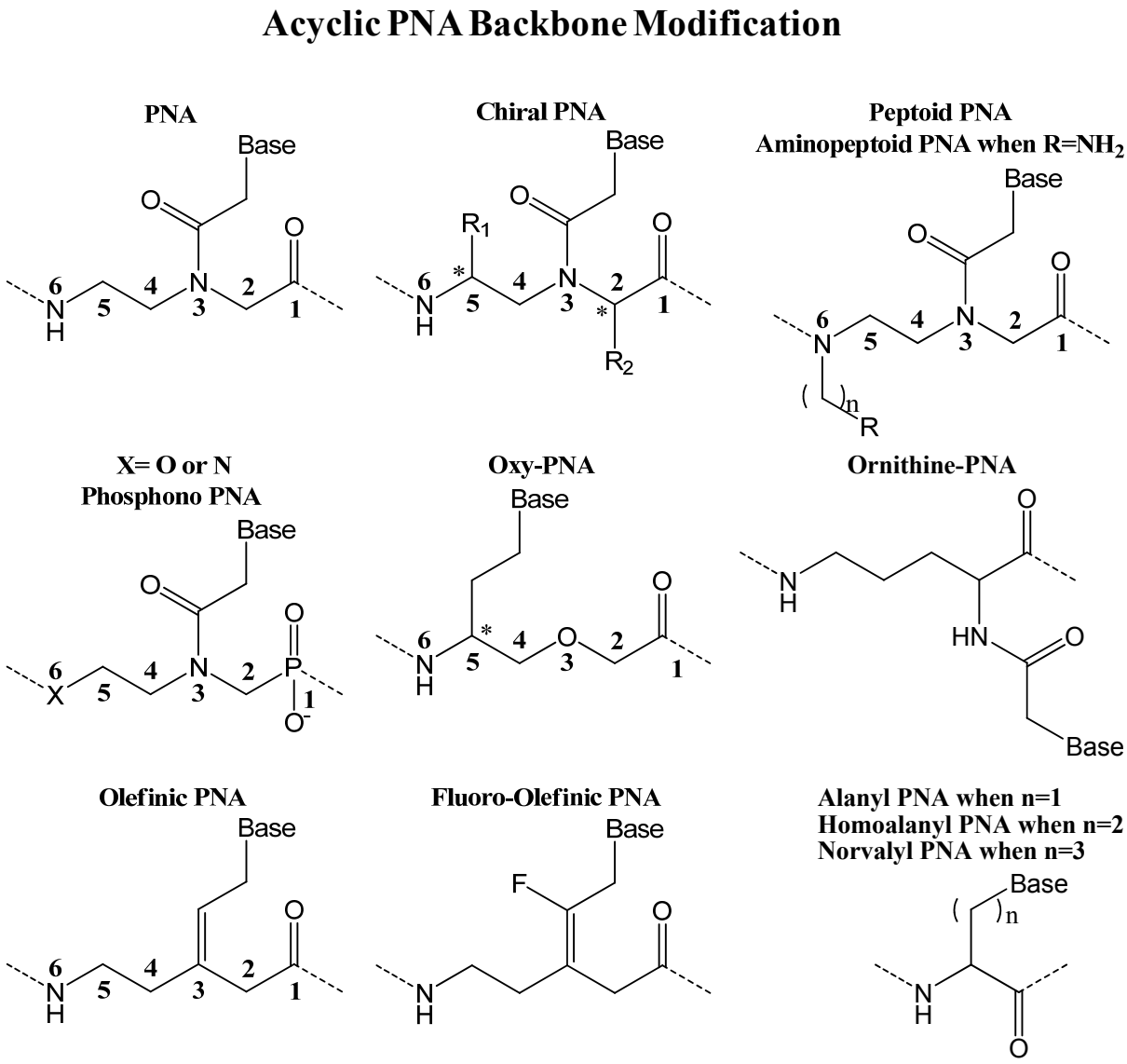


Figure 1-8: PNA backbone modifications based on acyclic structures

Ornithine PNAs, instead are based on the structure of ornithine, having the carboxymethyl nucleobase linked to the nitrogen in α, and the ornithine side chain linked by its amino group to the following monomer[46] (Figure 1-8). It was demonstrated that an omithine PNA analogue is a structural DNA mimic for recognizing RNA albeit not as effective as the original

aminoethyl glycine PNA. Using a poly-T ornithine-PNA, a triplex was formed with complementary RNA, while DNA was not bound[47].

Substituents inserted in the backbone are usually based on amino acid synthons, which are usually employed in order to have side chains either in position 2 or 5 (Figure 1-8). Monomers based on neutral, positively and negatively charged amino acids in position 2 have been synthesized and incorporated in PNAs[48,49], then tested in binding assays. The results clearly indicated the roles played by different parameters for tuning the DNA affinity of PNAs: charge, steric hindrance, stereochemistry. By analyzing the PNA-DNA stability, in fact, it is possible to observe that the steric hindrance introduced by bulky side chains (typical examples are leucine, tryptophan or phenylalanine)[50], as well as the electrostatic repulsion existing between negatively charged PNAs and DNA (for example when incorporating glutamic or aspartic acid)[48] led to less performing PNA probes. On the other hand, side chains bearing a positive charge (based on lysine or arginine)[48,49,51] inserted in the backbone positively contribute to the PNA-DNA duplex stability, due to the positive electrostatic interactions.

As far as stereochemistry was concerned, the analysis of the binding abilities of PNAs incorporating in position 2 enantiomeric monomers either based on D- or L-amino acids led to the conclusion that stereochemistry plays a very important role in DNA recognition. It was found that D-amino acid containing monomers increase the PNA-DNA duplex stability as compared to the enantiomeric counterparts, independently from the nature of the side chain. In order to further improve the PNA abilities, the combined use of positive electrostatic interactions and chiral properties led to the development of monomers containing a D-lysine side chain in position 2. Incorporation of such monomers in PNAs showed a great improvement in complementary DNA and RNA sequence recognition, both in terms of affinity and single mismatch discrimination.[52] When the lysine side chain is substituted with arginine, the binding properties are conserved, but in vivo experiments demonstrated better cellular uptake, allowing the application of these probes as antisense drugs[53]. The introduction of analogous side chains in position 5 has been used both to improve hybridization properties and to introduce fluorophore groups without affecting sequence recognition, obtaining the so called γPNAs[54].

Backbone modifications can be introduced not only on the carbon atoms, but also on the nitrogen ones. For example, peptoid and aminopeptoid PNAs (Figure 1-8) can be obtained if the aminoacidic side chains are introduced on the nitrogen in position 6[55]. Such PNAs have been synthesized with side chains bearing an amino group at the end, but different carbon

spacers between this group and the backbone. The results showed that, although short chain resulted in PNA-DNA duplex destabilization, four or longer carbon spacers did not destabilize the complex, which conserved the same selectivity of unmodified PNAs. The observation that also biotin-labeled side chains did not disturb the binding demonstrated that this method can be exploited in order to add another position for labeling the PNA probe.

Another type of backbone modification inspired by the peptide structures is that of the so-called alanyl-PNAs[56] (Figure 1-8), in which the backbone is a standard peptide structure including alternated alanyl units. The nucleobases are linked to the methyl side chain, in the β-position, the linker length can be changed obtaining homoalanyl and norvalyl PNA monomers. Preliminary studies carried out by hybridizing these molecules with similar structures demonstrated their ability to interact specifically via Watson-Crick and Hoogstein bonds, (even if non conventional pairing mode were observed in some cases) to form double strand structures; the system has been used to understand to role of side chains in the recognition processes. The results showed that a three atom linker (norvalyl-PNAs) has a reduced ability to form specific interactions due to its flexibility, while shorter carbon linkers show better performances.

The attempt to induce preorganization in PNAs, in order to obtain more stable duplex and more rigid and selective probes, is one of the crucial points in PNA modification. As said before, the introduction of cyclic structures within the PNA backbone is one of the conceptually (albeit not synthetically) simplest ways to obtain this effect. Several cyclic PNAs have been developed, starting from five or six member rings. Modifications based on five membered rings originated from the structure of proline or pyrrolidine[57]. Libraries of cyclic backbone modifications were also developed, but very few of them showed improvement in DNA or RNA recognition, as compared to unmodified PNAs: among these, aminoethylpyrrolidinone PNA (aepone-PNA)[58], pyrrolidine PNA and aminoethylprolyl PNA (aepPNA) showed significant hybridization properties[59] (Figure 1-9). Cyclopentane PNAs of various stereochemistry were also tested and among these, (S,S)-trans-cyclopentane PNA contains the correct stereochemistry for DNA binding[60].

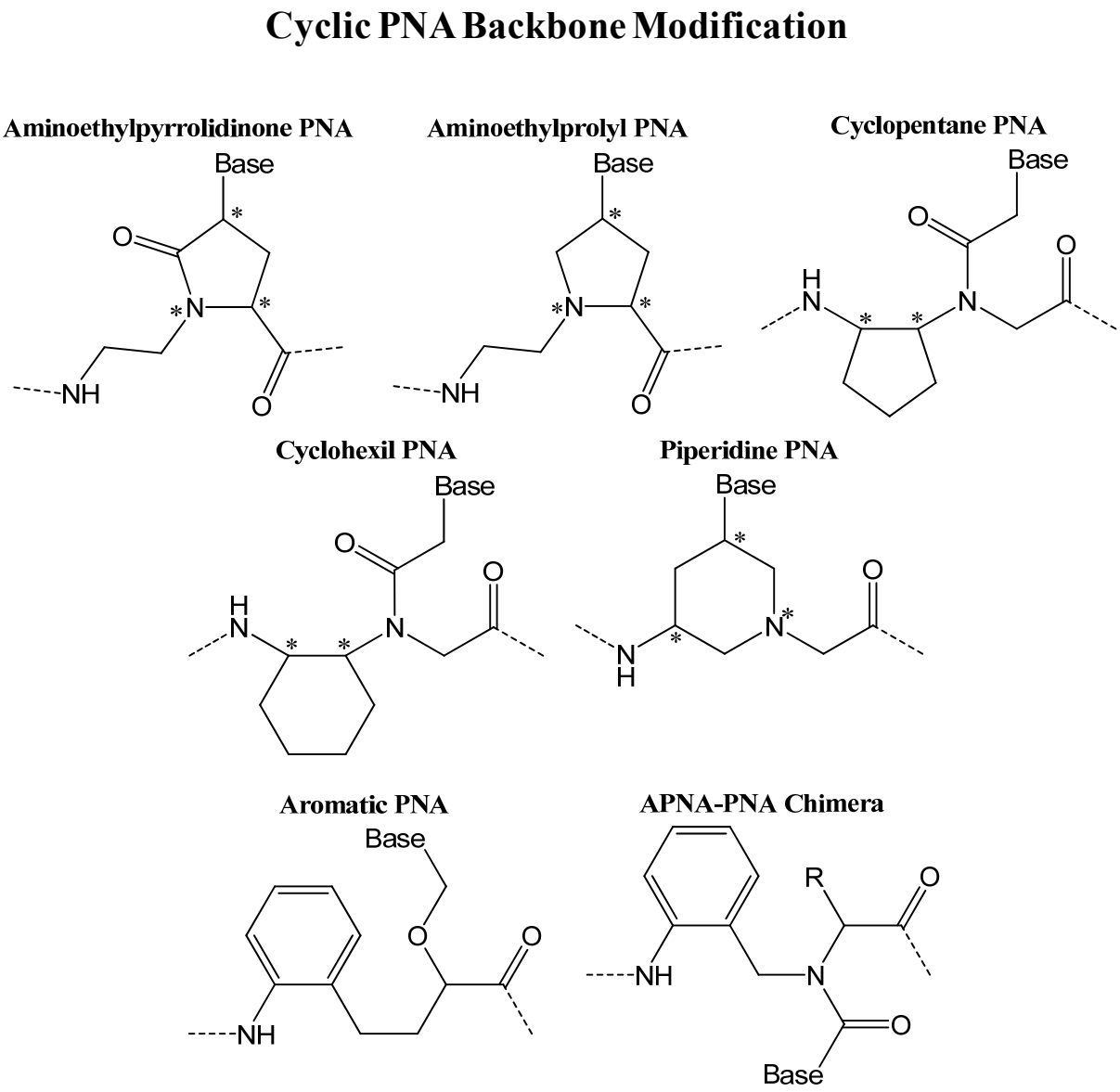


Figure 1-9: Strucures of the main PNAs bearing cyclic modifications in the backbones

Among PNAs containing six-membered rings, piperidine PNAs[61] (Figure 1-9) showed good improvements in DNA recognition forming more stable triplexes when hybridized to polyA complementary DNA, the effect being particularly evident when the modified monomer was placed at the C-terminus. Aminocyclohexylglycyl PNA (Figure 1-9)[57] showed good performance when the isomer SR or RS were used. In particular, the choice of the stereocenter configuration allowed to obtain PNAs more performing when the complementary DNA sequence had to be recognized, or when RNA had to be discriminated over DNA. While the insertion of one or two 1R-2S monomers increased the discrimination of a point mutation, the use of 1S-2R monomers increased the selectivity in poly-A RNA binding (compared to DNA). When using three modified monomers in the same sequence (one in the middle and

two and the opposite ends) PNAs showed an excellent discrimination of RNA mixed sequences over DNA, reaching 50°C of discrimination in the best case[62].

Aromatic PNAs (APNA) and the second generation N-(2-aminobenzyl)glycine or N-(2-aminobenzyl)-β-alanine (APNA-PNA Chimera) are another example of cyclic PNAs in which the aminoethyl part of the monomer is replaced by aromatic rings (Figure 1-9). Several preliminary experiments with different structures demonstrated that, although the substituents are very bulky and the PNA conformation is strongly influenced by the aromatic ring, when modifying the distances between the nucleobases, the PNA-DNA complexes are only slightly destabilized, maintaining the same selectivity[,63].

The effects of modifications introduced in the linkers between PNAs and nucleobases have also been studied, in order to understand the role of the tertiary amide in complementary sequence recognition. For this reason, the carboxylic linker has been replaced by a double bond-based linker, obtaining Olefin PNA (OPAs)[64]. The molecules were synthesized by controlling the double bond stereochemistry, in order obtain (E)-OPAs or (Z)-OPAs and to check the different influence of the nucleobase orientation on the DNA affinity. The observation that OPAs conserved the PNA recognition properties, but showed a highly decreased DNA affinity, demonstrated the importance of the amide unit in the duplex formation. The same study was further extended by modifying the linker, such as in Fluorinated Olefinic PNAs (F-OPNAs), in which the OPA were further modified by linking a fluorine atom to an olefinic carbon (Figure 1-8). Fluorine was used because it gives a dipole moment similar to that of carbonyl group, but it is not able to make hydrogen bonds. Strong dependence of the hybridization properties depending on the position of the modification was observed[65], excluding that the linker dipole had an effect in the hybridization process.

Another part that can be *ad hoc* modified to introduce new features in PNA is the chemical bond between two PNA monomers. Phosphono-PNA (pPNA, Figure 1-8) is a class of molecules characterized by the insertion of a phosphate ester or phosphonamide bond replacing the amide bond between monomers, mainly for solubility reasons. The insertion of negative charges, which could be a problem for DNA binding because of the electrostatic repulsion, was actually demonstrated to be almost ininfluent on the affinity of some mixed PNA-pPNA probes for the complementary DNA sequence, which did not change as compared to the unmodified probe[66]. Another type of modification introduced is that of Oxy-PNA, another class of modified PNA molecules obtained using the monomer -NH-CH(CH_2-CH_2-Base)-CH_2-O-CH_2-CO-. PNAs containing these modified structures have been synthesized and studied by UV spectrophotometry, demonstrating that they can hybridize

DNA and RNA complementary sequences showing a melting curve sharper than standard DNA-PNA duplexes and a comparable stability[67]. Finally, also deoxyribose or riboseamide PNA have been obtained, in which the phophodiester bond from DNA was substituted by an amide bond. Some PNA probes containing one or more of these modified monomers were synthesized and tested, although the performances were not particularly noteworthy[68]. The development of hydrazino-PNAs, probes having the primary terminal amino group replaced by a hydrazine moiety, took place projecting a monomer having two orthogonal protecting groups on the two nitrogen atoms of the hydrazine unit, being able to tune the reactivity of the molecule during the oligomerization[69].

As mentioned before, synthetic efforts have been devoted not only to the modifications of the backbone, but also to the introduction of new groups in the structures of the nucleobases, mainly with the aim of increasing the affinity for the complementary sequences. This was usually done in two ways: by increasing the aromatic part, in order to increase stacking interactions, or by increasing the possibility to form hydrogen bonds[70]. The synthesis of bicyclic or tricyclic analogues has been one of the most used ways to improve hydrophobic interactions. Many thymine analogues bearing an extra cycle have been developed: 7-chloro-1,8-napthyridin-2(1H)one (7-Cl-bT, Figure 1-10) turned out to increase the PNA affinity towards complementary DNA[71], and an also tryciclic cytosine based on benzothiazine-pyrimidine (Figure 1-10) structure showed good performance as well, since it increased the stability of the duplex and was highly fluorescent[72]. The same structure was used in order to add an alkyl chain with a tertiary amine, able to form an extra hydrogen bond when guanine has to be bound, obtaining the so-called G-clamp (Figure 1-10); this modification increased the duplex stability as well as increased sequence selectivity[73]. Another field in which the modifications introduced into the nucleobases can have a big impact is the formation of PNA-DNA_2 triple helices. Since standard cytosine only forms Hoogsteen interaction when protonated, thus making its performances strongly pH-dependent, the use of pseudoisocytosine (J base), permanently protonated at the Hogsteen donor site,[74] in place of cytosine, eliminates the necessity of having an acidic pH during triple helix formations; 1,8-napthyridin-2,7-(1,8H)-dione (K base) was a further improvement in this field: due to its increased stacking contribution, formation of triplex at neutral pH was favoured[75](Figure 1-10).

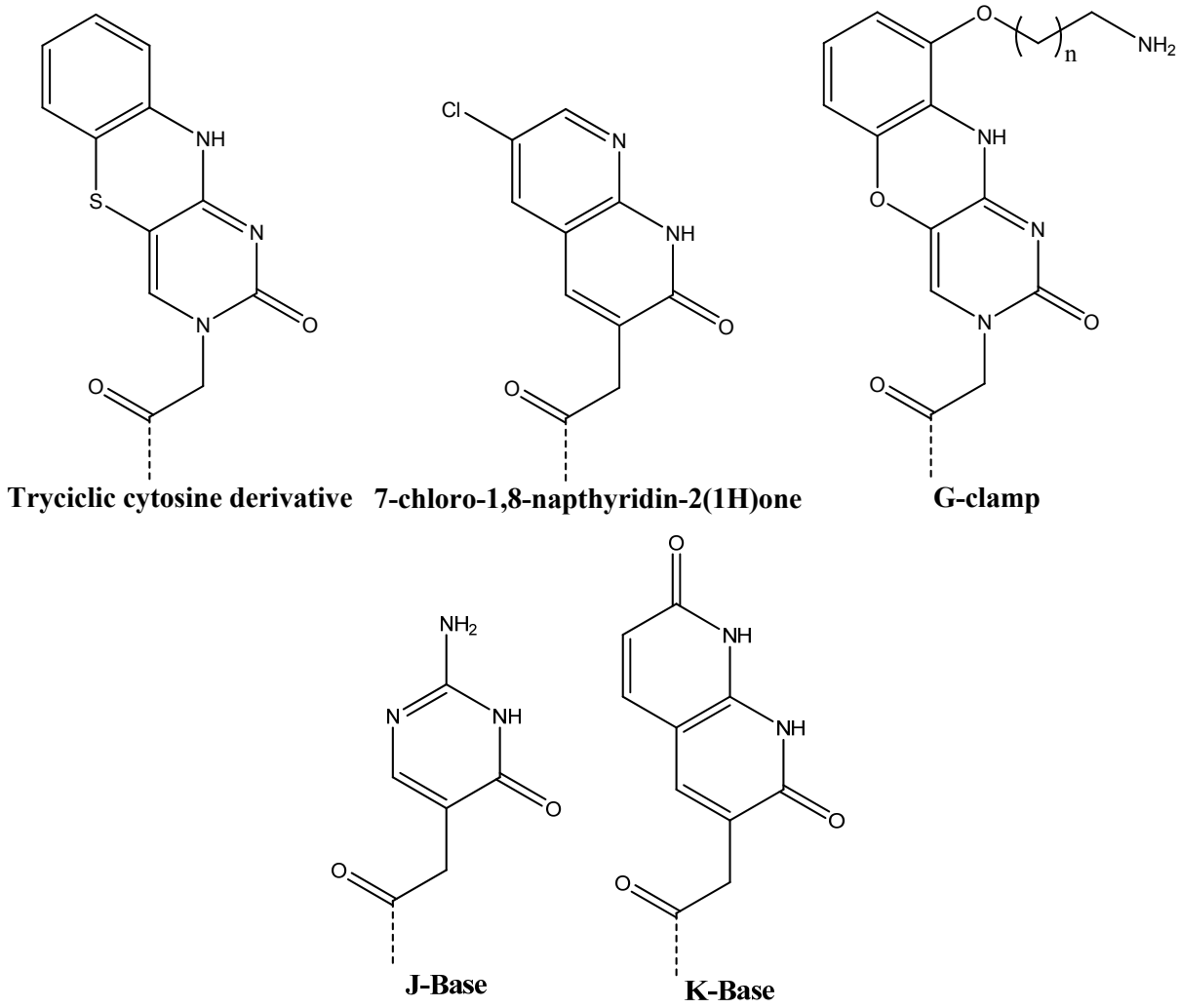


Figure 1-10: Chemical structures of some of the most successful modified nucleobases developed to increase Watson-Crick (upper row) or Hoogsteen (lower row) interactions

In general, the development of new modifications led to the synthesis of a large number of different bases, characterized by different properties. The introduction of alkyl chains ending with reactive groups has also been fully exploited in order to introduce fluorophores after the synthesis of PNAs or by directly synthesizing PNAs with bases bearing pre-linked fluorophores.

Development of non-discriminating nucleobases (nucleobases which may recognize with the same stability A, G, T or C) is another field of studies that led to the synthesis of derivatives able to recognize all the nucleobases by stacking interactions, rather than by hydrogen bonds, obtaining PNA probes which may be used when sequences only differing for a point mutation have to be targeted with the same affinity[70].

1.7 Chiral PNAs

Among all these classes of modified PNAs, the introduction of stereogenic centers into the backbone is undoubtedly the best way to tune the PNA properties towards the desired functions. Suitably designed chiral PNAs are always those giving the best results in terms of DNA affinity, mismatch recognition, RNA/DNA selectivity, ability to perform strand invasion, cellular uptake, solubility and so on. As described before, chirality can be introduced into the PNA backbone in many different ways. In this paragraph we will discuss more thoroughly the properties of chiral PNAs having a backbone based on aminoacidic side chains inserted in position 2 or 5 or both. The earliest work was done on chiral PNAs modified in position 2. As outlined above, side chains based on different amino acids were tested, and the best results were obtained by using PNAs including monomers synthesized starting from D-lysine[76], which showed an increased affinity towards the complementary sequences as well as a better selectivity in their recognition. Indeed, a PNA that allowed to obtain outstanding results in terms of selectivity was obtained by inserting three chiral 2D-Lys monomers in adjacent positions, obtaining the so-called "chiral-box" PNA[77]. This probe was able to perfectly discriminate antiparallel DNA vs. parallel DNA in duplex formation, a recognition not affordable with standard PNAs. Moreover selectivity experiments showed that this probe also had a perfect selectivity in point mutation discrimination. A crystal structure obtained by hybridizing a "Chiral Box" PNA with the complementary DNA, allowed to investigate the contribution of the chiral modifications to the duplex structure.[78]

The introduction of positively charged side chains was also exploited for opening DNA double helices in order to perform double strand invasion. Gel-shift experiments demonstrated that when the pseudocomplementary nucleobases are coupled with chiral monomers bearing D-lysine side chains in position 2, double strand invasion ability was improved and applicable also to difficult sequences[79].

Further modifications were subsequently introduced not only in position 2, but also in position 5. These molecules are called γPNAs, in order to distinguish them from the previous ones, called α-PNAs. It was demonstrated that when L-lysine side chains are introduced in this position, the recognition performances improve[80]. The lysine side chain can also be exploited for introducing fluorescent groups or peptides, since the increased affinity towards the complementary sequences are preserved also when the amino end is acetylated or derivatised; the stability decreases only when bulky fluorescent dyes are introduced[81].

PNAs including monomers containing D- or L-Lysine side chains in position 2 and 5 were also synthesized and studied during the hybridization of complementary sequences. As predictable, the best configurations turned out to be 2D-5L, and the affinity and the selectivity where the highest for these probes.

The reasons for the high performances showed by chiral PNAs were investigated by studying chiral PNA-PNA duplexes by circular dichroism, also by using cyanine dyes such as $Disc_2(5)$ dye[82], which is able to form aggregates into the minor groove of a duplex PNA-PNA or PNA-DNA. The formation of such complexes can be detected by UV-Vis spectrophotometry, due to an ipsochromic effect, or by circular dichroism, due to the exciton coupling effect of the aggregate, indicative of its handedness, which is dictated by that of the double helix. These data clearly indicated that, while achiral PNA-PNA duplexes are an equimolar mixture of right-handed and left-handed helices, 2D-PNA and 5L-PNA preferentially formed right-handed helices when hybridized to complementary achiral PNAs. Thus, 2-D and 5L-PNAs prefer the same handedness as DNA. This preference makes the formation of the duplex with complementary DNA easier and more stable[83].

Since chirality is of crucial importance in these probes, the eventual racemization has to be checked during the synthesis of such probes. Since the chiral center in position 2 can undergo racemization during standard peptide synthesis, as it is known for amino acids, a new strategy, called submonomeric strategy, was developed in order to preserve the enantiomeric purity. This synthetic approach allowed to reduce the racemization in this position from 30-50% to 2-6%. This strategy consists in the synthesis of a submonomer that bears a Fmoc protecting group on the nitrogen in position 3 instead of the nucleobase. The carbammate formed with the Fmoc group is less electron withdrawing than the amide formed with the nucleobase, and this allows to reduce the acidity of the hydrogen in position 2 and thus the racemization during the carboxylic group activation. The Fmoc group can be removed after the coupling and the nucleobase is introduced by a standard coupling directly on the resin (Figure 1-11)[84].

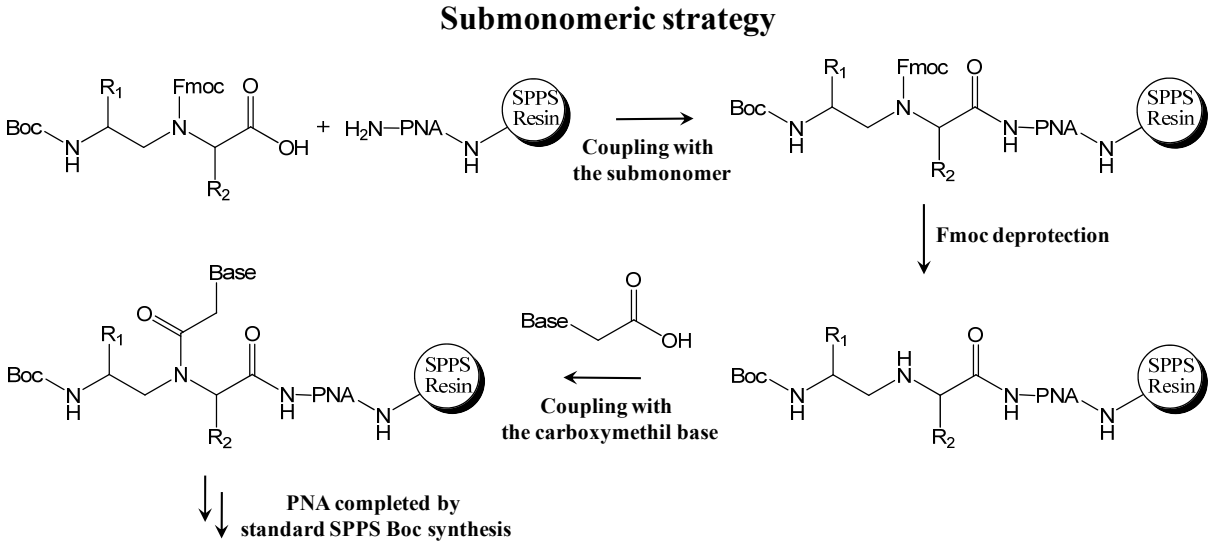


Figure 1-11: Submonomeric strategy used to avoid racemization during the coupling of a chiral PNA monomer

1.8 PNA Applications

Since their invention, it has been clear that the outstanding properties of PNAs could turn these molecules into powerful tools for life scientists, in all the fields where the selective recognition of complementary sequence is required, like biomedical or diagnostic applications.

When used as drugs, PNAs can be exploited to target DNA or RNA. Pharmaceutical applications that require DNA targeting have usually the purpose to block the replication of a gene related with the onset of certain illness such as cancer; this approach is the so-called antigene therapy. Alternatively, RNA can be targeted in the cytoplasm, in order to block the expression of selected proteins; in this case, the approach is called antisense. PNAs are also used in analytical applications, when specific sequences have to be identified, in order to predict a risk of illness onset, or to recognize the presence of biological contamination in complex matrices. Usually, the detection of sequences having some biological meaning is to be performed in a fast and selective way.

1.8.1 Analytical Applications

The recognition of a DNA sequence in a biological sample usually takes place in three steps: the extraction and purification of the DNA from the sample, the amplification by PCR of the genomic region containing the sequence to be detected, and the recognition of the sequence.
Although DNA sequencing is often possible, the necessity to develop new assays based on probes that rapidly recognize a specific sequence is also necessary, because the sequencing is time-consuming, expensive and often made impossible by the low amounts of DNA. The use of specific probes can make the recognition more robust, faster and cheaper. PNAs are particularly suitable for these applications, because of their chemical stability, the affinity for DNA which enhances the sensitivity, the possibility of forming duplexes at low ionic strength that allows the hybridization in conditions where the DNA does not form secondary structures, and the selectivity, especially in the recognition of point mutations.
DNA detection can be performed in solution or on surface. In solution assays, the PNA probes and the DNA to be analyzed are in homogeneous solution; a great number of different assays have been developed, being based on different readout signals. The easiest signal that can be exploited is the change of color of the sample, in colorimetric assays, when the this happens upon the nucleic acid hybridization. Molecules such as cyanine $DiSC_2(5)$ dye, able to interact with PNA-DNA double helix but not with PNA or DNA single strands, giving an ipsochromic effect due to aggregation which result in an instantaneous color change, have been exploited for these kinds of essays[85]. A colorimetric test using Disc2(5) dye, PNA and Succ-β-CyD was successfully applied for the detection of SNPs correlated to the apolipoprotein E gene, which have been linked to the Alzheimer disease[86]. Another effect causing changes in the color of a solution is the aggregation state of nanoparticles; the use of PNAs with gold nanoparticles was demonstrated to cause their collapse, that is reversible when complementary DNA is added, causing a different absorption band[87].
The possibility to link the PNA to a superparamagnetic iron oxide nanoparticles (MNP) obtaining MNP-conjugated PNAs (MPNA). These nanoparticles have been used for the detection of complementary DNA sequences, exploiting their capability of MPNA to enhance the relaxation time T2 in aqueous solutions when specific hybridization takes place[88].
More sensitive assays are those based on fluorescence, by exploiting the appearance of a signal upon PNA-DNA duplex formation. These particular PNAs are designed to be non fluorescent when non hybridized, and to have a conformational change when the hybridization takes place, leading to the lighting up of a fluorescent signal. PNA molecular

beacons belong to a class of molecules used for this purpose: when they are not hybridized, their hairpin-like structure keeps the fluorophore (linked to one end of the probe) next to his quencher (linked to the opposite end), whereas when hybridization takes place the molecule has to stretch, moving the fluorophore away from the quencher, thus inducing fluorescence switch on[89].

Another kind of probes are the PNAs derivatised with thiazol orange, which are not usually fluorescent in solution, but when the duplex is formed, the dye can interact with the helix increasing its rigidity and modifying its electronic environment, so that the fluorescence is turned on[90].

The lack of charge of PNA probes has been exploited in applications like Southern Blot (for DNA detection) or Northern Blot (for RNA detection), where the use of labeled PNA can simplify the methods and speed them up. PNAs are hybridized with DNA before the gel separation: since PNA-DNA duplexes are characterized by having a different charge-mass ratios, they can be separated from free DNA, and be visualized through fluorescent, immuno-enzymatic or radioactive signals, obtaining information about sequence and strand size at the same time[91,92].

A similar property has been exploited also in capillary electrophoresis (CE) in order to recognize complementary sequences and discriminate point mutations with a good selectivity. If a DNA strand is recognized and hybridized by the probe, a shift in its migration time is observed, allowing to obtain good performances in a short time, especially when highly specific chiral-box modified PNAs are used[93]; this allowed to recognize the presence of the R553X point mutation of the cystic fibrosis, using fluorescently labeled DNA oligonucleotide or amplified DNA[93].

The same effect can be exploited with the IE-HPLC technique, obtaining a robust and easy identification of DNA. When this method is performed by using fluorescently labeled DNA, the technique becomes more sensitive, since a fluorescence-based chromatogram is observed[94]. Recently a PNA Molecular-Beacon designed to recognize a sequence diagnostic of Roundup Ready soybean has been used in IE-HPLC[95] using non labeled DNA, obtaining the same detection limits affordable with standard PNA and fluorescent DNA[94].

The strength of complexes formed between PNA and DNA is very useful for applications where the PNA molecule has to compete with other ligands for the hybridization with the complementary DNA strand; this happens, for example, when PNAs are coupled with PCR technique, such as in PCR-clamping[96]: in this technique the binding of the PNA probe to the complementary strand inhibits the amplification process, because it competes with the binding

of the primers, or of the polymerase, also at high temperature. Such a system allowed the detection of genetically modified organism in food samples[97].

PNAs can also be used in real time PCR, where the amplification process can be followed online by monitoring a fluorescence signal that increases during the amplification process: PNA probes can be designed for giving a fluorescence signal when the amplification takes place, in particular PNA-molecular beacons have recently been applied since they hybridize the amplified sequences creating a fluorescence signal during the process[98].

Diagnostic techniques on solid phase are heterogeneous systems in which the PNA probe is linked to a solid support, while the DNA to be analyzed is in solution, and is captured on the surface when recognized by the probe. PNAs can be linked to a wide range of materials such as gold, glass, plastic etc., exploiting many kind of interactions, such as covalent bonds, host-guest or gold-thiol interactions etc. Microarray technology is a technique exploited in combination with PNAs. It is based on the deposition of the probe on flat surfaces in precise and well controlled positions using robotic systems based on contact printing or ink-jet deposition, obtaining probe spots in the range of some hundred micrometer. Chips fabricated in this way are hybridized at controlled temperature with pre-amplified DNA that can be labeled or label-free, non-hybridized sequences are washed away afterwards, and the sample can be analyzed using different effects. Signal are observed only where PNAs hybridize complementary sequences, and this allows to understand which sequence is in the sample. The size of the spots obtained by this technique allows to link to the surface a large number of different PNA probes in a very small area, giving the possibility to perform many assays at the same time using the same chip. A recent work demonstrated the realization of PNA microarrays to simultaneously detect the presence of hazelnut and peanut, by recognizing specific sequences, in raw materials and commercial products[99]. Another work shows the fabrication of Arg-PNA microarrays for a selective discrimination of SNPs on solid surface. The system thus fabricated allowed the selective and simultaneous recognition of two SNPs related to the apolipoprotein E gene, and so the discrimination of six different genotypes[100].

Other techniques that are based on bigger, more robust, devices, exploit the use of multi-well plates derivatized with PNAs in connection with the use of fluorescence detector: thus it is possible to read many wells in which different samples are analyzed at the same time, and to detect where hybridization is taking place. In order to analyze non labeled DNA samples this technique has been coupled with enzymatic systems, more typical of protein detection. When a DNA sequence is recognized by the probe on the surface, it forms a duplex. The duplex is, afterwards, recognized (by a non hybridized part of the target DNA sequence) by another

DNA strand labeled with a group that can be recognized by an enzyme. The enzyme recognition starts a series of reactions that eventually produce a fluorescence signal[101].

PCR-free techniques are the very ultimate goal and a very difficult challenge for scientists that work in the field of DNA recognition, so that many efforts have been put in the realization of systems able to perform such assays. One of these systems is based on Surface Plasmon Resonance (SPR), a phenomenon which induces a change in the refractive index due to the increase of mass on the surface. If a PNA probe is linked to the surface, hybridization with the DNA/RNA complementary sequences will increase the mass laying on the surface giving rise to an SPR signal. If the target DNA is labeled with bulky groups (such as gold nanoparticles), or a sandwich assay is performed, using a further probe linked to gold nanoparticles, this method can become very sensitive, thus allowing the detection of DNA without the necessity of pre-amplification[102]. This technique has been applied using a 15-mer PNA sequence specifically designed to identify Roundup Ready (RR) genetically modified soybean, obtaining a selective sequence discrimination with a femtomolar sensitivity[102]. Sandwich assays like the ones described above have been used for label-free DNA detection, exploiting the hybridization of the target sequences with the PNA probe on one side and with a revealing labeled PNA probe on the other side.

Also other interactions have been exploited in order to form such sandwiches for introducing different types of labeling groups. The interaction between a DNA strand hybridized to the PNA and a Zr^{4+} ion was used for introducing a rhodamine label that interact with the ion that is coordinated by DNA phosphates[103]. This fluorophore can be detected by Surface Enhanced Raman Scattering techniques on gold and silver derivatised silicon surfaces.

Finally, QCM microbalance is another technique that can be exploited to detect non labeled nucleic acids, since the signal is given by the increase of mass linked to the sensor, which increases when a DNA strand hybridizes with the PNA probe[104,105].

Another critical point in the analysis of nucleic acid sequences is the matrix interferences found in the biological samples. DNA extraction and purification from a real sample is not always an easy step, and for this reason PNAs linked to surfaces have been applied in DNA purification by hybridization. PNAs, in fact, can be used to complex DNA molecules in complex matrices, separating them from other interferents. In this way, a concentration effect can be achieved, and DNA can be purified in a suitable way for subsequent PCR amplification and detection[106].

DNA extraction is not always necessary for diagnostic applications in vivo, since sometimes hybridization can be performed directly in the cells. Fluorescent PNA oligomers labeled with

different fluorophores, were used to hybridize complementary DNA or RNA in the cell and revealed by fluorescence microscopy. Fluorescence microscopy allows to recognize which fluorescent probe stays in the cell after the washings, obtaining information about the sequence hybridized. This analysis is called Fluorescence In Situ Hybridization (FISH) and has been largely exploited in biology for recognizing specific type of cells by their DNA; this approach can be used to identify different bacteria in the same cell culture, performing also multiple recognition at the same time thanks to the combination of many fluorophores[107]. The use of PNA probes increases the quality of the technique, since PNAs enter the cells more easily than oligonucleotides, are more resistant in vivo, and hybridize better to the complementary sequences[108].

1.8.2 Biomedical Applications

When a therapy has DNA or RNA sequences as targets, selectivity is the main goal to achieve. Since in most cases the therapy requires a large number of cells to be killed, the possibility to act only against the target cells may allow to cure the illness without affecting the host body. Moreover, the possibility to specifically interact with the genetic code of a cell is a strong tool that can be used in order to control its life, since it allows to block some genetic mechanisms or to stimulate others. Many efforts have been done in the development of this approach, called genetic therapy, designing oligonucleotides able to target DNA or RNA within the cell; however, great limitations have been found when using standard oligonucleotides[1]. The introduction of DNA analogues improved the potential of these applications. In particular, as far as PNAs are concerned, the enzymatic stability of these molecules, together with their affinity and specifity in the recognition of the complementary sequences, makes them highly suitable tools for biomedical applications. In any case, when DNA is targeted, a series of problems are to be solved for making the strategy effective. Since DNA is present in the cell as a double strand helix, the hybridization can be performed only if the helix can be opened. The necessity to hybridize a sequence in double strand form, makes PNAs suitable for this application, because of their ability to perform strand invasion under certain circumstances; however the choice of the sequence is not always straightforward and chemical modifications may be necessary to perform invasion[109]. The fact that the DNA is located in the nucleus and protected by the nuclear membrane, which is permeable only by molecules specifically recognized by defined receptors, is another problem to be solved when

this approach is chosen. The solution has often been found by conjugating PNAs with carrier molecules which behave as keys able to recognize the lock and open the door to the inner part of the nucleus. One of the most common solution is the coupling with positively charged peptides, usually rich in lysine or arginine residues, such as NLS or Tat sequence, which also promote strand invasion because of the positive charges[110].

Recently, a new type of DNA target has been considered, the G-Quadruplex, a particular DNA structure formed in GC rich zones. Many studies have demonstrated the importance of this areas because many transcription factors recognize and bind to these kind of sequences. PNAs have been used to target and block GC-rich zones, thanks to their ability to perform strand invasion[111].

The application of PNA in antisense therapy can increase the performance of the method, since the PNA molecules are not hydrolyzed in the cell, as it happens for standard oligonucleotides[112]. Compared to antigene applications, the antisense approach does not have to deal with the nuclear uptake of the molecule, because RNA targeting takes place in the cytoplasm and not in the nucleus. Although the hybridization process may appear easier because RNA is in single strand form, secondary structures may occur in RNA, making its targeting difficult PNAs facilitates the recognition, since the stability of PNA-RNA duplexes is usually enough to open the secondary structures. Unlike DNA, that exists as a single copy, RNA is produced in more than one copy, and its production continues during the treatment, making the time necessary for blocking it effectively longer.

Another approach linked to the antisense therapy is the targeting of micro-RNAs, short RNA sequences 20-30 nucleotides long. MiRNAs are implicated in post-transcriptional gene regulation, which plays a vital role in cellular processes such as developmental control, metabolism and apoptosis. PNAs showed excellent performances in these applications, also when compared to other DNA analogues such as LNA or PS-DNA[113,114].

1.9 References

[1] D. A. Braasch, D. R. Corey, *Biochemistry*, **2002**, 41, 14, , 4053
[2] E. T. Kool, *Chem. Rev.*, **1997**, 97, 1473
[3] F. Pellestor, P. Paulasova, *European Journal of Human Genetics*, **2004**, 12, 694
[4] E. De Clercq, F. Eckstein,T. C. Merigan, *Science*, **1969**, 165, 1137
[5] S. Gryaznov, J. K. Chen, *J. Am. Chem. Soc.*, **1994**, 116, 3143
[6] D. A. Braasch,D. R. Corey, *Chem. Biol.*, **2001**, 8, 1
[7] A. Arzumanov, A. P. Walsh, V. K. Rajwanshi, R. Kumar, J. Wengel, M. J. Gait, *Biochemistry*, **2001**, 40, 14645
[8] J. Heasman, *Dev. Biol.*, **2002**, 243, 209
[9] J. Wang, B. Verbeure, I. Luyten, E. Lescrinier, M. Froeyen, C. Hendrix, H. Rosemeyer, F. Seela, A.van Aerschot, P. Herdewijn, *J. Am. Chem. Soc.*, **2000**, 122, 8595
[10] D. Renneberg, C. J. Leumann, *J. Am. Chem. Soc.*, **2002**, 124, 5993
[11] M. J. Damha, C. J. Wilds, A. Noronha, I. Bruckner, G. Borkow, D. Arion, M. A. Parniak, *J. Am. Chem. Soc.*, **1998**, 120, 12976
[12] J. Kurreck, *Eur. J. Biochem.*, **2003**, 270, 1628
[13] P. E. Nielsen, M Engholm, R. Berg, O. Buchardt, *Science*, **1991**, 254, 1497
[14] B, Hyrup, P. E. Nielsen, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 1, 5
[15] V. V. Demidov, V. N. Potaman, M. D. Frank-Kamenetskii et al., *Biochem. Phrmacol*., **1994**, 48, 1310
[16] F. P. Schwarz, S. Robinson, J. M. Butler, *Nucleic Acids Research*, **1999**, 27, 24, 4792
[17] M. Eghlom, O. Buchardt et al., *Nature*, **1993**, 365, 566
[18] P. E. Nielsen, *Pure Appl. Chem.*, **1998**, 70, 105
[19] M. Eghlom, O. Buchardt et al., *Nature*, **1993**, 365, 566
[20] V. V. Demidov, M. D. Frank-Kamenetskii, *Trends Biochem. Sci.*, **2004**, 29, 62
[21] P. E. Nielsen, M. Egholm, O. Buchardt, *Bioconjugate Chem.*, **1994**, 5, 3
[22] A. RAY, B. Nordén, *The FASEB Journal*, **2000**, 14, 1041
[23] M. Egholm, P. E. Nielsen, O. Buchardt, R. H. Berg, *J. Am. Chem. Soc.*, **1992**, 114, 9677
[24] P. E. Nielsen, L. Christensen, *J. Am. Chem. Soc.*, **1996**, 118, 2287
[25] J. Lohse, O. Dahl, P. E. Nielsen, *Proc. Natl. Acad. Sci. U.S.A.*, **1999**, 96, 11804.
[26] K. Kaihatsu, R. H. Shah, X. Zhao, D. R. Corey, *Biochemistry*, **2003**, 42, 13996
[27] G. He, S. Rapireddy, R. Bahal, B. Sahu, D. H. Ly, *J. Am. Chem. Soc.*, **2009**, 131, 12088
[28] T. Ishizuka, J. Yoshida, Y. Yamamoto et al., *Nucleic Acids Research*, **2008**, 36, 5, 1464
[29] T. Ishizuka, K. Otani, J. Sumaoka, M. Komiyama, *Chem. Comm.*, **2009**, 1225
[30] T. Ishizuka, T. Tedeschi, R. Corradini, M. Komiyama, S. Sforza, R. Marchelli, *ChemBioChem*, **2009**, 10, 2607
[31] P. E. Nielsen, M. Egholm, R. H. Berg, *Science*, **1991**, 254, 5037, 1497
[32] S. A. Thomson, J. A. Josey, S. A. Noble et al., *Tetrahedron*, **1995**, 51, 6179
[33] So-Yeop Han and Young-Ah Kim, *Tetrahedron*, **2004**, 60, 2447
[34] L. D. Mayfield, D. R. Corey, *Analytical Biochemistry*, **1999**, 268, 401
[35] M. Planas, E. Bardajì, K. J. Jensen and G. Barany, *J. Org. Chem.*, **1999**, 64, 7281
[36] D. W. Will, G. Breipohl, D. Langner, J. Knolle, E. Uhlmann, *Tetrahedron*, **1995**, 51, 12069
[37] F.Debaene, N. Winssinger, *Org. Lett*., **2003**, 5, 4446
[38] L. Bialy, J. J. Dìaz-Mochòn, E. Specker, L. Keinicke, M. Bradley, *Tetrahedron*, **2005**, 61, 8295
[39] G. Upert, M. Mehiri, M. Goddard, A. Di Giorgio, R. Benhida, R. Condom and N. Patino, *Tetrahedron Letters*, **2005**, 46 4081
[40] J. M. Heemstra and D, R. Liu, *J. Am. Chem. Soc.*, **2009**, 131, 11347
[41] H. Lee, J. H. Jeon, J. C. Lim, H. Choi, Y. Yoon and S. K. Kim, *Org. Lett.*, **2007**, 9, 3291

[42] C. G. Simmons, A. E. Pitts, L. D. Mayfield, J. W. Shay, D. R. Corey, *Bioorganic & Medicinal Chemistry Letters*, **1997**, 7, 23, 3001
[43] D. W. Will, G. Breipohl, D. Langner, J. Knolle, E. Uhlmann, *Tetrahedron*, **1995**, 51, 12069
[44] R. Corradini, S. Sforza, T. Tedeschi, F. Totsingan, R. Marchelli, *Curr. Topic Med. Chem.*, **2007**, 7, 681
[45] B. Hyrup, M. Egholm, P. E. Nielsen, P. Wittung, B. Nordén, O. Buchardt, *J. Am. Chem. Soc.*,**1994**, 116, 18, 7964
[46] K. H. Petersen, O. Buchardt, P. E. Nielsen, *Bioorganic & Medicinal Chemistry Letters*, **1996**, 6, 7, 793
[47] S. Sforza, G. Galaverna, A. Dossena, R. Corradini, R. Marchelli, *Chirality*, **2002**, 14, 591
[48] G. Haaima, A. Lohse, O. Buchardt, P. E. Nielsen, *Angew. Chem. Int. Ed*., **1996**, 35, 1939
[49] S. Sforza, G. Haaima, R. Marchelli, P. E. Nielsen, *Eur. J. Org. Chem*., **1999**, 197
[50] A. Puschl, S. Sforza, G. Haaima, O. Dahl, P. E. Nielsen, *Tetrahedron Lett.*, **1998**, 39, 4707
[51] P. Wittung, M. Eriksson, R. Lyng, P. E. Nielsen, B. Norden, *J. Am. Chem. Soc.,* **1995**, 117, 10167
[52] S. Sforza, R. Corradini, S. Ghirardi, A. Dossena, R. Marchelli, *Eur. J. Org. Chem.*, **2000**, 2905
[53] P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner, D. H. Ly, *J. Am. Chem. Soc.*, **2003**, 125, 6878
[54] E. A. Englund, D. H. Appella, *Angew. Chem. Int. Ed.*, **2007**, 46, 1414
[55] Xiao-Wei Lu, Y. Zeng, Chuan-Fa Liu, *Org. Lett.,* **2009**, 11, 2329
[56] U. Diederichsen, D. Weicherdinga, N. Diezemanna, *Org. Biomol. Chem*., **2005**, 3, 1058
[57] T. Govindaraju, V. A. Kumar, K. N. Ganesh, *J. Org. Chem.*, **2004**, 69, 1858
[58] N. K. Sharma, K. N. Ganesh, *Chem Comm*, **2003**, 2484
[59] V. A. Kumar, *Eur. J. Org. Chem*., **2002**, 2021
[60] J. K. Pokorski, M. A. Witschi, B. L. Purnell, D. H. Appella, *J. Am. Chem. Soc.*, **2004**, 126, 15067
[61] P. S. Lonkar, V. A. Kumar, *Org. Biomol. Chem*., **2004**, 14, 2147
[62] T. Govindaraju, V. A. Kumar, K. N. Ganesh, *J.Am. Chem. Soc.*, **2005**, 127,4144
[63] L. D. Fader, E. L. Myers, Y. S. Tsantrizos, *Tetrahedron*, **2004**, 60, 2235
[64] R. Schutz, M. Catin, C. Roberts, B. Greiner, E. Uhlmann, C. Leumann, *Angew. Chemie Int. Ed.*, **2000**, 39, 1250
[65] M. Hollenstein, C. J. Leumann, *Org. Lett*., **2003**, 5, 1987
[66] V. A. Efimov, M. V. Choob, A. A. Buryakova, A. L. Kalinkina, O. G. Chakhmakhcheva, *Nucleic Acids Research*, **1998**, 26, 566
[67]M. Kuwahara, M. Arimitsu, M. Shigeyasu, N. Saeki, M. Sisido, *J. Am. Chem. Soc.*, **2001**, 123, 4653
[68] V. A. Kumar, *Eur. J. Org. Chem*., **2002**, 2021
[69] P. Cerea, C. Giannini, S. Dall'Angelo, E. Licandro, S. Maiorana, R. Marchelli, *Tetrahedron*, **2007**, 63, 4108
[70] F. Wojciechowski, R. H. E. Hudson, *Current Topics in Medicinal Chemistry*, **2007**, 7, 667
[71] A. B. Eldrup, C. Christensen, G. Haaima, P. E. Nielsen, *J. Am. Chem. Soc.*, **2002**, 124, 3254
[72] A. B. Eldrup, B. B. Nielsen, G. Haaima et al., *Eur. J. Org. Chem.*, **2001**, 9, 1781
[73] K. G. Rajeev, M. A. Maier, E. A. Lesnik, M. Manoharan, *Org. Lett.*, **2002**, 4, 4395
[74] M. Egholm, L. Christensen, K. L. Dueholm, O. Buchardt, J. Coull, P. E. Nielsen, *Nucleic Acid Research*, **1995**, 23, 217
[75] C. Christensen, A.B. Eldrup, G. Haaima, P. E. Nielsen, *Bioorg. Med. Chem.*, **2002**, 12, 3121
[76] G. Haaima, A. Lohse, O. Buchardt, P. E. Nielsen, *Angew. Chem. Int. Ed.*, **1996**, 35, 17, 1939
[77] S. Sforza, R. Corradini, S. Ghirardi, A. Dossena, R. Marchelli ,*Eur. J. Org. Chem.*, **2000**, 2905
[78] V. Menchise, G. De Simone et al., *PNAS*, **2003**, 100, 21, 12021
[79] T. Ishizuka, J. Yoshida, *Nucleic Acids Research*, **2008**,. 36,. 5, 1464
[80] E. A. Englund, D. H. Appella, *Org. Lett.*, **2005**, 7, 16, 3465
[81] E. A. Englund, D. H. Appella, *Angew. Chem. Int. Ed.*, **2007**, 46, 1414
[82] J. O. Smith, D. A. Olson, B. A. Armitage, *J. Am. Chem. Soc.,* **1999,** 121, 2686
[83] S. Sforza, T. Tedeschi, R. Corradini, R. Marchelli, *Eur. J. Org. Chem.,* **2007**, 5879
[84] S. Sforza, T. Tedeschi, R. Corradini, D. Ciavardelli, A. Dossena, R. Marchelli, *European Journal of Organic Chemistry*, 6, **2003**, 1056
[85] M. M. Ali, Y. Li, *Angew. Chem. Int. Ed*., **2009**, 48, 3512

[86] T. Tedeschi, S. Sforza, S. Ye, R. Corradini, A. Dossena, M. Komiyama, R. Marchelli, *J. Biochem. Biophys. Methods*, **2007**, 70, 735
[87] X. Su, R. Kanjanawarut, *ACS Nano*, **2009**, 3, 9, 2751
[88] G. Prencipe, S. Maiorana, P. Verderio, M. Colombo, P. Fermo, E. Caneva, D. Prosperi, E. Licandro, *Chem. Comm*., **2009**, 6017
[89] H. Kuhn, V. V. Demidov, J. M. Coull et al., *J. Am. Chem. Soc.*, **2002**, 9, 124, 6, 1097
[90] D. V. Jarikote, O. Köhler, E. Socher, O. Seitz, *Eur. J. Org. Chem.*, **2005**, 3187
[91] H. Perry-O'keefe, X. W. Yao, J. M. Coull, M. Fuchs, M Egholm, *Proc. Natl. Acad. Sci. USA*, **1996**, 93, 14670
[92] A. T.-Salazar, J. Dhawan, A. Lovejoy, Q. A. Liu, A. N. Gifford, *Anal. Biochem.*, **2007**, 360, 92
[93] T. Tedeschi, M. Chiari, G. Galaverna, S. Sforza, M. Cretich, R. Corradini, R. Marchelli, *Electrophoresis,* **2005**, 26, 4310
[94] S. Rossi, F. Lesignoli, A. Germini, A. Faccini, S. Sforza, R. Corradini, R. Marchelli, *Journal of Agricultural and Food chemistry*, **2007**, 55, 7, 2509
[95] F. Totsingan, S. Rossi, R. Corradini et al., *Organic & Biomolecular Chemistry*, **2008**, 6, 7, 1232
[96] H. 0rum, P. E.Nielsen, M. Egholm et al., *Nucleic Acids Research*, **1993**, 21, 23, 5336
[97] C. Peano, F. Lesignoli, M. Gulli, R. Corradini, M.C. Samson, R. Marchelli, N. Marmiroli, *Anal. Biochem.*, **2005**, 344, 174
[98] K. Petersen, U. Vogel, E. Rockenbauer, K. V. Nielsen, S. Kølvraa, L. Bolund, B. Nexø, *Molecular and Cellular Probes*, **2004**, 18, 117
[99] S. Rossi, E. Searavelli, A. Germini, R. Corradini, C. Fogher, R. Marchelli, *European Food Research and Technology*, **2006**, 223, 1, 1
[100] A. Calabretta, T. Tedeschi, G. Di Cola, R. Corradini, S. Sforza, R. Marchelli, *Molecular Biosystems*, **2009**, 5, 11, 1323
[101] N. Zhang, D. H. Appella, *J. Am. Chem. Soc.*, **2007**, 129, 8424
[102] R. D'Agata, R. Corradini, G. Grasso, R. Marchelli, G. Spoto, *ChemBioChem*, **2008**, 9, 13, 2067
[103] C. Fanga, A. Agarwal, K. D. Buddharaju et al., *Biosensors & Bioelectronics*, **2008**, 24, 216
[104] C. Yao, T. Zhub, J. Tang, *Biosensors & Bioelectronics*, **2008**, 23, 879
[105] J. Wang, *Biosensors & Bioelectronics*, **1998**, 13, 757
[106] D. P. Chandler, J. R. Stultus, S. Cebula,B. L. Schuck, D. W. Weaver, K. K. Anderson, *Applied and Enviromental Microbiology*,**2000**, 3438
[107] H. Perry-O'Keefe, S. Rigby, K. Oliveira, D. Sørensen, H. Stender, J. Coull, J.J. Hyldig-Nielsen, *Journal of Microbiological Methods*, **2001**, 47, 281
[108] L. Cerqueira, N. F. Azevedo, C. Almeida, T. Jardim, C. W. Keevil, M. J. Vieira, *Int. J. Mol. Sci.*, **2008**, 9, 1944
[109] J. Hu, D. R. Corey, *Biochemistry*, **2007**, 46, 7581
[110] A. Faccini , A. Tortori, T. Tedeschi et al., *Chirality*, **2007**,20, 494
[111] M. I. Onyshchenko, T. I. Gaynutdinov, E. A. Englund, D. H. Appella,R. D. Neumann and I. G. Panyutin, *Nucleic Acids Research*, **2009**, 1
[112] J. Kurreck, *Eur. J. Biochem.*, **2003**, 270, 1628
[113] S. Y. Oh, Y. S. Ju, H. Park, *Molecules and Cells*, **2009**, 1
[114] M. M. Fabani, J. G. Michael, *RNA*, **2008**, 14, 336

Chapter 2

Label-free detection of Single Nucleotide Polymorphisms (SNPs): PNA Molecular Beacons

In this chapter the application of modified PNAs for SNP detection is shown. PNA beacons allow to detect complementary DNA sequences without the necessity to label it before or after PCR amplification. Upon hybridization, in fact, these molecules emit a fluorescence signal which is highly enhanced if compared to that of the free unhybridized beacon. The probes here presented were designed in order to recognize a SNP which is diagnostic for the olive cultivar "Ogliarola Leccese". Three beacons of different length were synthesized, in order to test the recognition performances in relation to the PNA length. The application in AE-HPLC, together with solution assays, allowed to identify the ideal length for specific and efficient DNA hybridization and to verify the PNA probes performances. After optimization of the method, the probe was applied to recognize the SNP in a sample of DNA extracted from olive leaves and amplified by PCR. It has been shown that this methodology can be applied to real samples.

2.1 Introduction

In the last years we have assisted to a fast development of selective and sensitive bioassays aimed at recognizing genetically significant DNA sequences with methods at the same time robust and simple[1]. Most of the research on DNA markers is related to genetic diseases, with the aim of providing efficient tools to assess the risk of an illness, also when symptoms are not yet present[2,3]. Actually, most polymorphisms are silent and do not have any pathological implication, but their identification can be exploited for identifying an organism or an individual (such as in forensic science)[4]. In food analysis, the recognition of DNA extracted from a defined ingredient can be extremely useful for tracing the origin of a food product or for evidencing ingredients undeclared, even if present in very small amounts[5,6]. Among the different DNA polymorphisms that can be usually exploited for these purposes, most studies concern the Single Nucleotide Polymorphisms (SNPs)[7], which consists in a single nucleobase change, and microsatellite sequences, small repeating non-coding sequences differing in the number of repeats. Single Nucleotide Polymorphisms are the major source of variability within genomic DNA and may have a biological meaning or not, according to the position in which they are located in a given genome[7,8].

Although SNP recognition is of extreme importance, classical methods for their detection are time consuming and expensive. These methods usually imply DNA extraction and purification, amplification by polymerase chain reaction (PCR) and sequencing of the amplicons. Although sequencing has been improved by the use of automated techniques, these methods are still expensive and cumbersome, especially when more than one polymorphism in different genomic locations has to be detected, since the PCR-sequencing cycle has to be repeated for each single mutation. For this reason, new methods based on many different techniques have been developed in order to perform faster and cheaper analyses. The new methods are usually based on the synthesis of probes complementary to the sequence to be recognized, hybridization of the probes with amplified DNA and detection of a reading out signal when selective hybridization occurs. Although these assays look promising, many improvements are still possible in this field: the necessity to perform high throughput analysis, the possibility to develop PCR-free and label-free methods and the possibility to perform such assays with robust, reproducible and cheap techniques still represents a challenge.

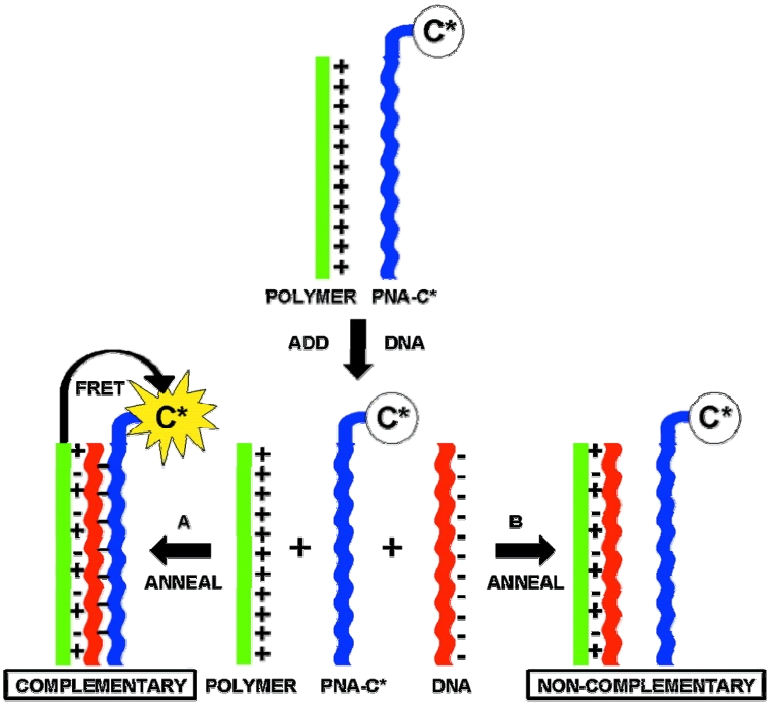


Figure 2-1: Schematic representation for the use of water-soluble cationic polymers with a specific PNA-C* optical reporter probe to detect a complementary ssDNA sequence (B. S. Gaylord et al., *Proc. Nat. Acad. Sci*., 2002, 10954)

The probes used for such applications can be simple DNA oligonucleotides, but the necessity to achieve high selectivity for point mutation introduced the necessity to use different DNA analogues, and PNAs turned out to be among the best performing molecules. Actually, the PNA properties have been fully exploited in the development of several DNA bioassays[9,10].

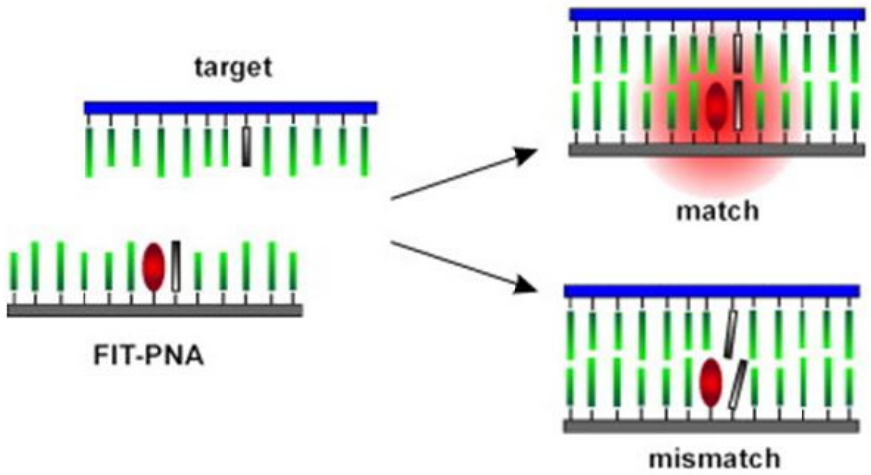


Figure 2-2: Representation of the FIT probe mechanism (E. Socher et al., *Anal. Biochem.*, 2008, 375, 318)

For example, the possibility to perform PNA-based analysis for recognizing non labeled DNA has been deeply investigated, leading to the development of assays based on many different analytical techniques. Mass spectrometry is one of these, since it can be easily exploited to perform label-free DNA recognition, detecting duplex formation directly by ESI mass spectrometry only when the probe recognizes the complementary DNA[11,12]. However, mass spectrometry can fail in identifying long sequences of amplified DNA.

The use of fluorescent PNA probes together with cationic conjugated fluorescent polymers, extended label-free detection also to optical methods (Figure 2-1)[13], where DNA labeling is usually required. The polymers bind the PNA-DNA duplex by electrostatic interaction and emit by Fluorescence Resonance Energy Transfer (FRET) from the dye on the PNA, allowing SNP recognition with good selectivity[14].

With the aiming of performing detection of unlabelled amplified DNA a series of modified PNAs able to produce a signal when hybridized to the complementary sequences was developed. PNA labelled with modified cyanine which emits fluorescence when hybridized or PNA molecular beacons are some of the probes used for this purpose. Conjugation of PNAs with different types of thiazole orange (TO) groups linked to the backbone in place of a nucleobase led to the development of FIT probes (Forced Intercalation of Thiazole orange, figure 2-2)[15, 16], molecules which are not fluorescent when not hybridized, whereas upon hybridization with the complementary sequence, a fluorescence light up is observed on account of the forced intercalation of TO inside the duplex. The performance of these molecules made them suitable for solution assays[17] as well as for real time PCR[18].

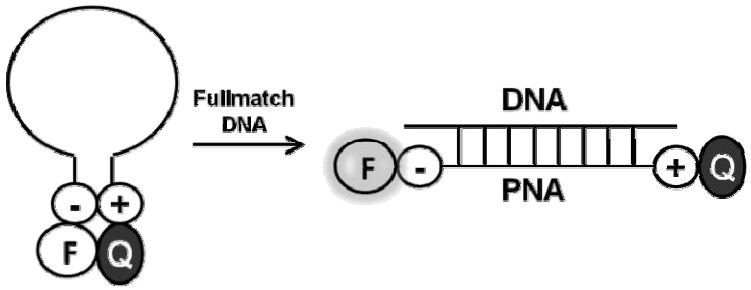


Figure 2-3: Schematic representation of the PNA beacon mechanism when hybridized to fullmatch DNA

PNA molecular beacons have been developed on the same accounts of the corresponding DNA-based probe. These molecules are obtained by linking a fluorophore, such as fluorescein, and the corresponding quencher, such as dabcyl, at the opposite ends of the molecule; when the probe is in solution, the conformation of the beacon keeps the ends close to each other causing the quenching of the fluorescence (Figure 2-3); when complementary

DNA is added, the hybridization causes a change in conformation that switches on the fluorescence since the quencher moves away from the fluorescent unit[19,20]. Depending on the type of beacon, different strategies are used to keep the probe in the closed form when not hybridized: DNA beacons are usually kept closed thanks to a stem obtained using two short complementary sequences at the ends of the probe. PNA molecular beacons may be maintained closed by an electrostatic interaction exerted by two amino acids bearing opposite charges at both ends of the probe.

PNA molecular beacons have been used together with many techniques for the recognition of complementary sequences obtaining a good signal-to-noise ratio, since the flexibility of the backbone allows a ready closure when no complementary sequence is hybridized. By coupling these molecules with PNA openers, i.e. PNAs able to strand invade duplex DNA, it was possible to hybridize not only single stranded DNA, but also double helices making the target sequence accessible to the PNA. The modified probes display a high selectivity in sequence recognition, being able to recognize single nucleotide polymorphisms[19]. In order to increase PNA selectivity, which is necessary in some applications, chiral centers within the PNA backbone were introduced[21]. A monomer bearing a L-lysine side chain in position 5 has been introduced in the middle of the sequence, as previous works demonstrated that enhanced binding performances are due to this modification[22]. Moreover, the side chain could be used for linking the PNA probe to functional active groups on surfaces or to nanoparticles[23]. The features offered by these probes allowed their application as useful tools in many analytical techniques. The fluorescent signal produced by the probe after hybridization is very useful in real time PCR, since it allows to monitor the amplification process in real time[24]. Good performances were obtained also by IE-HPLC[21, 25], by combining the discriminative power offered by the chromatographic technique.

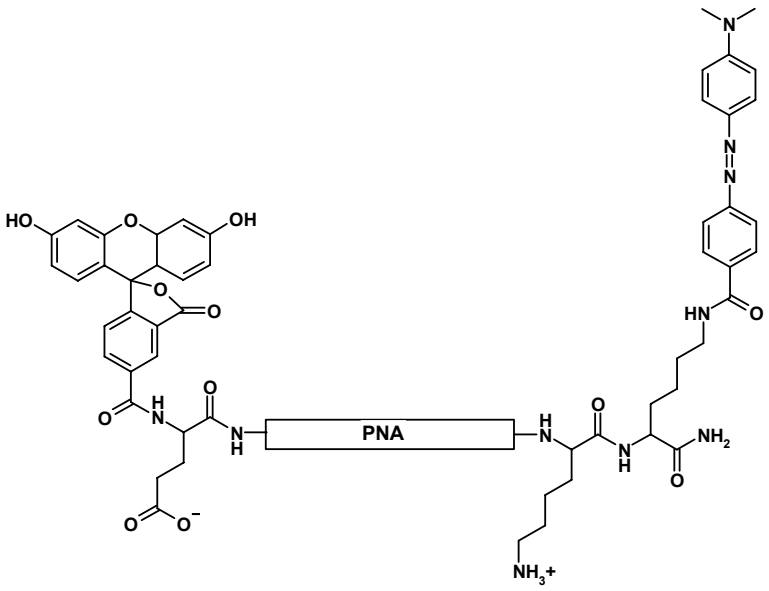


Figure 2-4: Chemical structure of a PNA Molecular Beacon (F. Totsingan, et al., *Org. Biomol. Chem.*, 2008, 6, 1232)

2.2 Results and Discussion

In this chapter, PNA molecular beacons are designed in order to be used in the field of food science, in particular in connection with the authenticity of monocultivar extra virgin olive oil. In fact, the recognition of SNPs characteristic of a specific cultivar of olive can allow to certify a monocultivar product. The probes used here had been developed in a previous work[26] in order to recognize a specific cultivar of olive among twelve different cultivars. In particular, the cultivar taken in consideration was "Ogliarola Leccese". For this cultivar, in fact, a thymine substitutes an adenine in the position 60 of the gene Actin. The PNA probes were synthesized in order to be complementary to the sequence containing the thymine, thus able to recognize this particular SNP.

Table 2-1. PNA sequences and melting temperatures of the PNA:DNA antiparallel fullmatch/mismatch duplexes in phospate buffer (pH=7) at a 5μm concentration for each strand

PNA	Sequence	Tm (°C) PNA/DNA fullmatch	Tm (°C) PNA/DNA mismatch	ΔTm (°C)
Beacon 11 mer	Fluo-Glu-ACTC**A**TTCACC-Lys-Lys-(Dabcyl)-NH_2	53	36	17
Beacon 13 mer	Fluo-Glu-TTACTC**A**TTCACC-Lys-Lys-(Dabcyl)-NH_2	54	37	17
Beacon 15 mer	Fluo-Glu-GGTTACTC**A**TTCACC-Lys-Lys-(Dabcyl)-NH_2	65	51	14
PNA 13 mer	H-TTACTC**A**TTCACC-NH_2	54	35	19

The monomer in bold corresponds to the SNP identifying the cultivar "Ogliarola Leccese"

Three stemless PNA beacons, as previously described[21] (Figure 2-4), were designed only differing for the length of the sequence: 11, 13 and 15mer; with the mismatch position in the middle. The scope was to study the effect of the length on the efficiency of the probe. The melting temperature values of the three PNAs with the complementary and mismatched DNA oligonucleotides are reported in Table 2-1 and compared with a 13-mer standard PNA with the same sequence. As expected, the selectivity decreased with the increase of the length. It is to be noted that, whereas the 11-mer and 13-mer PNA probes showed a certain similarity in

the affinities towards oligonucleotides, the 15-mer PNA presented quite different and much higher stability values.

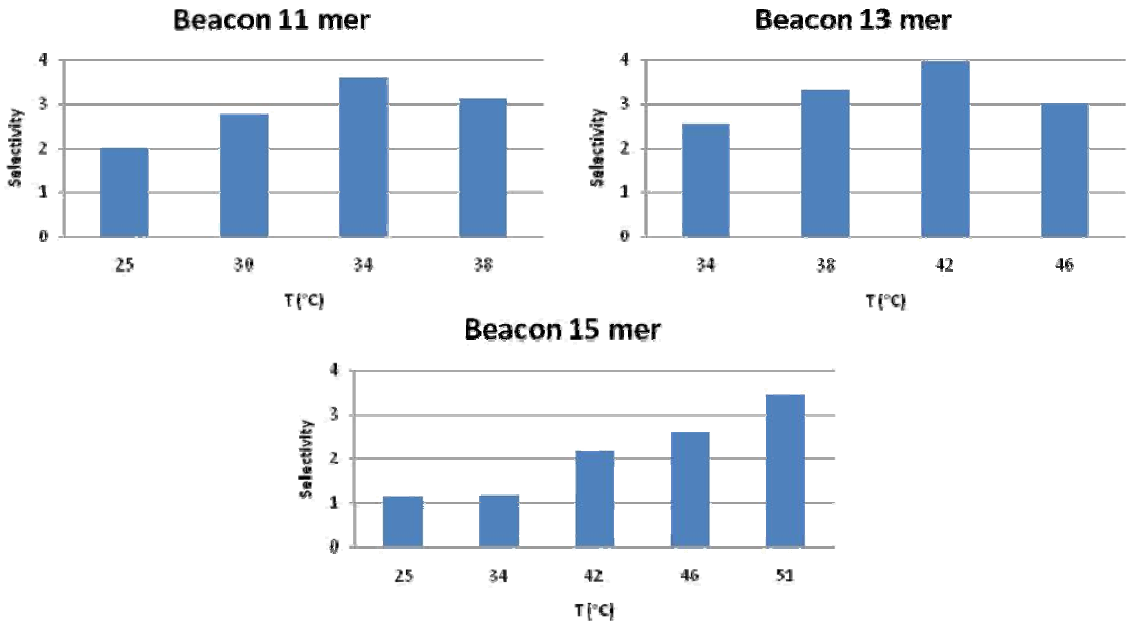


Figure 2-5: Selectivity of PNA molecular beacons at different temperatures measured by fluorescence spectroscopy. Selectivity has been obtained calculating the ratio between emitted fluorescence at 522 nm of fullmatch and mismatch hybrid with excitation wavelength at 497nm.

Discrimination properties were tested in solution by evaluating the fluorescence switching on in the presence of fullmatch and mismatch oligonucleotides for a 1μM solution in Tris buffer at pH=8. The results shown in figure 2-5 were obtained by measuring the fluorescence emission at 522 nm for fullmatched and mismatched hybrids and calculating the ratio between the two values at different temperatures. In general, all probes show optimal discrimination above room temperature, suggesting that recognition experiments should be carried out in partially denaturing conditions. As expected, the optimum temperature for the probe performance differs for the three PNAs according to the probe length: the longer the PNA, the higher the optimum temperature. In particular, the 15-mer probe could discriminate the point mutation with reasonably good performances only at high temperature, confirming that this probe is not ideal for analytical purposes, while smaller probes seemed to be more efficient in the mismatch recognition especially at lower temperatures. Comparing the performances offered by the two shorter probes, it appeared that the 13 mer probe had a slightly better selectivity in the conditions of analysis.

A drawback of the method emerged from the consideration that the probe in the closed form had a residual fluorescence that decreases the selectivity. A way to separate the aspecific

fluorescence contribution due to the non hybridized beacon from the specific signal given by the hybrid formation, is to use the beacon in HPLC, where the two species may be chromatographically separated. Moreover, under the conditions in which HPLC analyses are performed, a partial denaturation of duplexes occurs, which has been demonstrated to be optimal for maximizing the selectivity. In particular, anion exchange HPLC has been demonstrated to be the optimal technique to separate PNA-DNA duplexes from free DNA and PNA. The eluents used in this method are at a slightly basic pH, which maximizes the fluorescence intensity of fluorescein. Moreover, the ionic strength stabilize the closed conformation of the unbound PNA-beacon, minimizing the aspecific fluorescence signal. The only species that could interfere with the detection is the free probe, but if this molecule is well separated from the peak corresponding to the duplex, there is no risk of interference from other molecules, since free DNA or residues from the PCR mixture are not fluorescent. This behavior allows to develop fast gradients. The performances in the HPLC system of the different PNA probes with different lengths were studied in the present work.

Hybrids with fullmatched and mismatched DNA oligonucleotides having the same length as the probe were tested and analyzed by anion exchange HPLC, using a TSK gel DNA-NPR column. The analyses were performed with a gradient from Tris 0.02M (pH= 9) to Tris 0.02M, NaCl 1M (pH=9). The chromatograms were obtained by using a fluorescence detector (λex= 497nm; λem= 520nm), as reported in previous works[25]. For each probe, the duplexes were prepared in a 1:1 ratio with the fullmatch DNA, and the concentration was decreased until it was possible to observe a clear chromatographic peak, in order to find the lowest concentration detectable. At these concentrations, selectivity experiments were carried out in the optimized conditions; for this reason, fullmatch and mismatch hybrids were prepared and analyzed as previously described.

The chromatograms obtained with 11 mer and 13 mer probes showed a clear and sharp peak corresponding to the PNA-DNA hybrids (Figure 2-6), when hybrids are formed using fullmatch DNA. The 11 mer probe showed a good sensitivity, since the duplex could be clearly detected down to a concentration of 30 nM (Figure 2-6 A). A broad peak eluting before the duplex was also present: this signal can be attributed to the free probe and had not a constant intensity, probably due to small changes in the eluent. When the 13-mer probe was used, none of these broad signal could be detected (Figure 2-6 C), probably on account of the higher stability of the corresponding PNA-DNA duplex. The experiments performed using decreasing concentrations of probes and target sequences showed that the probe stabilities and the sensitivity offered by the fluorescence detector allow to use very small amounts of probes.

In particular, the 11mer probe could be efficiently set at the concentration of 30 nM (Figure 2-6 B), while the higher stability offered by the 13mer probe allowed to work at a concentration of 10 nM (Figure 2-6 D), reaching limits which could not be afforded in previous similar works[21, 25].

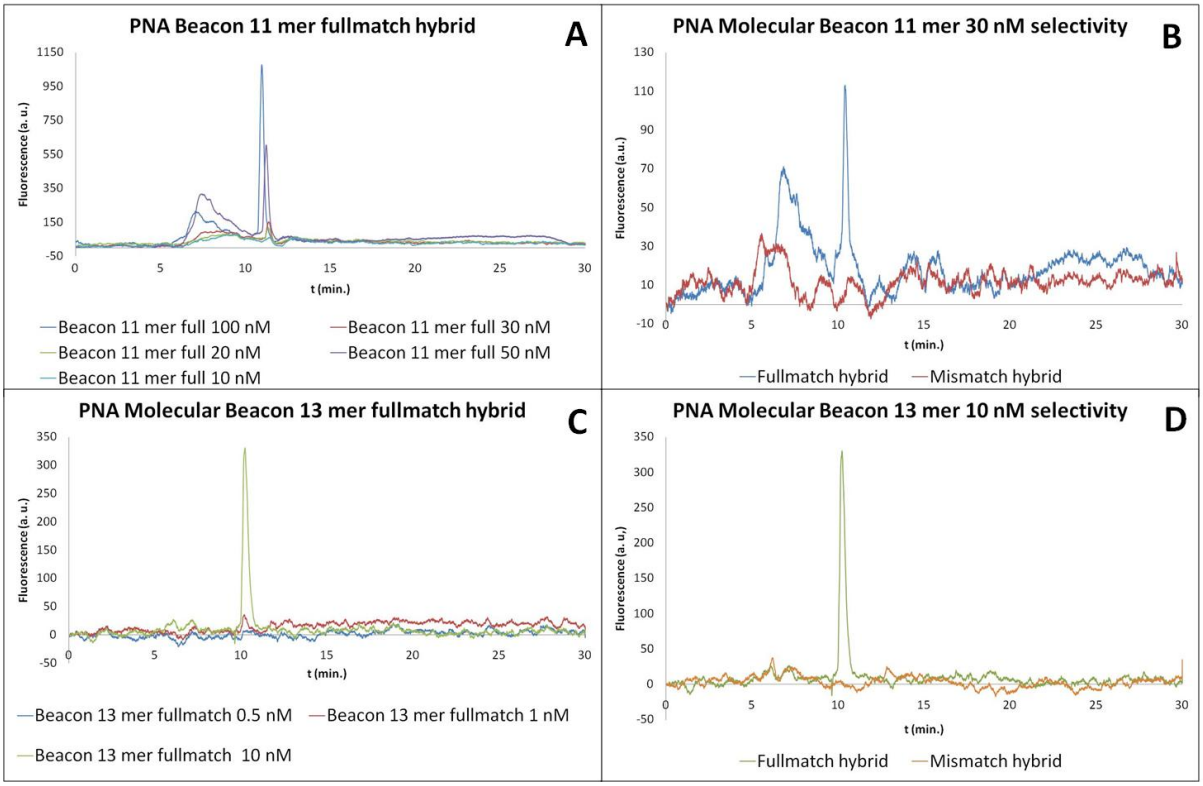


Figure 2-6: AE-HPLC-FD chromatograms (λex= 497nm; λem= 520nm) obtained for PNA beacon-DNA fullmatch hybrids at various concentrations with 11-mer (A) and 13-mer (C) probes. Chromatograms obtained with fullmatch DNA and single mismatch DNA at concentrations of 30 nM for 11-mer (B) and 10 nM for 13-mer (D) just above the limit of detection are shown. Conditions: TSK gel DNA-NPR column with a gradient from 100% A (Tris 0.02M, pH= 9) to 100% B (Tris 0.02M, NaCl 1M pH=9).

The lowest concentrations detectable were then used to perform selectivity tests, by using oligonucleotides containing one mismatch exactly corresponding to the SNP characteristic of other olive varieties. The chromatograms obtained (Figure 2-6) show that both probes did not give rise to any peak when mismatched oligonucleotides were used, allowing to recognize the mutation with an excellent selectivity at room temperature.

The data obtained with the 15 mer PNA beacon, exactly mirroring the results in solution, were less clear than those with the shorter probes. The chromatograms always showed a double peak of unknown origin corresponding to the duplex signal, that could not be attributed to impurities or byproduct since either PNA and DNA were HPLC-purified and MS characterized. Moreover, the recognition experiments performed with this probe showed a

lower sensitivity, and significant peaks could be obtained only by using relatively higher concentrations (200nM, Figure 2-7). Finally, although some good chromatograms were obtained, a high variability in the peak intensity was noticed, making the probe not particularly suitable for this assay. To make bad things worse, a PNA-DNA hybrid could be clearly detected also when the beacon was hybridized with the mismatched oligonucleotides.

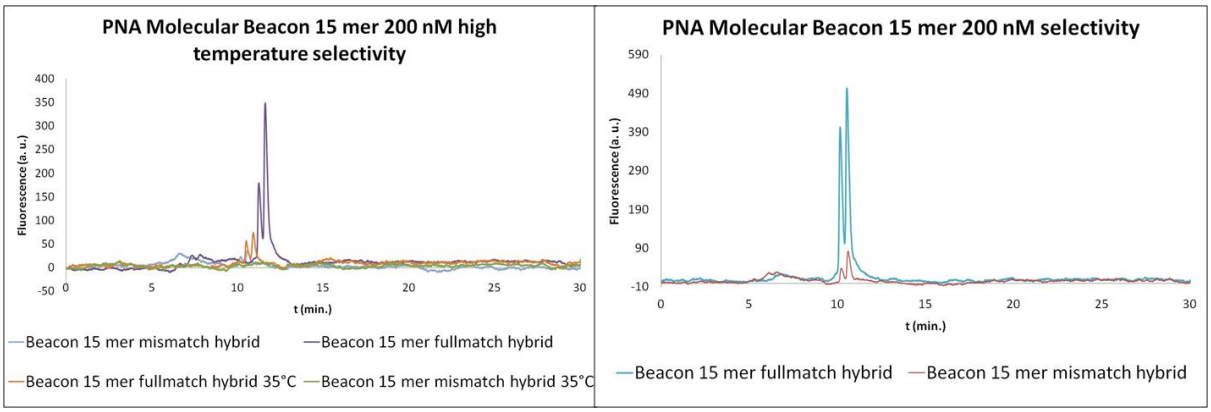


Figure 2-7: Chromatogram obtained for fullmatched and mismatched hybrids using PNA Molecular Beacon 15 mer 200nM at different temperatures. The analytical conditions are the same, as described above

Since the only difference between this PNA and the other previously reported is the length, these problems should be related to this parameter. The increased length may cause self-aggregation in solution, due to the increased hydrophobicity, and a slower rate in the hybridization process also with complementary sequences. Moreover, the 15-mer beacon is less selective on account of the higher stability of the fullmatch and mismatch PNA-DNA hybrids at room temperature. In order to completely denaturate the mismatched duplex, the column temperature was increased. The chromatograms in figure 2-7 show that warming up to 35°C was necessary in order to get rid of the aspecific signal, but at this temperature the fullmatch hybrid was also highly destabilized, as demonstrated by its strongly decreased signal.

Since all the results suggested that the optimal probe, in terms of selectivity and sensitivity, was a PNA beacon 13 bases long, this probe was used in the optimized conditions for the recognition of the DNA samples extracted from olive leaves and amplified by PCR. DNA extracted from leaves of two different cultivars of olive were used: one from “Ogliarola Leccese”, bearing the SNP at position 60 (thymine versus adenine) of the actine gene, for which the probe has been designed; the other from “Canino”, which was chosen as negative control, since it has an adenine at position 60 as other eleven cultivars. The extracted DNA was amplified by Polymerase Chain Reaction (PCR) and purified with an affinity column.

The sample thus obtained was hybridized with the 13 mer PNA beacon 100nM and analyzed by AE-HPLC using the same conditions optimized with synthetic oligonucleotides.

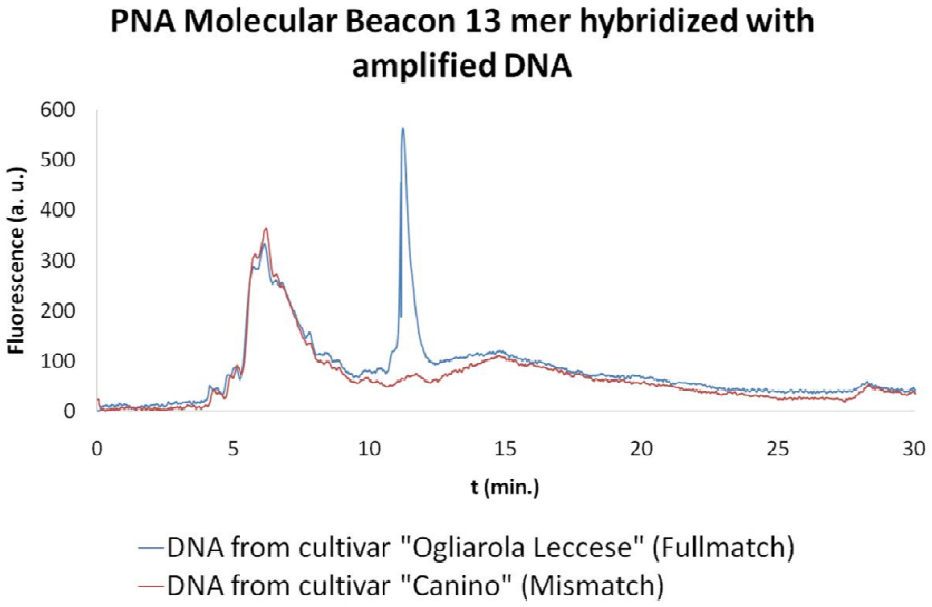


Figure 2-8: Chromatograms obtained hybridizing amplified DNA extracted from olive leaves with PNA Beacon 13 mer

The chromatograms obtained (Figure 2-8) show a very good recognition of the complementary DNA. The DNA extracted from leaves coming from "Ogliarola Leccese" gave a clear peak, whereas the other sample, containing the DNA extracted from leaves coming from "Canino", with a mismatched base, did not give any signal, confirming the good performances of this PNA beacon-based HPLC methodology.

2.3 Conclusions

In this work an extensive study has been done on PNA molecular beacon performances for SNP detection of oligonucleotides and PCR products of DNA extracted from olive plants. On the basis of the results obtained it is evident that the design of the PNA beacon is a crucial point, and in particular the length is an important factor for its performance. Binding affinity and selectivity assays demonstrate that in this case a 13mer probe showed the best performances. The probe was applied to the identification of SNPs in real sample allowing a good discrimination of DNA from different olive cultivars. The method here developed can be considered a fast, robust and sensitive assay for DNA analysis, which does not require DNA labeling and is performed on a commonly available instrument (HPLC).

2.4 Experimental Part

2.4.1 Materials and reagents

PNA monomers were from Applera (Milan, Italy); MBHA Rink amide resin was from Novabiochem (Inalco spa, Milan, Italy); O-(benzotriazolyl)-N,N,N,N-tetramethyluronium hexafluorophosphate (HBTU) and N,N-diisopropylethylamine (DIEA) were fromAldrich (Milan, Italy) and N-methylpyrrolidone (NMP) was from Advanced Biotech Italia srl (Seveso, Italy). All solvents used for HPLC were of chromatographic grade. Doubly-distilled water was produced by Millipore Alpha-Q purification module. Oligonucleotides used for melting temperature measurement were purchased from Thermoelectron (Ulm, Germany), and their purity was checked by ion-exchange HPLC. DNA was extracted from certified olive leaves by Dr. Enzo Perri (CRA, Rende) and by Dr. Luciana Baldoni (CNR, Perugia).

2.4.2 PNA Beacon Synthesis

The synthesis was performed on an ABI 433A peptide synthesizer with software modified to run the PNA synthetic steps (scale: 5 micromol), using Fmoc chemistry and standard protocols with HBTU–DIEA coupling[25]. A MBHA-Rink amide resin (29 mg, 0.64 mmol active sites per g) was downloaded to 0.23 mmol g^{-1} with Fmoc-Dabcyl-Lys-OH. To this a second lysine unit (Fmoc(Boc)Lys) was coupled using a 5-fold excess and HBTU–DIEA. The PNA oligomer was subsequently synthesized, after Fmoc deprotection, according to the procedures previously described[9,10]

After completion of the PNA part, a Fmoc(OtBu)Glu-OH residue was coupled at the N-terminal PNA monomer and deprotected. A solution of 25 μmol of carboxyfluorescein in NMP was pre-mixed with a solution of 25 μmol of DHBtOH in NMP and 25 μmol of DIC and activated for 15 minutes. This solution was then added to the resin and stirred overnight[25]. After the coupling, the resin was washed with NMP and DCM and dried under vacuum. The PNAs were cleaved from resin by using a TFA/m-cresol (9: 1) mixture; the cleaving solution was filtered on sintered glass, the filtrate was concentrated under a nitrogen stream, and the crude products were precipitated by addition of Et_2O. The mixture was cooled at −20°C for 2 hours, followed by centrifugation (15min at 3500 rpm, twice) and removal of solvent by

pipetting to afford the crude PNA products as a red precipitate. Residual ether was removed under a stream of nitrogen. The crude PNAs were purified on RP-HPLC with UV detection at 260 nm. A semi-preparative C18 (5 lm, 250 × 10 mm, Jupiter Phenomenex, 300 A) column was utilized, eluting with 0.1% TFA in water (eluent A) and 0.1% TFA in water–acetonitrile 60 : 40 (eluent B); elution gradient: from 100% A to 100% B in 35 min, flow: 4 ml min^{-1}. HPLC analysis for all PNAs was carried out by LC-MS by using an XTerra anlytical C18 column (3x250 mm, 5 μm, flow 0.5 ml/min), gradient elution from 100% H_2O (0.2% HCOOH, eluent A) to 60% H_2O and 40% CH_3CN (0.2%HCOOH, eluent B) in 30 min. MS detector set in the positive ion mode, capillary voltage 3kV, cone voltage 30V, full scan acquisition from 150 to 1500 m/z.

PNA Beacon 11 mer. Yield (after purification): 9.2%. calculated MW: 3894.3; ESI-MS. m/z 779.9 (MH_5^{5+}), 650.1 (MH_6^{6+}); 557.3 (MH_7^{7+}); 487.8 (MH_8^{8+}); 433.7 (MH_9^{9+}) found: 779.8, 650.0, 557.2, 487.7, 433.7

PNA Beacon 13 mer. Yield (after purification): 14.3%. calculated MW: 4425.5; ESI-MS. m/z 886.1 (MH_5^{5+}), 738.6 (MH_6^{6+}); 633.2 (MH_7^{7+}); 554.2 (MH_8^{8+}); found: 886.3, 738.8, 633.4, 554.4

PNA Beacon 15 mer. Yield (after purification): 1.3%. calculated MW: 5008.6; ESI-MS. m/z 1002.7 (MH_5^{5+}), 835.8 (MH_6^{6+}); 716.5 (MH_7^{7+}); 627.1 (MH_8^{8+}); 557.5 (MH_9^{9+}) found: 1002.6, 835.7, 716.4, 627.1, 557.5

2.4.3 UV melting analysis

The PNA beacons were dissolved in water and their concentrations were determined by UV absorption at 260 nm on an UV/Vis Lambda Bio 20 spectrophotometer (Perkin Elmer) using the following molar absorptivities: Beacon 11 mer 149636 L mol^{-1} cm^{-1}; Beacon 13 mer 166836 L mol^{-1} cm^{-1}; Beacon 15 mer 190236 L mol^{-1} cm^{-1}, calculated using the following ε260 (M^{-1} cm^{-1}) for the nucleobases: T 8600, C 6600, A 13700, G 11700 and molar absorptivity of the carboxyfluorescein and dabcyl units.

For thermal melting measurements, solutions of 1: 1 DNA–PNA were prepared in pH= 7.0 sodium phosphate buffer (100 mM NaCl, 10 mM phosphate, 0.1 mM EDTA). Concentrations were 5 μM for each component. Thermal denaturation profiles (Abs vs. T) of the hybrids were measured at 260 nm with an UV/Vis Lambda Bio 20 Spetrophotometer equipped with a Peltier Temperature Programmer PTP6 which is interfaced to a personal computer, using a

temperature ramp of 1°C min^{-1} from 25°C to 90°C. The melting temperature (Tm) was determined from the maximum of the first derivative of the melting curves.

2.4.4 Fluorescence measurements

Fluorescence measurements were performed on a Perkin Elmer Luminescence Spectrometer LS 55. All solutions were prepared in Tris buffer (0.25 mM $MgCl_2$, 10 mM Tris, pH= 8.0). Concentrations were 1 μM for each component. All samples were excited at 497 nm and the emission was monitored at 520 nm. Triplicate measurements were performed for each composition.

2.4.4 HPLC measurements

All experiments were carried out using an Alliance 2690 Separation Module HPLC system (Waters), equipped with a temperature controller, dual k absorbance detector 2487 (Waters) and scanning fluorescent detector 474 (Waters). Anion-exchange chromatographic measurements were carried out using the column TSK-gel DNA-NPR 4.6 mm ID × 7.5 cm. Binary linear gradient was used: eluent A= Tris 0.02M pH 9; B= Tris 0.02M, NaCl 1M pH= 9. flow: 0.5 mL min−1. Linear gradient: from 100% A to 100% B in 20 min; Fluorescence detector λ_{ex}= 497 nm, λ_{em} = 520 nm.

The chromatograms obtained by the fluorescence detector were corrected by subtraction of the baseline obtained with injection of water in order to compensate for the baseline drift. For each probe the concentration used varied from 200nM to 1nM with the fullmatched oligonucleotides. Selectivity experiments were performed using the lowest concentration possible (30nM for Beacon 11mer, 10nM for Beacon 13mer, 200nM for Beacon 15mer)

2.4.5 PCR amplification

PCR was performed in a PCRsprint Thermal Cycler (Thermo Hybaid, Basingstoke, UK) using the following conditions: 1 cycle of DNA denaturation and Blue Taq activation at 95°C for 5 min; 30 cycles consisting of DNA denaturation at 95°C for 50 s, primer annealing at 58°C for 50 s and elongation at 72°C for 50 s; one step of final elongation at 72°C for 10 min.

The mastermix for the PCR was prepared using Buffer 1X, $MgCl_2$ 2mM, dNTPs 200 µM, Primer For and Rev 400 nM and Taq 0.05U/µl. The primer uses were:

Forward 5'-TCTGTGGAGAAGAGCTACGAGTTG-3', Reverse: 5'-AGGCTGGTAAAGAACCTCAGGACA-3'

The PCR products were immediately analyzed by 2% agarose gel in 0.5× TBE or stored at −20°C until use. In the unbalanced PCR a first amplification was performed as described above, then a small amount (2–5 µL) of the reaction mixture was amplified in the second run using a fresh mastermix solution of the same composition except that a 10 fold excess of the primer on the target sequence was used.

In order to prepare the samples for the HPLC analysis, two tubes containing 50 µl of PCR product each, were purified by Genuine PCR Clean-up Kit from Sigma-Aldrich and concentrated redissolved in 50 µl of water. 15 µl of this DNA solution was hybridized with PNA Beacon 13mer 100nM with a final volume of 30µl. Before the hybridization with the beacon, the sample was warmed up to 90°C for 3 minutes and cooled to 50°C for 3 minutes.

2.5 References

[1] F.R.R. Teles, L.P. Fonseca, *Talanta*, **2008**, 77, 606

[2] Y. S. Min, J. H. Choi, S. Y. Lee, N. C. Yoo, *J. Microbiol. Biotechnol.*, **2009**, 19, 7, 635

[3] Y. Nakamura, *Journal of Human Genetics*, **2009**, 54, 1

[4] B. Budowle, A. van Daal, *Biotechniques*, **2008**, 44,5, 603

[5] A. Germini, E. Scaravelli, F. Lesignoli, S. Sforza, R. Corradini, R. Marchelli, *Eur. Food Res. Technol.*, **2005**, 220, 619

[6] A. Germini, A. Mezzelani, F. Lesignoli, *J. Agric. Food Chem.*, **2004**, 52, 4535

[7] F. S. Collins, L. D. Brooks, A. Chakravarti, *Genome Res.*, **1998**, 8, 1229

[8] A. J. Brookes, *Gene*, **1999**, 234, 177

[9] P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science*, **1991**, 254, 1497

[10] P.Wittung, P. E. Nielsen, O. Buchardt, M. Egholm and B. Nord´en, *Nature*, **1994**, 368, 561

[11] S. Sforza, T. Tedeschi, R. Corradini, A. Dossena and R. Marchelli, *Chem. Commun*., **2003**, 1102

[12] T. Tedeschi, M. Chiari, G. Galaverna, S. Sforza, M. Cretich, R. Corradini, R. Marchelli, *Electrophoresis*, **2005**, 26, 4310

[13] B. S. Gaylord, A. J. Heeger, G. C. Bazan, *Proc. Natl. Acad. Sci.*, **2002**, 10954

[14] H. A. Al Attar, J. Norden, S. O'Brien, A. P. Monkman, *Biosensors & Bioelectronics*, **2008**, 23, 1466

[15] O. Seitz, F. Bergmann, D. Heindl, *Angew. Chem. Int. Ed.*, **1999**, 38, 15, 2203

[16] D. V. Jarikote, O. Köhler, E. Socher, O. Seitz, *Eur. J. Org. Chem.*, **2005**, 3187

[17] K. Li, B. Liu, *Analytical Chemistry*, **2009**, 81, 10, 4099

[18] E. Sochera, D. V. Jarikote, A. Knoll, L. Röglin, J. Burmeister, O. Seitz, *Analytical Biochemistry*, **2008**, 375, 318

[19] H. Kuhn, V. V. Demidov, J. M. Coull, *J. Am. Chem. Soc.*, **2002**, 124, 6, 1097

[20] O. Seitz, *Angew. Chem. Int. Ed.*, **2000**, 39, 18, 3249

[21] F. Totsingan, T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Chirality*, **2009**, 21, 245
[22] E. A. Englund, D. H. Appella, *Org. Lett.*, **2005**, 7, 16, 3465
[23] E. A. Englund, D. H. Appella, *Angew. Chem. Int. Ed.*, **2007**, 46, 1414
[24] K. Petersena, U. Vogel, E. Rockenbauer et al., *Molecular and Cellular Probes*, **2004**, 18, 117
[25] F. Totsingan, S. Rossi, R. Corradini et al., *Org. Biomol. Chem.*, **2008**, 6, 1232
[26] A Calabretta, *Master Thesis*,**2006**

Chapter 3

A new class of Chiral PNAs: Arginine PNAs

This chapter presents a new class of modified PNAs: chiral arginine-based PNAs. The design and the synthesis of submonomers and monomers based on the structure of arginine are shown. The modified monomers have then used for the synthesis of a chiral PNA containing a 2D,5L-Arg monomer, to be compared with homologous probes based on lysine, and of a PNA containing three modified monomers (2D; 2D,5L; 5L).The scope is to explore the effect that modifications based on chiral positively charged amino acids have on PNA hybridization properties. The properties of these PNAs have been tested by measuring the melting temperatures of the corresponding PNA-DNA duplexes and comparing them with the previous generations of chiral PNAs.

3.1 Introduction

As discussed in chapter 1, the possibility to increase PNA recognition properties by introducing chemical modifications has always been intensely investigated. As previously shown, modifications of the PNA structure, either in the backbone[1] or in nucleobases[2], can deeply modify the recognition properties, obtaining better performances in some cases, but hampering the recognition of complementary sequences in other cases. Modifications of the PNA backbone based on the side chains of amino acids showed excellent properties[1,3]. As explained above, aminoacidic side chains can be introduced in position 2 or 5 of the backbone (Figure 3-1). In particular, modifications based on the structure of lysine showed increased affinity towards DNA mainly due to the positive charge present on its side chain[4,5]. The effect that different configurations of the stereogenic centers introduced in the PNA structures have on the hybridization properties towards complementary DNA were studied, allowing to define the correct configurations for improved DNA affinity. In particular it has been defined that modifications based on D-lysine in position 2[6] and on L-lysine in position 5[7] are improving the PNA affinity for complementary DNA sequences. This is due to the favored right-handedness that these configurations induce into the PNA strands: PNAs preferring right-handed helices show an improved DNA affinity[8] being the DNA right-handed. The combination of the two favorable modifications in the same monomer led to the development of PNAs with a 2D,5L-Lysine monomer, obtaining a new class of modified PNAs that showed enhanced affinity for the complementary sequence, as well as a good selectivity in mismatch recognition[9].

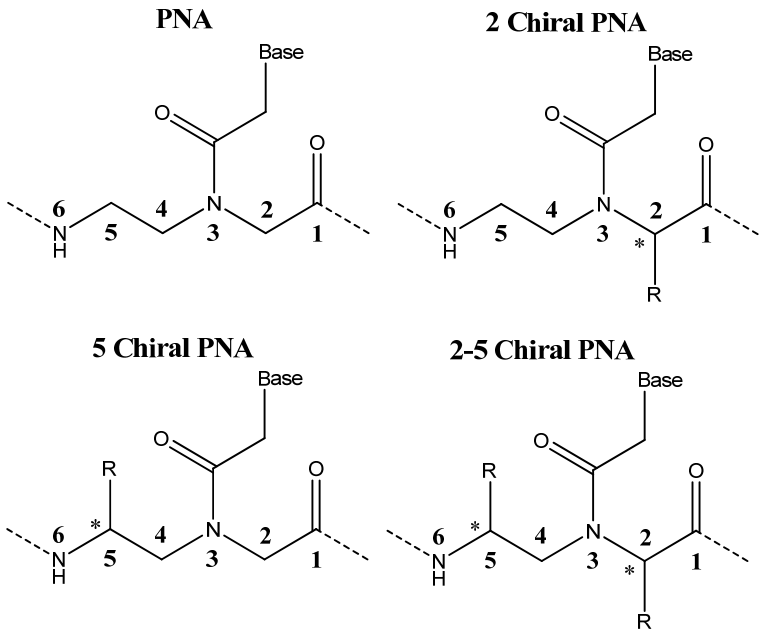


Figure 3-1: Chemical structure of chiral PNAs

The introduction of multiple modified monomers was investigated with PNAs bearing 2D-Lys modified units in different positions of the molecule. The introduction of three adjacent 2D-Lysine

monomers in the middle position of a PNA strand resulted in an excellent PNA probe (called the "chiral box" PNA), with good affinity and excellent selectivity[10,11]. "Chiral box" PNAs have been used in several applications for the discrimination of point mutations, obtaining excellent results in assays based on mass spectrometry and electrophoresis[12,13].

Unfortunately, it is difficult to apply of these molecules on surface devices, such as PNA microarrays, since the immobilization of the probes on the surface usually exploits the reaction between the terminal amino group of the PNAs and a reactive group on the surface[14]. The presence of the lysine primary amino groups, on its side chain, would thus interfere with this reaction.

In this chapter a new class of chiral PNAs will be presented, mainly intended for surface applications, such as the development of chiral PNA microarrays. The general idea was to substitute the side chain of lysine with that of arginine, which also contains a positive charge but does not bear a primary amino group. A PNA with one 2D,5L-Arg modified monomer in the middle, to be compared with the analogous lysine-based PNAs, and a PNA with a series of chiral monomers resulting in four adjacent arginine side chains have been synthesized, and their binding properties towards complementary oligonucleotides have been studied.

3.2 Results and Discussion

3.2.1 Sequence Design

The need to develop probes able to perform DNA recognition on surface determined the necessity to substitute the previously used lysine, in the development of chiral PNAs. In fact, the strategy usually employed to link the PNAs to glass surfaces is based on the reaction between a reactive group on the surface and the terminal amino group of the PNA[14]. The presence of the amino group of the lysine side-chains would thus compete with the terminal one in this reaction, making the PNA deposition less reproducible. For this reason, chiral modifications based on the structure of arginine were chosen, since the guanidium groups are much less nucleophilic than primary amines, but still bear a positive charge at physiological pH.

Since it has been demonstrated that PNAs containing one residue with two lysine-derived side chains having configurations 2D and 5L had a highly improved affinity and selectivity in the recognition of complementary DNA sequences, a PNA was first designed bearing a 2D,5L-arginine monomer in the middle (PNA 1, Figure 3-2), in order to compare its binding abilities

with those of the analogous PNA bearing in the middle a chiral monomer 2D,5L based on lysine.

Moreover, since the introduction of several adjacent positively charged monomers in the sequence was demonstrated to improve the binding selectivity, another PNA was designed combining the features of the "chiral box" PNA and those of 2D,5L PNA. This new PNA was designed with a 2D,5L arginine-based monomer in the middle, which should determine an enhanced affinity for the complementary DNA sequence, flanked by 2D-modified and a 5L-modified arginine-based monomers, which should improve the PNA selectivity in mismatch

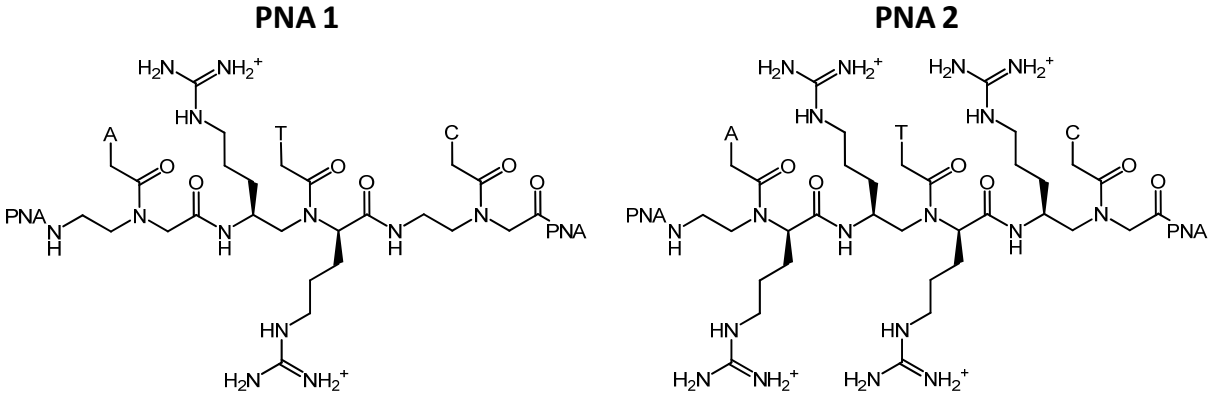


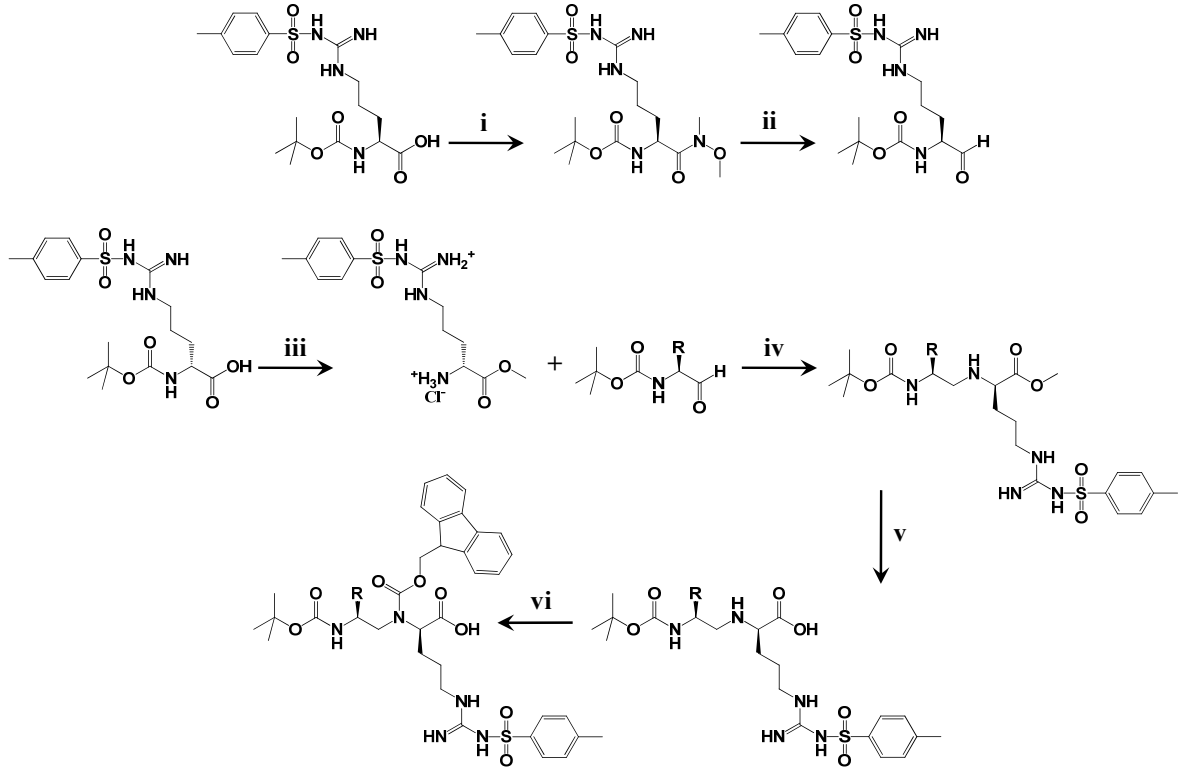
Figure 3-2: Chemical structure of PNA 1: 2D,5L-Arg PNA and of PNA 2: 2D-Arg_2D,5L-Arg_5L-Arg PNA (right)

recognition (PNA 2, Figure 3-2). The sequence chosen for both probes was a standard decameric sequence used as model for the development of other modified PNAs, in order to compare the performances of the new probes with those given by other modifications[10].

3.2.2 PNA synthesis

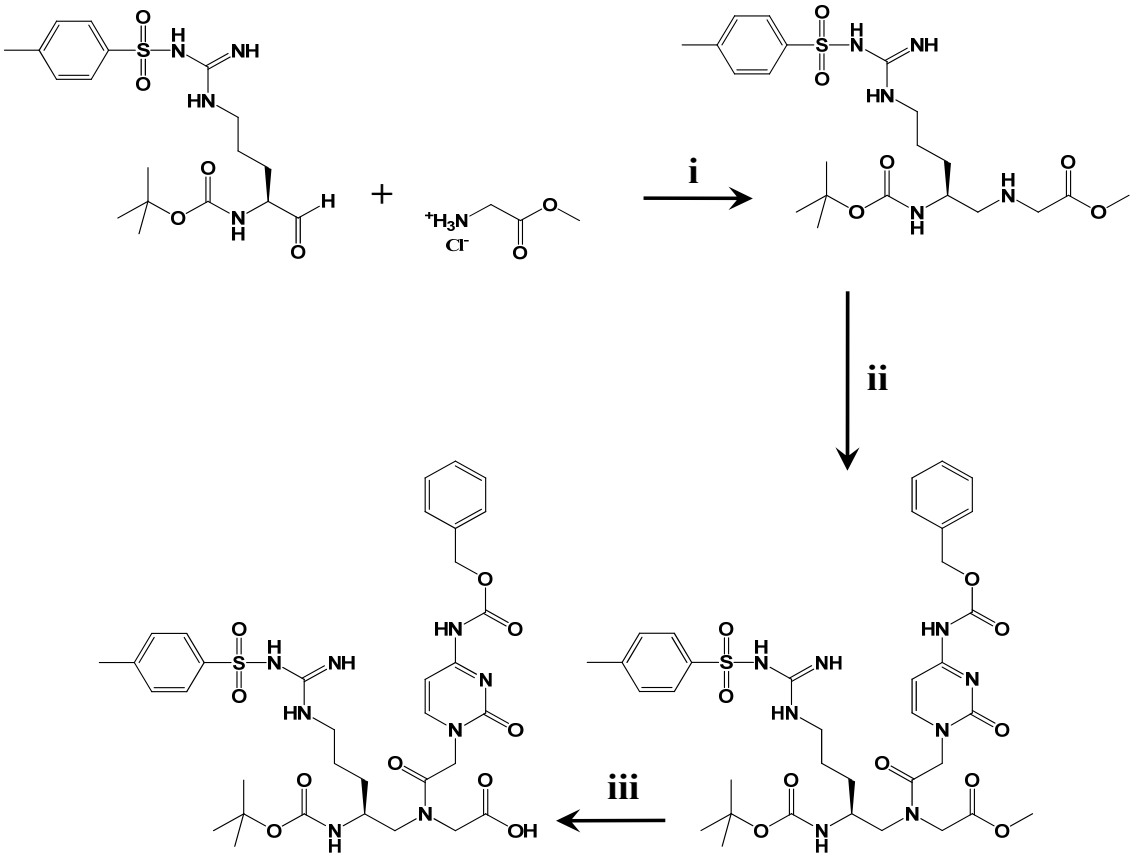
PNAs were synthesized by using the common SPPS protocol based on the Boc strategy for the standard monomers and for the monomer bearing the modification on the carbon atom in position 5, whereas the Boc submonomer strategy was used for the monomers bearing the substituents in position 2 or 2 and 5, in order to preserve the optical purity at the 2 position[15].

The synthesis of the PNAs required the preparation of three different modified units: a 2D-Arg submonomer, a 2D,5L-Arg submonomer, and a 5L monomer. The 2D,5L-Arg submonomer and the 2D-Arg submonomer were prepared, as reported in Scheme 3-1, starting from the commercial D- and L-Boc-Arginine(Tos)-OH. Boc-L-Arg(Tos)-OH was transformed into the corresponding Weinreb amide and then reduced to the aldehyde. Boc-D-Arg(Tos)-OH was transformed in the methyl ester and Boc deprotected. The PNA backbone was then synthesized by reductive amination of D-Arg(Tos)-OMe with the Boc-L-Arg(Tos) aldehyde (for 2D,5L subomonomer) or Boc aminoacethaldehyde (in the case of 2D submonomer). The methyl ester was hydrolyzed and the secondary amine was protected by the introduction of a Fmoc group (Scheme 3-1).



Scheme 3-1: i) 0.97 eq. N-methyl-N-methoxyamine.HCl, HBTU, DIPEA, DMF η: 80%; ii) 4.6 eq. LiAlH4 (1M in THF), THF, η: 94%; iii) $SOCl_2$ 1M in CH_3OH, η: 98% iv) 1 eq. D-Arg(Tos)OMe, 1 eq. $NaBH_3CN$, 1.1 eq. CH_3COOH, CH_3OH, η: 51% (2D,5L), 48% (2D); v) 10eq. NaOH THF: H_2O = 1:1, η: 87% (2D,5L), 75% (2D) vi) 2eq. BSA, 1.5 eq. DIPEA, 2 eq. FmocCl, CH_2Cl_2 η: 30% (2D,5L), 38% (2D). R= H for 2D submonomer; **R=$CH_2CH_2CH_2NHC(NH\text{-}Tos)NH$** for 2D,5L submonomer

The Arginine modified monomer was linked to a Cytosine, obtaining a 5L-Arg-Cytosine(Z) monomer. The molecule was prepared, as reported in scheme 3-2. The PNA backbone was then synthesized by reductive amination of Gly-OMe (hydrochloride) with the Boc-L-Arg(Tos) aldehyde. The methyl ester was coupled with the carboxymethyl-(Z)-cytosine by



Scheme 3-2: i) 1 eq. Gly-OMe·HCl, 1 eq. $NaBH_3CN$, 1.1 eq. CH_3COOH, CH_3OH, η: 50%; ii) 2eq. carboxymethyl-(Z)-cytosine, 2 eq EDC·HCl, 2 eq DhBTOH, 3 eq DIPEA, DMF η: 83%.iii) 2eq. BaOH THF: H_2O = 1:1, η: 75%

activating the carboxylic group with N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC)/ 3-Hydroxy-1,2,3-benzotriazin-4(3H)-one (DhbtOH). Finally the methyl ester was hydrolyzed, obtaining the desired monomer (Scheme 3-2). All the above compounds were characterized by ^{1}H and ^{13}C NMR and ESI mass spectrometry.

According to previously reported procedures[15], the chiral submonomers units were inserted by manual coupling with the HATU/DIEA protocol and, after Fmoc deprotection, the nucleobase residues were introduced by a double coupling with DIC/DhBTOH. All the PNA oligomers were purified by RP-HPLC and characterized by ESI-mass spectrometry (Figure 3-3).

The analysis of HPLC-MS data in figure 3-3 shows that PNA 1 was obtained with good purity (>90%), whereas PNA 2, even after HPLC purification, showed ~28% of a byproduct identified by MS as the PNA2 without a carboxymethyladenine moiety. This byproduct was originated because of the low coupling efficiency between the carboxymethyladenine and the PNA in the last 2D chiral monomer, probably due to the steric hindrance exerted by the previous monomers. The iteration of the same coupling many times, as well as the use of other coupling agents or different reaction conditions, did not allow to obtain a quantitative

yield in this reaction. Moreover, since the byproduct had a retention time very similar to that of the desired PNA molecule, the purification turned out to be very difficult, thus PNA 2 was obtained as a mixture with this byproduct.

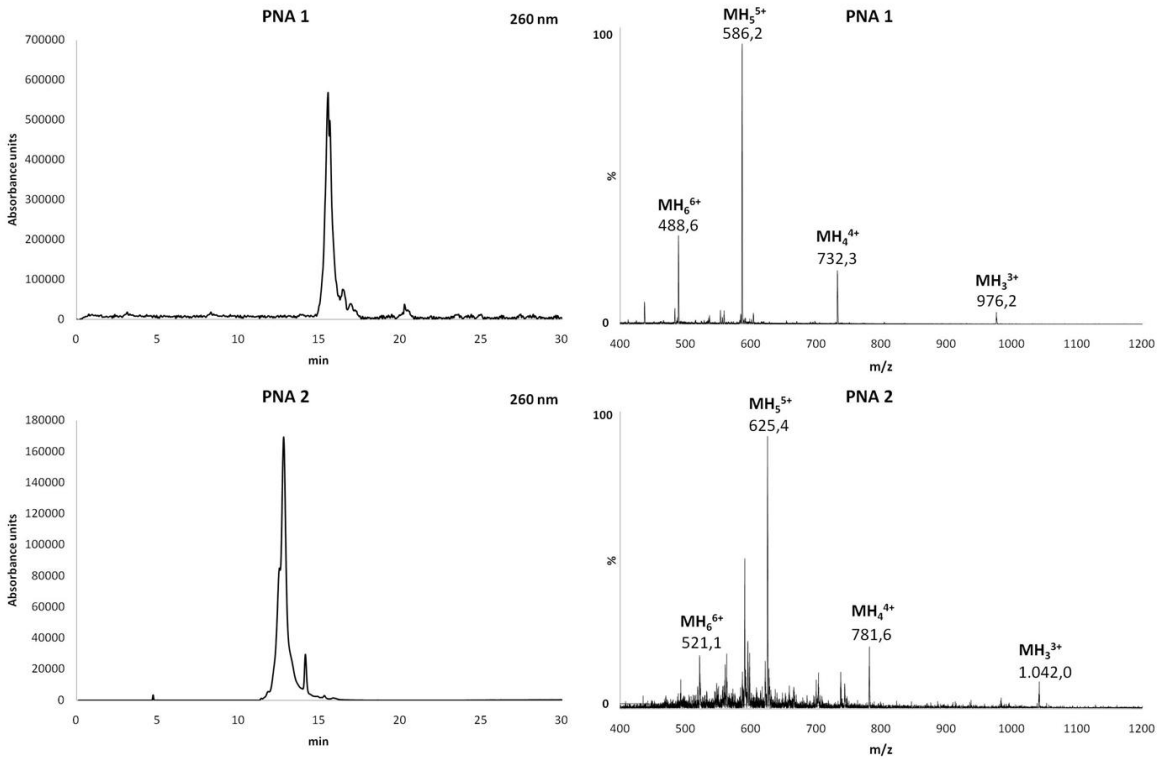


Figure 3-3: HPLC profiles and mass spectra of PNA1 and PNA 2

3.2.3 Selectivity of binding

In order to verify the affinity of the synthesized probes for complementary and mismatched DNA sequences, melting temperatures were measured for the fullmatch hybrids and the mismatch ones, the latter using an oligonucleotide with a guanine replacing the adenine corresponding to the central modified PNA monomer. In the case of PNA 2, the presence of the byproduct was taken into consideration for the calculation of the real concentration of the molecule, assuming that the stability of the byproduct bound to DNA was too low to interfere with the melting temperature of the complete probe. Melting temperatures were evaluated as the first derivatives of the UV absorption curves at 260 nm in a temperature range from 20 to 90°C. The values obtained are shown in Table 3-1, together with those of the corresponding achiral PNA and of the PNA containing a 2D,5L Lysine modified monomer in the middle, which are reported in order to compare the results obtained with the new PNA probes[16].

Table 3-1. PNA sequences and melting temperatures of the PNA:DNA antiparallel fullmatch/mismatch duplexes in phospate buffer (pH=7) at a 5μm concentration for each strand			
Sequence	Tm (°C) PNA/DNA Fullmatch	Tm (°C) PNA/DNA Mismatch	ΔTm (°C)
H-GTAGATCACT-NH_2	50	40	10
H-GTAGA($T_{2D,5L\text{-}Lys}$)CACT-NH_2	60	43	17
PNA 1: H-GTAGA($T_{2D,5L\text{-}Arg}$)CACT-NH_2	60	41	19
PNA2: H-GTAG($A_{2D\text{-}Arg}$)($T_{2D,5L\text{-}Arg}$)($C_{5L\text{-}Arg}$)ACT-NH_2	60	40	20
Oligonucleotide sequences: **Fullmatch**: 5'-AGTG**A**TCTAC-3' **Mismatch**: 5'-AGTG**G**TCTAC-3'			

The melting temperatures in Table 3-1 confirm that the substitution of lysine side chains with arginine side chains did not impair the binding abilities of PNAs. The stability of the fullmatch PNA-DNA hybrids, indeed, was the same for both new PNAs, identical to those measured for the analogous Lys-based PNA[11] and much better than that shown by the unmodified PNA. Even the selectivity was not affected: no selectivity decrease was observed for the Arg-PNAs, and even a small increase could be observed for these new probes, making it even more attractive for diagnostic applications. Thus, these new molecules are perfectly suitable for applications on surface, preserving all the good features of lysine-based PNAs without the presence of reactive amino groups. Quite unexpectedly, PNA 2, albeit the presence of an extended series of four arginine side chains flanking the nucleobase corresponding to the mismatch, showed for fullmatch and mismatch hybrids stabilities fully comparable to those obtained by using PNA 1, which only had two arginine side chains. Although PNA 2 presents two more positive charges, the fullmatch hybrid stability did not change, and the increased sterical hindrance did not significantly decrease the stability of the mismatch hybrid. This can be explained according to previously published works[16], by supposing that most of the contribution to the stability of the fullmatch duplex is given by the 2D,5L monomer: both the positively charged arginine side chains and the correct configurations imparting a right-handedness to the PNA strand increase the DNA affinity. Increasing the number of arginine side chains probably does not further affect the preferred handedness, and the increased number of positive charges, which should enhance electrostatic attraction for the complementary DNA strand, is probably balanced by the increased hindrance introduced in the middle of the PNA strand. The same counteracting effects

(increased electrostatic attraction vs. increased sterical hindrance) probably contribute to the lack of improvements in mismatch recognition.

3.3 Conclusions

In this chapter we have demonstrated that it is possible to modify the structure of lysine-based PNAs, whose properties made them suitable for diagnostic application in solution, without any impairment in their binding properties, by using substituents based on the structure of arginine. We have designed and synthesized prototypical PNAs of a new class of modified PNAs: Arg-PNAs. These molecules are to be considered fully suitable for diagnostic applications on surfaces, thanks to the lack of primary amino groups, which would compete with the PNA amino terminus during surface derivatization, and for their binding properties, comparable or even slightly better than those of lysine-based PNAs. Finally, it has been demonstrated that increasing from two to four the number of arginine side chains in the middle of the PNA strand does not increase the PNA binding properties.

3.4 Experimental Section

3.4.1 Materials and Reagents

PNA N-Boc protected standard monomers, N-[1H-1,2,3-(benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU), N-[(dimethylamino)-1H-1,2,3-triazol[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU), dichloromethane, N–methylpyrrolidone (NMP) and N,N-dimethylformamide (DMF) were purchased from Applied Biosystem (Foster City, CA, USA). Carboxymethyl-O-benzyl-guanine was purchased by ASM (Hannover, Germany). Boc-Arg(Tos)-OH, Boc-D-Arg(Tos)-OH and m-cresol were from Fluka (Buchs, Switzerland). (4-Methylbenhydryl)amine (MBHA) PS resin was from Novabiochem (Laufelfingen, Switzerland). Carboxymethylthymine, diisopropylethylamine (DIPEA), N,O-Bistrimethylsilyl acetamide (BSA), trifluoromethane sulfonic acid (TFMSA), trifluoroacetic acid (TFA), 3-hydroxy-1, 2,3-benzotriazin-4-(3h)-one (DhBtOH), N-(3-

Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and diisopropylcarbodiimide (DIC) were from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides (guaranteed oligos grade) were purchased from ThermoFisher Scientific (Ulm, Germany) and used without further purification.

3.4.2 Chemical Synthesis

D-Arg-N$^{\omega}$-Tosyl methyl ester hydrocloride. N-Boc-N$^{\omega}$-(Tosyl)-D-Arginine-OH (2g, 4.67 mmol) was dissolved in methanol (80ml) with magnetic stirring. The mixture was cooled at 0°C and thionyl chloride (5.8ml, 80mmol, 1M in methanol) was added dropwise. The solution was stirred for 6h at room temperature. The methanol was evaporated and the residue was washed few times with methanol to yield the product as a white foam. Yield: 98%. ^{1}H-NMR (300MHz, [D_6]-DMSO, 25°C): δ(ppm): 10.37 (sb, 3H, N-H amine), 8.64 (sb, 1H, N$^{\eta}$-H arginine side chain), 7.64 (d, 2H, J= 6 Hz aromatic tosyl group), 7.38 (sb, 1H, N$^{\varepsilon}$-H arginine side chain), 7.29 (d, 2H, J= 6 Hz aromatic tosyl group), 6.79 (sb, 1H, N$^{\eta}$-H arginine side chain), 3.99-3.97 (m,1H, C-Hα), 3.71 (s, 3H, methyl ester), 3.1-3.05(m, 2H, CH_2 arginine side chain), 2.34 (s, 3H, CH_3 tosyl group), 1.77-1.72 (m, 2H, CH_2 arginine side chain), 1.49-1.44 (m, 2H, CH_2 arginine side chain). ^{13}C NMR (75 MHz, $CDCl_3$):δ(ppm) 169.6,156.6, 141.3, 141.0,129.0, 125.6, 52.6, 51.4, 48.4, 27.0, 24.4, 20.8. ESI-MS (CH_3OH, positive ions): calculated m/z: 343.1 (MH^+) found: 343.3. HRMS (ESI^+): calcd m/z for $C_{14}H_{23}N_4O_4S$ (MH^+): 343.1440, found: 343.1443

N$^{\alpha}$-Boc-L-Arg-N$^{\omega}$-Tosyl-N,O-dimethylhydroxyamide. N-Boc-N$^{\omega}$-(Tosyl)-L-Arg-OH (2g, 4.67 mmol) was dissolved in DMF. The solution was cooled at 0°C and HBTU was added. After 15 minutes DIEA and N,O-dimethylhydroxyamine were introduced. The reaction was warmed at room temperature and left stirring for two hours. DMF was evaporated under reduced pressure and the residue was recovered with ethyl acetate and washed with saturated potassium hydrogen sulphate (twice) and sodium hydrogen carbonate (twice). The combined organic phases were dried over magnesium sulphate and filtered, and solvent was evaporated under vacuum. The product was obtained as white foam. Yield: 80% ^{1}H-NMR (300MHz , $CDCl_3$): δ (ppm) 7.73 (d, 2H J(H,H)= 9 Hz, CH aromatic tosyl group), 7.22 (d, 2H, J(H,H)= 9 Hz, CH aromatic tosyl group), 6.47 (sb, 2H, N$^{\eta}$H arginine side chain), 3.71 (s, 3H, N-metoxy group), 3.35 (sb, 1H C-Hα), 3.17 (s, 3H, N-methyl group), 2.38 (s, 3H, CH_3 tosyl group), 1.69-1.59 (m, 4H, CH_2 arginine side chain), 1.45-1.43 (m, 11H, CH_2 arginine side

chain + Boc methyl groups). ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 172.5, 156.6, 156.3, 142.0, 140.6, 129.2, 126.0, 80.2, 61.5, 49.2, 40.8, 32.1, 30.8, 28.3 , 24.7, 21.4. ESI-MS (CH_3OH, positive ions): calculated m/z: 472.2 (MH^+), 494.2 (MNa^+), 510.2 (MK^+), found: 472.5, 494.5, 510.4. Elemental analysis: calculated C 50.83%, H 7.25%, N 14.82%, found: C 50.46%, H 6.98%, N 14.71%

N^{α}-Boc-L-Arg-N^{ω}-Tosyl-aldehyde N^{α}-Boc-Arg-N^{ω}-Tosyl-N,O-dimethylhydroxyamide (0.8g 1.7 mmol) was dissolved in dry THF (20 ml). A solution of $LiAlH_4$ (1M in THF, 2ml, 2 mmol) was added dropwise at 0°C. The mixture was then allowed to warm at room temperature and left stirring for one hour. The reaction was then diluted by adding ethyl acetate and evaporated. The residue was dissolved in DCM and washed with saturated potassium hydrogen sulphate (twice) and sodium hydrogen carbonate (twice). The combined organic phases were dried over magnesium sulphate and filtered, and solvent was evaporated under vacuum. The product was obtained as a colourless oil. Yield: 94%. ^{1}H-NMR (300MHz, $CDCl_3$): δ(ppm): 9.41 (s, 1H, CH aldehyde), 7.62 (d, 2H, J(H,H)= 7.6 Hz, CH aromatic tosyl group), 7.12 (d, 2H, J(H,H)= 7.6 Hz, CH aromatic tosyl group), 6.80 (bs, 1H, N^{η}-H arginine side chain), 5.58 (bs, 1H, N^{ε}-H arginine side chain), 5.06 (bs, 1H, N^{η}-H arginine side chain), 4.77 (bs, 1H, N-H carbamate), 3.65-3.42 (m, 2H, CH_2 arginine side chain), 3.03-2.99 (m, 1H, C-Hα), 2.28 (s, 3H, CH_3 tosyl group), 1.60-1.54 (m, 4H, CH_2 arginine side chain), 1.32 (s, 9H, CH_3 Boc group). ^{13}C-NMR (75 MHz, $CDCl_3$): δ(ppm): 200.4, 156.2, 155.2, 142.1, 140.1, 129.2, 125.8, 79.4, 50.3, 38.9, 28.3, 24.9, 24.4, 21.3. ESI-MS (CH_3OH, positive ions): calculated m/z: 413.2 (MH^+), 435.2 (MNa^+), 451.2 (MK^+), found: 413.4, 435.4, 451.3. HRMS (ESI^+): calcd m/z for $C_{18}H_{29}N_4O_5S$ (MH^+): 413.1859, found: 413.1855.

N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)-D-Arg-((N^{ω}-Tosyl)] methyl ester. Arg-N^{ω}-Tosyl methyl ester hydrochloride (0.57g, 1.52 mmol) and DIPEA (0.26ml) were dissolved in methanol (20 ml) at room temperature and the mixture was stirred for 20 minutes. N^{α}-Boc-Arg-N^{ω}-Tosyl-aldehyde (0.63g, 1.52mmol) was then added and the solution was stirred for 30 min. at room temperature, then cooled 0°C and $NaBH_3CN$ (0.1g, 1.52mmol) and CH_3COOH (0.096ml, 1.67mmol) were added. After 2h the methanol was evaporated. The residue was dissolved in DCM and washed with saturated potassium hydrogen sulphate (twice) and sodium hydrogen carbonate (twice). The combined organic phases were dried over magnesium sulphate and filtered, and solvent was evaporated under vacuum. The crude product was purified by flash chromatography (eluent ethyl acetate:methanol= 95:5) and obtained as colourless oil. Yield: 51%. ^{1}H-NMR (300MHz, $CDCl_3$): δ(ppm) 7.70 (d, 4H, J(H,H) = 7.2 Hz, CH aromatic tosyl

group), 7.19 (d, 4H, J(H,H) = 7.2 Hz, CH aromatic tosyl group), 6.52 (bs, 2H, N^{η}-H arginine side chain), 3.67-3.60 (m, 4H, CH_3 methyl ester + C(2)Hα), 3.18-3.00 (m, 3H, ψ CH_2 + C(5)Hα), 2.74-2.64 (m, 4H, CH_2 arginine side chain), 2.36 (s, 6H, CH_3 tosyl group), 1.54-1.45 (m, 8H, CH_2 arginine side chain), 1.37 (s, 9H, CH_3 Boc group). ^{13}C-NMR (75 MHz, $CDCl_3$, some signals are missing due to the pseudoequivalence of the arginine side chains): δ(ppm) 174.3, 156.9, 156.6, 142.0, 140.6, 129.2, 125.9, 79.7, 60.8, 52.3, 52.1, 50.4, 40.7, 29.8, 28.3, 25.4, 21.4. ESI-MS (CH_3OH, positive ions): calculated m/z: 739.3 (MH^+), 761.3 (MNa^+), 777.3 (MK^+), found: 739.7, 761.7, 777.7. HRMS (ESI^+): calcd m/z for $C_{32}H_{51}N_8O_8S_2$ (MH^+): 739.3271, found: 739.3272.

N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)-D-Arg-((N^{ω}-Tosyl)]-OH. N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)-D-Arg-((N^{ω}-Tosyl)] methyl ester (0.3g, 0.4 mmol) was dissolved in THF:H_2O = 1:1 (10ml). NaOH (0.16g, 4mmol) was added and the mixture was stirred at room temperature for 1 hour and 30 min. THF was evaporated and the product was precipitated as a white solid at pH= 6.5, filtered and dried under vacuum. Yield: 87%. ^{1}H-NMR (300MHz , CD_3OD): δ(ppm) 7.72 (d, 4H, J(H,H)= 6 Hz, CH aromatic tosyl group), 7.27 (d, 4H, J(H,H) = 8 Hz, CH aromatic tosyl group), 3.59-3.55 (m, 1H, C(2)-H α), 3.30-3.29 (m, 2H ψ CH_2) 3.16-3.12 (m, 4H CH_2 ariginine side chain), 2.93-2.86 (m, 3H, C(5)Hα), 2.36 (s, 6H, CH_3 tosyl group), 1.89-1.82 (m, 4H, CH_2 ariginine side chain), 1.64-1.51 (m, 4H CH_2 side chain ariginine) 1.43 (s, 9H, CH_3 Boc group). ^{13}C-NMR (75 MHz, $CDCl_3$, some signals are missing due to the pseudoequivalence of the arginine side chains): δ (ppm) 173.5, 159.2, 159.0, 143.9, 142.4, 130.6, 127.4, 81.3, 63.8 , 53.4, 50.2, 41.5, 31.2, 28.8, 26.9, 21.8. ESI-MS (CH_3OH, positive ions): calculated m/z: 747.3 (MNa^+), 763.3 (MK^+), found: 747.5, 763.2. HRMS (ESI^+): calcd m/z for $C_{31}H_{49}N_8O_8S_2$ (MH^+): 725.3115, found: 725.3117.

N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)-D-Arg-(N^{α}-Fmoc)-(N^{ω}-Tosyl)]-OH.

Bis(trimethylsilyl)acetamide (BTSA) (0.21ml, 0.86 mmol) and DIPEA (0.05ml, 0.29 mmol) were added to N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)-D-Arg-(N^{ω}-Tosyl)]-OH (0.21g, 0.29 mmol) suspended in DCM (15 ml), with exclusion of humidity by a $CaCl_2$ drying tube. When the solution was nearly clear (10-15 min were usually required), Fmoc-Cl (0.1g, 0.58 mmol) was added and the mixture was stirred for 2 hours at room temperature. Methanol (1 ml) was carefully added and the mixture was stirred for an additional 15 min, diluted with 15 ml of DCM, washed with 1M potassium hydrogen sulphate (3 times) and brine (times), dried over magnesium sulfate and evaporated to dryness. The residue was purified by flash chromatography (dichlomethane:methanol = 9:1) and the product was obtained as a white

foam. Yield: 30%. ^{1}H NMR (600MHz, CD_3OD): δ (ppm) 7.79-7.74 (m, 2H, CH aromatic Fmoc group), 7.72-7.70 (m, 4H CH aromatic tosyl group), 7.59-7.57 (m, 2H CH aromatic Fmoc group), 7.36-7.33 (m, 2H, CH aromatic Fmoc group), 7.27-7.22 (m, 6H CH aromatic Fmoc group + CH aromatic tosyl group), 4.55-44.36 (m, 2H CH_2 Fmoc), 4.25-4.15 (m, 1H CH Fmoc), 3.36 (bs, 1H C(2)-H α), 3.30-3.29 (bs, 1H, C(5)Hα), 3.14-3.07 (m, 2H, ψ CH_2), 2.33-2.30 (m, 10H, CH_2 arginine side chain + CH_3 tosyl group), 1.34-1.24 (m, 17H, CH_2 arginine side chain + CH_3 Boc group). ^{13}C-NMR (150 MHz, CD_3OD, the CH_3 signals of the tosyl groups have identical chemical shifts due the pseudoequivalence of the arginine side chains): δ(ppm) 178.1, 159.4, 158.8, 158.5, 158.4, 145.7, 145.5, 143.8, 143.0, 142.9, 142.5, 130.6, 129.2, 128.7, 127.8, 127.4, 126.4, 126.2, 121.3, 80.4, 68.8, 68.5, 64.2, 53.1, 51.2, 48.8, 42.2, 31.9, 29.2, 28.8, 28.4, 27.3, 21.8. ESI-MS (CH_3OH, positive ions): calculated m/z: 969.4 (MNa^+), found: 969.3. HRMS (ESI^+): calcd m/z for $C_{46}H_{58}N_8O_{10}NaS_2$ (MNa^+): 969.3615, found: 969.3624.

N^{α}-Boc-Glyψ-[D-Arg(N^{ω}-Tosyl)] methyl ester. D-Arg-N^{ω}-Tosyl methyl ester hydrochloride (0.57g, 1.52 mmol) and DIPEA (0.26ml) were dissolved in methanol (20 ml) at room temperature and the mixture was stirred for 20 minutes. Boc-Gly-aldehyde (0.24g, 1.52mmol) was then added and the solution was stirred for 30 min. at room temperature, then cooled 0°C and $NaBH_3CN$ (0.1g, 1.52mmol) and CH_3COOH (0.096ml, 1.67mmol) were added. After 2h the methanol was evaporated. The residue was dissolved in DCM and washed with saturated potassium hydrogen sulphate (twice) and sodium hydrogen carbonate (twice). The combined organic phases were dried over magnesium sulphate and filtered, and solvent was evaporated under vacuum. The crude product was purified by flash chromatography (eluent dicloromethane :methanol= 95:5) and obtained as white foam. Yield: 48%. ^{1}H-NMR (300MHz, DMSO-d_6): δ(ppm) 7.64 (d, 2H, J(H,H) = 8.1 Hz, CH aromatic tosyl group), 7.29 (d, 2H, J(H,H) = 8.0 Hz, CH aromatic tosyl group), 7.03 (bs, 1H, N^{η}-H arginine side chain), 6.72 (bs, 1H, NH-Boc), 6.56 (bs, 1H, N^{η}-H arginine side chain), 3.61 (s, 3H, CH_3 methyl ester), 3.34 (s, 1H, C(2)H α), 3.23-2.86 (m, 6H, CH_2 arginine side chain + Glyψ CH_2CH_2), 2.35 (s, 3H, CH_3 tosyl group), 1.61-1.43 (m, 4H, CH_2 arginine side chain), 1.37 (s, 9H, CH_3 Boc group). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm) 174.9, 156.4, 156.2, 141.2, 140.9, 128.9, 125.4, 77.3, 60.1, 51.2, 46.9, 40.6, 29.6, 29.2, 28.3, 25.5, 21.4. ESI-MS (CH_3OH, positive ions): calculated m/z: 486.6 (MH^+), 508.6 (MNa^+), 524.7 (MK^+), found: 486.5, 508.5, 524.4.

N^{α}-Boc-Glyψ-[D-Arg-(N^{ω}-Tosyl)]-OH. N^{α}-Boc-Glyψ-[D-Arg-((N^{ω}-Tosyl)] methyl ester (0.66g, 1.37 mmol) was dissolved in THF:H_2O = 1:1 (25ml). NaOH (0.42g, 10.5mmol) was

added and the mixture was stirred at room temperature for 3 hour. THF was evaporated and the product was precipitated as a white solid at pH= 7.5, filtered and dried under vacuum. Yield: 75%. ^{1}H-NMR (300MHz , DMSO-d^{6}): δ (ppm) 7.97 (bs, 1H, N-Hη arginine side chain), 7.64 (d, 2H, J(H,H)= 8 Hz, CH aromatic tosyl group), 7.29 (d, 2H, J(H,H) = 8 Hz, CH aromatic tosyl group), 7.18 (bs, 1H, N$^{\eta}$-H arginine side chain), 6.99 (bs, 1H, NH-Boc), 3.29-3.05 (m, 5H, C(2)Hα + Glyψ CH_2CH_2), 2.79-2.69 (m, 2H, CH_2 arginine side chain), 2.34 (s, 3H, CH_3 tosyl group), 1.73-1.40 (m, 4H, CH_2 arginine side chain), 1.38 (s, 9H, CH_3 Boc group). ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) 170.8, 156.8, 155.5, 141.6, 140.8, 128.9, 125.4, 77.9, 61.0, 45.9, 37.1, 28.0, 27.2, 25.0, 20.7. ESI-MS (CH_3OH, positive ions): calculated m/z: 472.6 (MH^+), 494.6 (MNa^+), 510.7 (MK^+), found: 472.4, 494.4, 510.4

N$^{\alpha}$-Boc-Glyψ-[D-Arg-(N$^{\alpha}$-Fmoc)-(N$^{\omega}$-Tosyl)]-OH. Bis(trimethylsilyl)acetamide (BTSA) (0.21ml, 0.86 mmol) and DIPEA (0.05ml, 0.29 mmol) were added to N$^{\alpha}$-Boc-Glyψ-[D-Arg-(N$^{\omega}$-Tosyl)]-OH (0.14g, 0.29 mmol) suspended in DCM (15 ml), with exclusion of humidity by a $CaCl_2$ drying tube. When the solution was nearly clear (10-15 min were usually required), FmocCl (0.1g, 0.58 mmol) was added and the mixture was stirred for 2 hours at room temperature. Methanol (1 ml) was carefully added and the mixture was stirred for an additional 15 min, diluted with 15 ml of DCM, washed with 1M potassium hydrogen sulphate (3 times) and brine (3 times), dried over magnesium sulfate and evaporated to dryness. The residue was purified by flash chromatography (dichlomethane:methanol = 9:1) and the product was obtained as a white foam. Yield: 38%. ^{1}H NMR (300MHz, DMSO-d_6):(some signals are splitted due to presence of rotamers) δ (ppm) 7.92-7.82 (m, 2H, CH aromatic Fmoc group), 7.70-7.59 (m, 4H CH aromatic tosyl group + CH aromatic Fmoc group), 7.45-7.18 (m, 6H CH aromatic Fmoc group + CH aromatic tosyl group), 4.33-4.00 (m, 3H CH_2CH Fmoc), 3.31 (bs, 1H, C(2)-Hα), 3.14-2.87 (m, 6H, CH_2 arginine side chain + Glyψ CH_2CH_2), 2.30 (s, 3H, CH_3 tosyl group), 1.66-1.38 (m, 13H, CH_2 arginine side chain + CH_3 Boc group). ^{13}C-NMR (75 MHz, DMSO-d_6,): δ(ppm) 175.0, 156.7, 155.9, 155.5, 143.8, 141.7, 140.8, 140.5, 128.8, 127.5, 127.1, 126.9, 125.4, 125.0, 119.9, 77.3, 66.4, 60.8, 48.3, 46.6, 45.2, 29.9, 28.1, 26.6, 20.7. ESI-MS (CH_3OH, positive ions): calculated m/z: 694.81 (MH^+), 716.81 (MNa^+), 732.9 (MK^+)found: 694.5, 716.4, 732.4.

N$^{\alpha}$-Boc-[L-Argψ-(N$^{\omega}$-Tosyl)]-Gly methyl ester. N$^{\alpha}$-Boc-L-Arg-N$^{\omega}$-Tosyl-aldehyde (686.7 mg, 1.66 mmol) and Gly-OMe•HCl (250.8 mg, 2.00 mmol) were dissolved in MeOH (20 ml). The reaction mixture was cooled to 0° C with an ice bath and $NaBH_3CN$ (132.1 mg, 2.00 mmol), and acetic acid (144 µl, 2.00 mmol) were added to the stirred solution. The reaction

was allowed to stir for 30 minutes at 0°C, then at room temperature for 4 hours. The solvent was evaporated and the residue was redissolved in EtOAc, and washed with saturated $NaHCO_3$ (3 times) and $KHSO_4$ (3 times). The organic layer was dried over Na_2SO4, filtered and evaporated to afford an oil. The oil was purified via column flash chromatography (from EtOAc to AcOEt/MeOH 9:1) to afford a colorless foam. Yeld: 50%. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm) 7.75 (d, 2H, J = 8.2 Hz, CH aromatic tosyl group), 7.22 (d, 2H, J = 8.2 Hz, CH aromatic tosyl group), 6.45 (bs, 3H, N–H), 5.00 (d, 1H, J = 8,4 Hz, NH-Boc), 3.72 (s, 3H, CH_3 methyl ester), 3.70-3.55 (m, 1H, C(5)Hα), 3.44 (d, 1H, J = 17,5 Hz, Gly CH_2), 3.35 (d, 1H, J = 17,5 Hz, C(2)H_2), 3.18 (bs, 2H, CH_2NH side chain), 2.70-2.50 (m, 2H, Argψ CH_2 pseudopeptide moiety), 2.38 (s, 3H, CH_3 tosyl group), 1.78 (bs, 1H, NH amine), 1.61-1.43 (m, 4H, CH_2CH_2 side chain),1.41 (s, 9H, CH_3 Boc group). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ(ppm) = 172.8, 156.8, 156.2, 141.8, 140.6, 129.1, 125.7, 79.0, 53.5, 52.9, 51.6, 50.3, 40.7, 30.1, 28.2, 25.6, 21.2. ESI-MS(CH_3OH, positive ions): calculated m/z 486.6 (MH^+), found 486.4.

N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)]-Gly[N^{α}-(Z)carboxymethylcytosine] methyl ester. N4-benzoxycarbonyl-N1-carboxymethylcytosine (CMC(Z)) (502.9 mg, 1.65 mmol) was dissolved in DMF (10 ml) at 0° C, together with DHBtOH (268.8 mg, 1.65 mmol) and DIPEA (423 µl, 2.56 mmol). EDC•HCl (314.4 mg, 1.64 mmol) was then added, and the solution was stirred for 10 minutes at 0° C, then for 20 minutes at room temperature; N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)]-Glymethyl ester (408.2 mg, 0.84 mmol) was then added to the mixture, and the solution was stirred overnight. After completion of the reaction the DMF was evaporated under vacuum. The residue was redissolved in AcOEt and washed with saturated $KHSO_4$ (3 times), and saturated $NaHCO_3$ (3 times). The organic layer was dried over Na_2SO_4 and filtered; the solvent was removed and the residue was purified via flash chromatography (AcOEt/MeOH = 95 : 5) to afford the product as a pale yellow foam. Yeld 95%. ^{1}H NMR (300 MHz, $CDCl_3$): major rotamer δ(ppm) 10.81 (bs, 1H, NH Cytosine), 7.69 (d, 2H, J = 8.1 Hz, CH aromatic tosyl group), 7.55 (bs, 1H, C(6)H Cytosine), 7.35-7.10 (m, 8H, C(5)H Cytosine, CH aromatic tosyl and benzyl group), 6.55 (bs, 2H, NH), 5.56 (bs, 1H, NH-Boc), 5.16 (s, 2H, CH_2 benzyl group), 4.90-4.45 (m, 2H, CO-CH_2-Cytosine), 4.40-3.85 (m, 2H, C(2)H_2), 4.00-3.70 (m, 2H, Argψ CH_2), 3.59 (s, 3H, CH_3 methyl ester), 3.41 (bs, 1H, C(5)H), 3.17 (bs, 2H, CH_2NH Arg side chain), 2.32 (s, 3H, CH_3 tosyl group), 1.80-1.30 (m, 13H, CH_3 Boc group and CH_2 side chain). ^{13}C NMR (75.4 MHz, $CDCl_3$): major rotamer δ(ppm) 169.5, 167.4, 163.3, 156.9, 156.2, 156.0, 152.5, 150.2, 141.8, 140.9, 135.0, 129.1, 128.6, 128.3, 128.2, 125.9, 95.6, 79.7, 77.2, 67.7, 52.8, 52.2, 50.9, 49.2, 48.9, 40.2, 28.8, 28.3, 25.6, 21.4.

ESI-MS(CH_3OH, positive ions): calculated 771.9 (MH^+), 793.9 (MNa^+), 810.0 (MK^+); found 771.8, 793.8, 809.9.

N^{α}-Boc-[L-Argψ-(N^{ω}-Tosyl)]-Gly[N^{α}-(Z)carboxymethylcytosine]-OH. To a stirred solution of N^{α}-Boc-[L-Arg-(N^{ω}-Tosyl)]ψ-Gly[N^{α}-(Z)carboxymethylcytosine] methyl ester (594.3 g, 0.77 mmol) in THF (40 ml) , a solution of $Ba(OH)_2 \bullet H_2O$ (363.9 mg, 1.15 mmol) in water (40 ml) was added. The reaction mixture was stirred for 30 minutes. The THF was then evaporated and the pH of the solution was lowered to 5 with a solution of HCl to induce the precipitation of the product. The solution was cooled at 4°C for 2 hours, then filtered and dried under vacuum to afford the desired product as a whithe solid. Yeld 72%. ^{1}H NMR (300 MHz, DMSO-d_6): major rotamer δ(ppm) 10.81 (bs, 1H, NH Cytosine), 7.81 (d, 1H, J = 7.2 Hz, Cytosine C(6)H), 7.64 (d, 2H, J = 8.1 Hz, CH aromatic tosyl group), 7.45-7.25 (m, 7H, CH aromatic tosyl and benzyl group), 7.02 (d, 1H, J = 7.2 Hz, Cytosine C(5)H), 6.90-6.60 (br m, 3H, NH side chain), 5.19 (s, 2H, CH_2 benzyl group), 4.90 (d, 1H, J = 15.9 Hz, CO-CH_2-Cytosine), 4.77 (d, 1H, J = 16.2 Hz, CO-CH_2-Cytosine), 4.03 (d, 1H, J = 17.1 Hz, Gly CH_2), 3.87 (d, 1H, J = 17.4 Hz, Gly CH_2), 3.70-2.90 (m, 5H, C(5)H, Argψ CH_2 and CH_2NH side chain), 2.34 (s, 3H, CH_3 tosyl group), 1.60-1.30 (m, 13H, CH_3 Boc group and CH_2 side chain). ^{13}C NMR (75.4 MHz, DMSO-d_6): major rotamer δ(ppm) 170.3, 167.1, 163.0, 156.5, 155.5, 154.9, 153.1, 150.5, 141.6, 141.0, 135.9, 129.0, 128.4, 128.1, 127.9, 125.5, 93.8, 77.9, 66.4, 51.4, 49.4, 48.5, 47.8, 40.2, 28.6, 28.1, 25.4, 20.8. HR-MS: calculated m/z for $C_{34}H_{45}O_{10}N_8S$ (MH^+): 757.2974, found: 757.3019; calculated for m/z for $C_{34}H_{44}O_8N_{10}NaS$ (MNa^+): 779.2793, found: 779.2831; calculated m/z for $C_{34}H_{43}O_{10}N_8S$ ($M\text{-}H^-$): 755.2817, found: 755.2819.

3.4.3 PNA synthesis

The synthesis of chiral PNAs was performed as described previously for similar molecules[17] by using manual synthesis for the chiral residues and automated Boc-SPPS protocols on a ABI 433A synthesizer, following the procedures provided from the company, for the achiral residues. The chiral submonomers were introduced by using HATU/DIEA coupling protocol, Fmoc group was deprotected by piperidine/NMP (20%) and carboxymethyl-Z-adenine, carboxymethyl-thymine [18] were linked to the submonomers on resin by using DIC/DhBtOH coupling protocol. Free PNAs were cleaved from the resin by using a 1:3 TFMSA/TFA mixture (10% thioanisole + 10% m-cresol) and precipitated by Et_2O. HPLC analysis for all

PNAs was carried out by LC-MS by using an XTerra anlytical C18 column (3x250mm, 5µm, flow 0.5ml/min), gradient elution from 100% H_2O (0.2% HCOOH, eluent A) to 60% H_2O and 40% CH_3CN (0.2%HCOOH, eluent B) in 30 min. MS detector set in the positive ion mode, capillary voltage 3kV, cone voltage 30V, full scan acquisition from 150 to 1500 m/z. HPLC purification for all PNAs was carried out on a semipreparative Jupiter (Phenomenex) C18 column (10x300mm, 5µm, flow 4ml/min); eluent A: 100% H_2O (0.1% TFA), eluent B: 60% H_2O and 40% CH_3CN (0.1%TFA). Specific preparative elution conditions for every PNA are given below.

PNA 1:H-GTAGA**T**$_{(2D,5LArg)}$CACT-NH_2. The synthesis was performed on 25mg of a preloaded Boc-T-MBHA-PS resin (loading 0.2mmol/g). Crude yield: 75%. The crude products were purified by semipreparative RP-HPLC, in gradient elution: from 100% A to 100% B in 30 min ESI-MS: calcd m/z: 976.0 (MH_3^{3+}), 732.2 (MH_4^{4+}), 586.0 (MH_5^{5+}), 488.5 (MH_6^{6+}) found m/z 976.2, 732.3, 586.2, 488.6. **PNA 2** H-GTAG**A**$_{(2D-Arg)}$**T**$_{(2D,5L-Arg)}$**C**$_{(5L-Arg)}$ACT-NH_2. Crude yield: 80%. The crude product was purified by RP-HPLC in gradient elution: from 100% A to 100%B in 30 minutes. ESI-MS calcd m/z: 1042.1 (MH_3^{3+}), 781.8 (MH_4^{4+}), 625.6 (MH_5^{5+}), 521.5 (MH_6^{6+}) found m/z 1042.0, 781.6, 625.4, 521.1.

3.4.4 Melting temperature measurements

Stock solutions of PNA **1**, **2**, and of oligonucleotides were prepared in doubly distilled water and their actual concentration calculated by UV absorbance using the following ε260 (M^{-1} cm^{-1}) for the nucleobases: T 8600, C 6600, A 13700, G 11700. By using these concentrations, hybrid solutions containing 5µM PNA-DNA duplexes were prepared. All the hybridisation experiments were carried out in 10mM phosphate buffer, 100mM NaCl, 0.1mM EDTA, pH= 7. All the hybrid samples reported were first incubated at 90°C for 5 minutes, then slowly cooled to room temperature. The samples were heated (1°C/min) and the UV signal variation at 260nm was recorded. Melting temperatures were measured as the maximum of the first derivatives of the melting curves.

3.5 References

[1] R. Corradini, S. Sforza, T. Tedeschi, F. Totsingan, R. Marchelli, *Current Topic in Medicinal Chemistry*, **2007**, 7, 681
[2] F. Wojciechowski, H. E. Hudson, *Current Topic in Medicinal Chemistry*, **2007**, 7, 667
[3] R. Corradini, S. Sforza, T. Tedeschi, R. Marchelli, *Chirality*, **2007**, 19, 269
[4] A. Puschl, S. Sforza, G. Haaima, O. Dahl, P. E. Nielsen, *Tetrahedron Lett.*, **1998**, 39, 4707
[5] P. Wittung, M. Eriksson, R. Lyng, P. E. Nielsen, B. Norden, *J. Am. Chem. Soc.,* **1995**, 117, 10167
[6] G. Haaima, A. Lohse, O. Buchardt, P. E. Nielsen, *Angew.Chem. Int. Ed.*, **1996**, 35, 17, 1939
[7] E. A. Englund, D. H. Appella, *Angew. Chem. Int. Ed.*, **2007**, 46, 1414
[8] S. Sforza, G. Haaima, R. Marchelli, P. E. Nielsen, *Eur. J. Org. Chem.*, **1999**, 197
[9] T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Letters*, **2005**, 46, 48, 8395
[10] S. Sforza, R. Corradini, S. Ghirardi, A. Dossena,R. Marchelli, *Eur. J. Org. Chem.*, **2000**, 2905
[11] V. Menchise, G. De Simone, T. Tedeschi, *PNAS*, **2003**, 100, 21, 12021
[12] T. Tedeschi, M. Chiari, G. Galaverna, S. Sforza, M. Cretich, R. Corradini, R. Marchelli, *Electophoresis*, **2005**, 26, 22, 4310
[13] S. Sforza, T. Tedeschi, R. Corradini, A. Dossena, R. Marchelli, *Chemical Communications*, **2003**, 9, 1102
[14] J. Weiler, H. Gausepohl, N. Hauser, O. N. Jensen, J. D. Hoheisel, *Nucleic Acids Research*, **1997**, 25, 14, 2792
[15] S. Sforza, T. Tedeschi, R. Corradini, D. Ciavardelli, A. Dossena, R. Marchelli, *Eur. J. Org. Chem.*, **2003**, 6, 1056
[16] S. Sforza, T. Tedeschi, R. Corradini, R. Marchelli, *Eur. J. Org. Chem.,* **2007**, 5879
[17] T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Lett.*, **2005**, 46, 8395
[18] S.A. Thomson, J.A. Josey, R. Cadilla, M.D. Gaul, C.F. Hassman, M.J. Luzzio, A.J. Pipe, K.I. Reed, D.J. Ricca, R.W. Wiethe, S.A. Noble, *Tetrahedron*, **1995**, 51, 6179

Chapter 4

Chiral PNA application on surface: Arginine-based PNA microarrays for ApoE genotyping

In this chapter PNAs containing a 2D-5L arginine monomer are utilized in diagnostic applications on surface. The design and synthesis of the modified PNAs by submonomeric strategy are described. The probes have been designed targeting sequences contained in the human gene coding for ApoE (a protein related to the cholesterol metabolism), in order to recognize six different genotypes corresponding to SNPs present in two different positions and related to the risk of the Alzheimer disease onset. After the synthesis, an innovative application is described, consisting in the fabrication of microarrays bearing chiral PNAs for a fast and precise genotype characterization.

4.1 Introduction

Single nucleotide polymorphisms (SNPs) are one of major sources of genetic variation in the human genome[1]. Many SNPs have been linked to several diseases or pathological conditions, such as the Alzheimer disease (AD), which was found to be related to SNPs located in the ApoE gene. This gene encodes for the apolipoprotein E, a protein involved in cholesterol metabolism. This protein has been found to exist in three main isoforms (ε2, ε3, ε4)[2] that differ for the substitution of amino acids at position 112 and 158: Cys112/Cys158 (ε2), Cys112/Arg158 (ε3) and Arg112/Arg158 (ε4). The combination of the variants on the two alleles generates six different genotypes[3]. An increased frequency of the ApoE ε4 allele in late-onset AD patients, both familiar and sporadic, has been demonstrated, and a gene dose effect for the ε4 allele was found in AD families, implicating that this allele is a major risk factor for AD[4].

Selective SNP recognition is useful both in diagnostic and therapeutic applications. For example, in the case of AD, having this disease origin in early-life[5] and given that no cures are at the moment available, accurate and early diagnosis of a genetic predisposition could be very important, since early therapeutic intervention, before severe cellular damages, would improve the prognosis and life quality of AD patients. Moreover, since AD diagnosis can be confirmed only by autoptic post-mortem analysis, a reliable genetic testing to be performed on all subjects suffering for dementia might help to distinguish between AD and non-AD subjects[6].

Recently, great efforts have been devoted to develop selective and specific methods for SNP analysis, yielding a large number of distinct approaches[7]. Usually, a PCR amplification of the desired SNP-containing region is initially performed in order to specifically increase the number of DNA molecules for detection. After PCR, allelic discrimination is done by different strategies: primer extension (nucleotide incorporation), ligation, enzymatic cleavage and hybridization[7]. Hybridization approaches exploit differences in thermal stability of double-stranded DNA to distinguish between matched and mismatched target-probe pairs. Thus, it is critical to perform assays under conditions where hybridization occurs only if the probe and the target are perfectly complementary.

Selective hybridization methods have been implemented not only in solution but also on surface-based methods, such as microarrays[8,9]. Such methods usually rely on the recognition of a DNA target by hybridization with a single strand oligonucleotide probe immobilized onto

a surface. Although methods performed in solution are usually more affordable and robust, surface systems display several features not achievable with other techniques. The concentration effect obtained on the surface where the probe is deposited usually results in a sensitivity increase of the method. Surface devices are fabricated with microsystems that allow to work with only very small amounts of probes, and small volumes of analyte solution for the hybridization process. The possibility to use multiple probes at the same time enables this technique to simultaneously give a large number of information and so to reduce the number of the necessary analyses. Finally, when the system has been used, it can be recycled by denaturating the duplex formed on the surface and thus leaving the probes linked to the surface ready to be further rehybridized.

The possibility to freely modify the surfaces allows to exploit different physical properties for revealing the duplex formation. The improvement of these techniques led to development of optical, electrochemical, or mass sensitive devices. Detection methods by fluorescence are mostly used being fast and very sensitive. In order to achieve optimal results, the probes should be highly selective[10]. Oligonucleotide probes suffer from two drawbacks: the strong dependence of the duplex stability upon the ionic strength[11] and the inability of the oligonucleotide probes to invade DNA secondary structures, which disfavours the target hybridization on the surface[12]. Therefore, these systems may be improved by substituting oligonucleotide molecules with other similar compounds with improved specificity and selectivity. Typical example of such compounds are Peptide Nucleic Acids (PNAs)[13].

PNA microarrays can be made by directly linking the probes to a surface (glass or polymeric) through a covalent linkage with suitably active functional groups. PNA microarrays can be built by deposition of pre-synthesized and purified probes[14], or by in-situ synthesis of PNAs[15]. PNA probes have particularly suitable features which allow them to be applied extensively for the analysis of particular DNA sequences[14,16], or for the detection of SNPs[15,17,18], obtaining good performances. First experiments in this field were carried out hybridizing amplified and labeled DNA samples[12] on surface and reading the fluorescence by laser scanners studied for these systems. This method is still the most used because it is robust and reproducible. Commercial PNA arrays exploit this detection method, albeit fluorescent labeling is not introduced directly, but in two steps; amplified DNA is labeled with a biotin and, after hybridization and washing are performed, a streptavidin-fluorophore is coupled and bound to the DNA[19]. Although fluorescent detection is still the most used, many efforts have been done for improving the detection performances, either by changing the detection method, or by improving the probe performances, introducing *ad hoc* modifications. Early works were

obtained by coupling array technology with mass spectrometry, exploiting the lack of phosphates in PNA backbone. The detection of phosphate group by TOF-SIMS mass spectrometry was, thus, used to determine whether the probe was hybridized with complementary DNA or not[20,21]. Conjugation of PNA-microarrays with fluorescent positively charged polymers was exploited to visualize hybridization with non-labeled DNA by fluorescence signals, exploiting the fact that positively charged polymers could interact with the duplex thanks to the negative charges from the DNA backbone, but not with PNA that has a neutral backbone[22,23]. The introduction of groups able to recognize selectively the PNA-DNA duplex has been done using gold nanoparticles bearing DNA sequences able to recognize the same strand hybridized by the PNA, enhancing the detection using silver and measuring the scattering of the light by the nanoparticle labeled spots[24].

Although many improvements in the readout signals led to very powerful methods, selectivity needs to be increased in order to achieve a better recognition of SNPs. For this reason it is important to have systems able to perfectly bind to matched targets and not to mismatched targets. PNAs have been modified to improve performances in the recognition of single point mutations. It has been demonstrated that this can be achieved by introducing suitable modifications in the PNA backbone[25]. Among modified PNAs, chiral amino acid-based analogues substituted at position 2 and 5 (Figure 4-1) have recently gained increasing attention, due to their peculiar DNA binding abilities easily tuneable according to the different configurations[26], as described in the previous chapter.

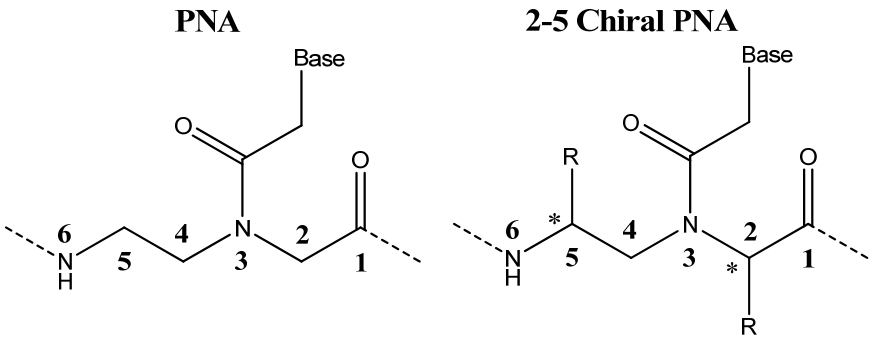


Figure 4-1: Chemical structure of chiral PNAs

Four modified PNA probes carrying one chiral monomer, derived from two arginine units, with two stereogenic centres in positions 2 and 5 having suitable configurations were synthesized in order to improve the specificity and the strength of DNA binding. The synthesized PNAs have then been used to build PNA microarrays for the genotypization of two different SNPs in the ApoE gene. The performances of these microarrays have been studied by using oligonucleotide mixtures simulating the different genetic profiles corresponding to the different SNPs in the ApoE gene.

4.2 Results and Discussion

4.2.1 Design and synthesis of modified chiral PNA probes

The properties of modified chiral PNAs containing lysine based monomers in the middle of the sequence have been showed in chapter 3, demonstrating that PNAs containing one residue with two lysine-derived side chains (2D,5L) had a highly improved affinity and selectivity in the recognition of complementary DNA sequences. An homologous PNA having the same modification but based on the structure of arginine maintained the same properties.

As explained in chapter 3, arginine has been used instead of lysine because the PNAs will be linked to surface for building PNA-microarrays. The presence of the guanidium group on the side chain of arginine allows to avoid any interference in the reaction between the terminal amino group of the probe and the reactive groups on the surface (which was possible if using lysine-based PNAs).

The PNA sequences to be synthesized had to be complementary to the two gene tracts bearing the SNPs, codifying for the three ApoE protein isoforms. Four undecameric PNAs (for two different SNPs in two different gene tracts) were designed (PNA 1-4, Table 4-1), with the chiral (2D,5L-Arg) unit located in the middle of the sequence, corresponding to the SNP positions. In both regions containing the codons for amino acid 112 and 158 in the ApoE protein, a T/C SNP was present, thus in both cases the PNA nucleobase corresponding to the SNP was an A or a G. It was also decided to place two spacer groups (aminoethoxyethoxyacetyl, AEEA) at the amino terminus in agreement with previous studies[27] which established the right distance between the probes and the surface of microarray for obtaining a good surface hybridization.

Table 4-1. PNA sequences and melting temperatures of the PNA:DNA antiparallel fullmatch/mismatch duplexes in phospate buffer (pH=7) at a 5μm concentration for each strand

PNA	Sequence[a]	Tm (°C) PNA/DNA Fullmatch[b]	Tm (°C) PNA/DNA Mismatch[b]	ΔTm (°C)
1	$H(AEEA)_2GCCGC\underline{\mathbf{A}_{(2D,5LArg)}}CACGT\text{-}NH_2$	78 (with **1'**)	61 (with **3'**)	17
2	$H\text{-}(AEEA)_2CAGGC\underline{\mathbf{A}_{(2D,5L\text{-}Arg)}}CTTCT\text{-}NH_2$	69 (with **2'**)	50 (with **4'**)	19
3	$H(AEEA)_2GCCGC\underline{\mathbf{G}_{(2D,5LArg)}}CACGT\text{-}NH_2$	85 (with **3'**)	69 (with **1'**)	16
4	$H\text{-}(AEEA)_2CAGGC\underline{\mathbf{G}_{(2D,5L\text{-}Arg)}}CTTCT\text{-}NH_2$	75 (with **4'**)	60 (with **2'**)	15

[a] The chiral monomer corresponding to the SNP position is underlined in bold

[b] Oligonucleotide sequences (SNP base is underlined in bold): **1'**: 5'-ACGTGTGCGGC-3', **2'**: 5'-AGAAGTGCCTG-3',**3'**: 5'-ACGTGCGCGGC-3', **4'**: 5'-AGAAGCGCCTG-3'

PNAs were synthesized by using common SPPS protocol based on the Boc strategy for the standard monomers, combined to the Boc submonomeric strategy for the chiral monomer only, since better suited to preserve the optical purity at the 2 position[28] and also because one single submonomer can be used for the synthesis of PNAs bearing different nucleobases on the chiral residue. The submonomer was prepared, as reported in Chapter 3, starting from the commercial D- and L-Boc-Arginine(Tos)-OH. Boc-L-Arg(Tos)-OH was transformed in the corresponding Weinreb amide and then reduced to the aldehyde. Boc-D-Arg(Tos)-OH was transformed in the methyl ester and Boc deprotected. The PNA backbone was then synthesized by reductive amination of D-Arg(Tos)-OMe with the Boc-L-Arg(Tos) aldehyde. The methyl ester was hydrolyzed and the secondary amine was protected by the introduction of a Fmoc group.

According to previously reported procedures[15], the chiral submonomers units were inserted by manual coupling with the HATU/DIEA protocol and, after Fmoc deprotection, the nucleobase residues were introduced by a double coupling with DIC/DhBTOH. All the PNA oligomers were purified by RP-HPLC and characterized by ESI-mass spectrometry (Figure 4-2).

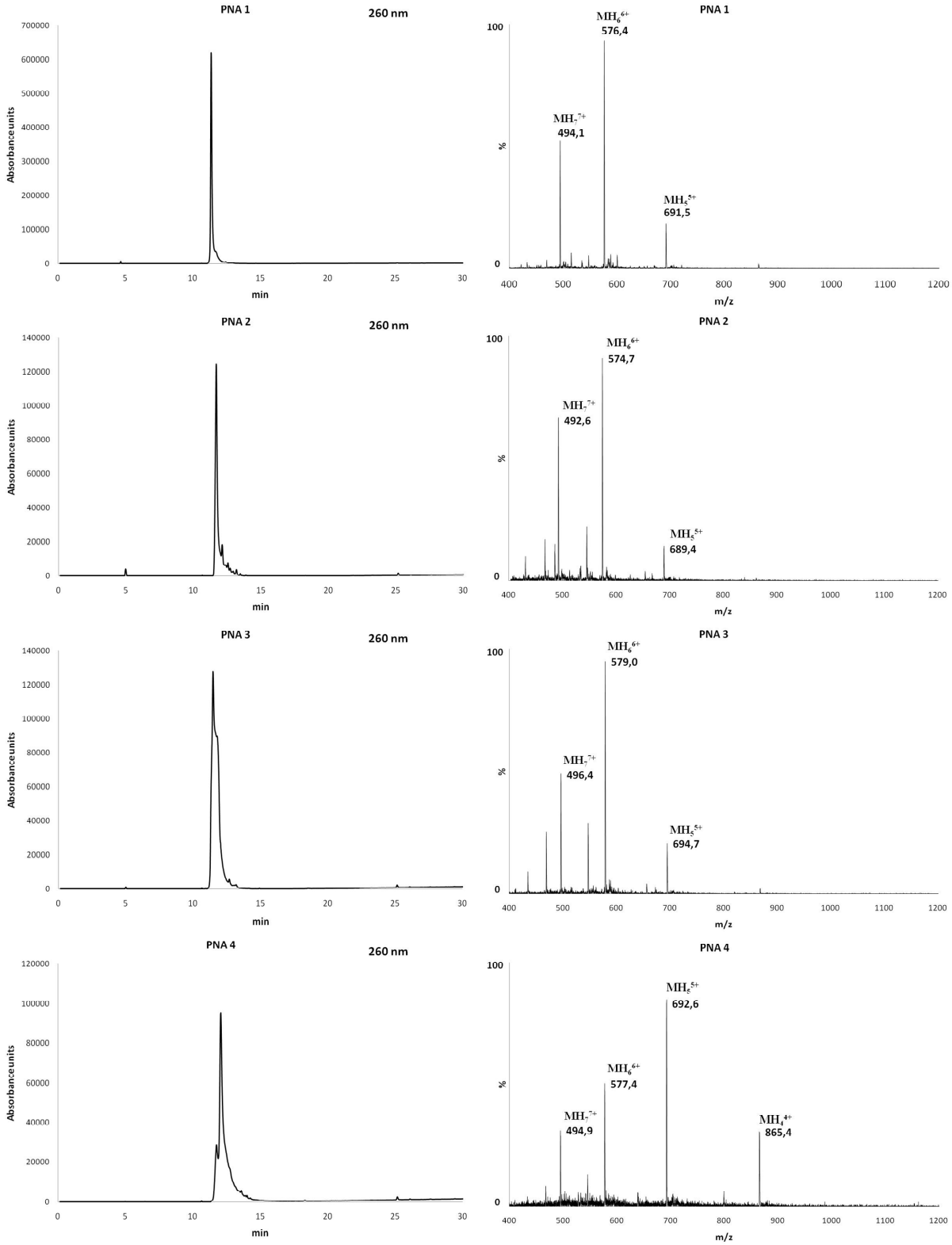


Figure 4-2: HPLC profiles and mass spectra of PNAs 1-4

4.2.2 DNA recognition by PNA probes in solution

Probe performances were initially tested in solution by measuring the melting temperatures of both fullmatch and mismatch PNA-DNA duplexes, by using oligonucleotides homologous to the ApoE sequences (Table 4-1). Melting temperatures were evaluated as the first derivatives of the UV absorption curves at 260 nm in a temperature range from 20 to 90°C.

The chiral PNA probes were found to bind with very high stability to complementary DNA sequences (Tm from 69 to 85°C) and also exerted very high sequence recognition, with differences between full match and mismatch melting temperatures ranging from 15°C to 19°C. The high stability of the full match complexes, together with the increased specificity, make feasible to find conditions, after PNA deposition on a microarray, in which it is possible to have good signals for full match duplexes and no signals for the mismatched ones.

4.2.3 PNA spotting on the array platform

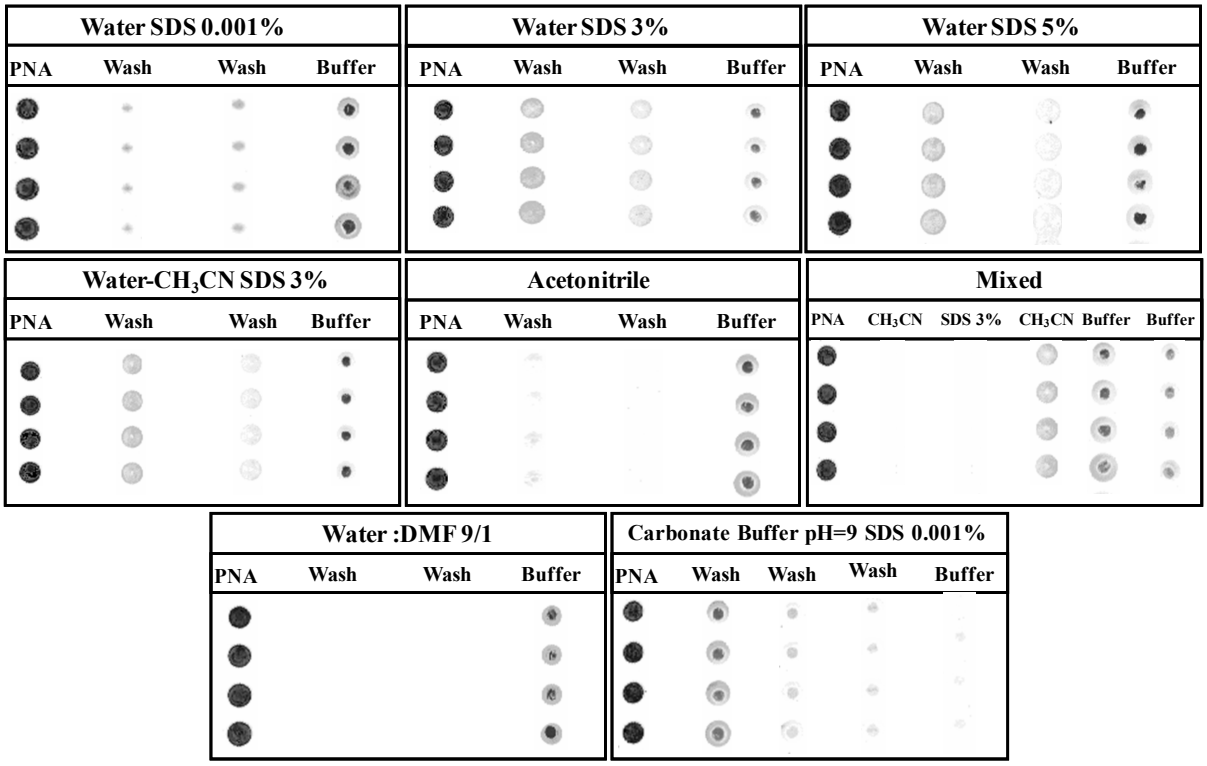


Figure 4-3: Optimization of washing procedure (effect of solution composition)

PNA-microarray platforms were built by using glass slides derivatised with N-hydroxysuccinimide active groups, which were allowed to react with the PNA terminal amino groups. The solutions for PNA spotting were prepared by dissolving the PNA probes (50 μM) in carbonate buffer (0.1 M, pH=9) containing SDS (0.001%). The deposition of the probes on the surface was done by using a pin system spotter.

Since PNAs are known to be strongly absorbed on many types of surfaces, three so called "washing-spotting" cycles had to be introduced after each PNA deposition, in order to avoid false positive signals due to the contamination of the pin. The optimization of the washing step was done by spotting a PNA, followed by two or three spotting of washing solutions, and finally spotting the same buffer used to spot the probe, in order to check if any residue contamination was present in the pin. The essays allowed to evaluate the "washing power" of different mixtures, looking for the one able to completely remove the undeposited PNA from the pin. Washing solutions were prepared using organic solvents (such as acetonitrile and dimethylformamide) and different mixtures of SDS (Figure 4-3). The effect of pH on the ability to solubilize the adsorbed probe on the pin was also tested using different buffer mixtures (Figure 4-4).

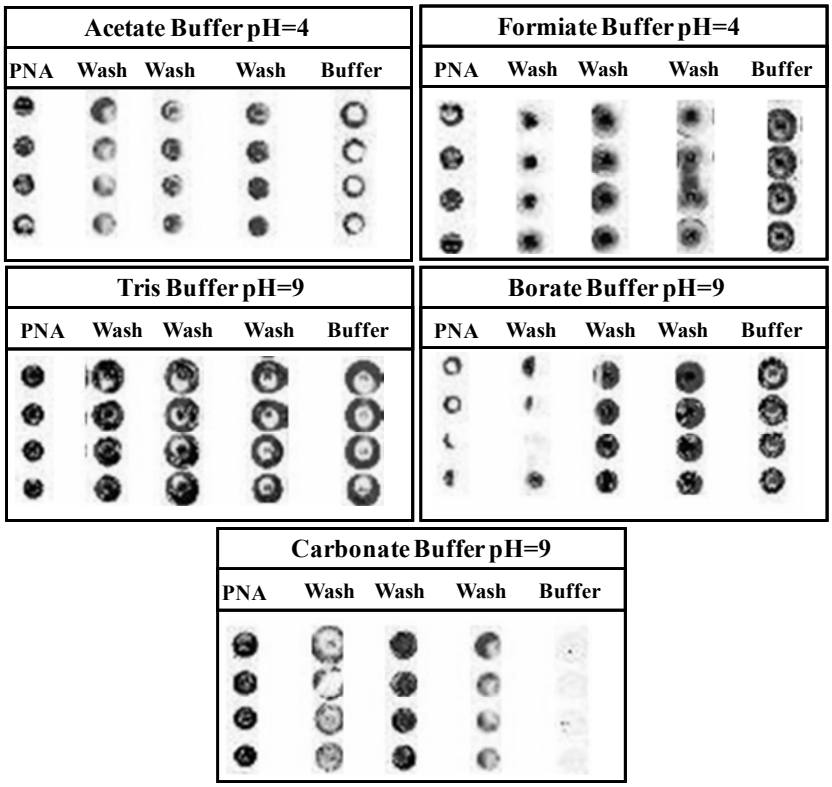


Figure 4-4: Optimization of washing procedure (effect of pH and buffer)

The results showed in figures 4-3 and 4-4 indicate that the best results in terms of lack of cross contamination is obtained with the same buffer used for depositing the PNAs, composed by 0.1 M sodium carbonate buffer at pH= 9 with SDS present at various concentration (0.2%, 1% and 0.001%); after these cycles no traces of PNAs were detected upon spotting (as confirmed by the lack of hybridization with the complementary Cy5-oligonucleotide). Deposition efficiency was checked in every

slide by spotting a fluorescent oligonucleotide derivatised with the Cy3 fluorophore. Thus the definitive protocol used for spotting the PNAs was the following: PNA deposition at the concentration of 50μM in SDS 0.001%, Carbonate 0.1M pH= 9, followed by three washing with Carbonate 0.1M and SDS 0.2%, SDS 1%, and SDS 0.002%

The spotting process was followed by an incubation step in a dark room with controlled humidity overnight, and by a capping process with, a 50 mM ethanolamine solution, in 0.1M TRIS buffer (pH=9) at 50°C for 1h, in order to block any unreacted active group on the slide, followed by washing of the slides with buffer and water.

4.2.4 Oligonucleotide hybridization

Several microarray slides, each bearing all four different PNAs, were then hybridised with 100 nM solutions of oligonucleotides derivatised with the Cy5 fluorophore.

In order to increase the hybridization efficiency and to refine shapes of the spots, an incubation-hydration step in SDS 0.1%, SSC buffer (0.3M NaCl, 0.03M sodium citrate, pH= 7), at 40°C for 30 minutes was added before the hybridization with oligonucleotide solutions[29]. This step was particularly required when using the slides several days after PNA spotting, allowing to maintain the efficiency of the devices, which could be used months after the preparation without significative differences.

The hybridation-incubation was carefully optimised in order to eliminate any signal due to mismatched PNA-DNA duplexes without depleting the signals due to full match duplexes: optimal results were obtained by incubation at 55°C for 2 hours. Every slide-bound PNA was singularly tested with the different oligonucleotides: in all cases, under the above reported conditions, a clear signal was detected for full match PNA-DNA duplexes, whereas no signal or only a very weak signal was detected for the mismatched complexes.

Table 4-2. Genotypes related to ApoE mutations	
Genotype	Oligonucleotide simulation[a]
ε2/ε2	**1' + 2'**
ε2/ε4	**1' + 2' + 3' + 4'**
ε3/ε4	**1' + 3' + 4'**
ε4/ε4	**3' + 4'**
ε2/ε3	**1' + 2' + 4'**
ε3/ε3	**1' + 4'**
[a] Oligonucleotide sequences (SNP base is underlined in bold): **1'**: 5'-ACGTGTGCGGC-3', **2'**: 5'-AGAAGTGCCTG-3', **3'**: 5'-ACGTGCGCGGC-3', **4'**: 5'-AGAAGCGCCTG-3'. All the oligonucleotides were derivatized with Cy5	

In order to simulate the different genotype combinations, further experiments were then performed by using oligonucleotide mixtures. The SNPs in the ApoE gene tracts coding for the 4 different isoforms are: a thymine in ε2 and ε3 and a cytosine in ε4 for the codon corresponding to the amino acid 112; a thymine in ε2 and a cytosine in ε3 and ε4 for the codon corresponding to the amino acid 158. By considering all the allelic combinations, the 6 different genotypes were simulated by mixing the corresponding oligonucleotides, as reported in Table 4-2.

The oligonucleotide mixtures were then incubated in six independent experiments with the arginine-PNA microarrays, in the same conditions reported before. The results are reported in figure 4-4.

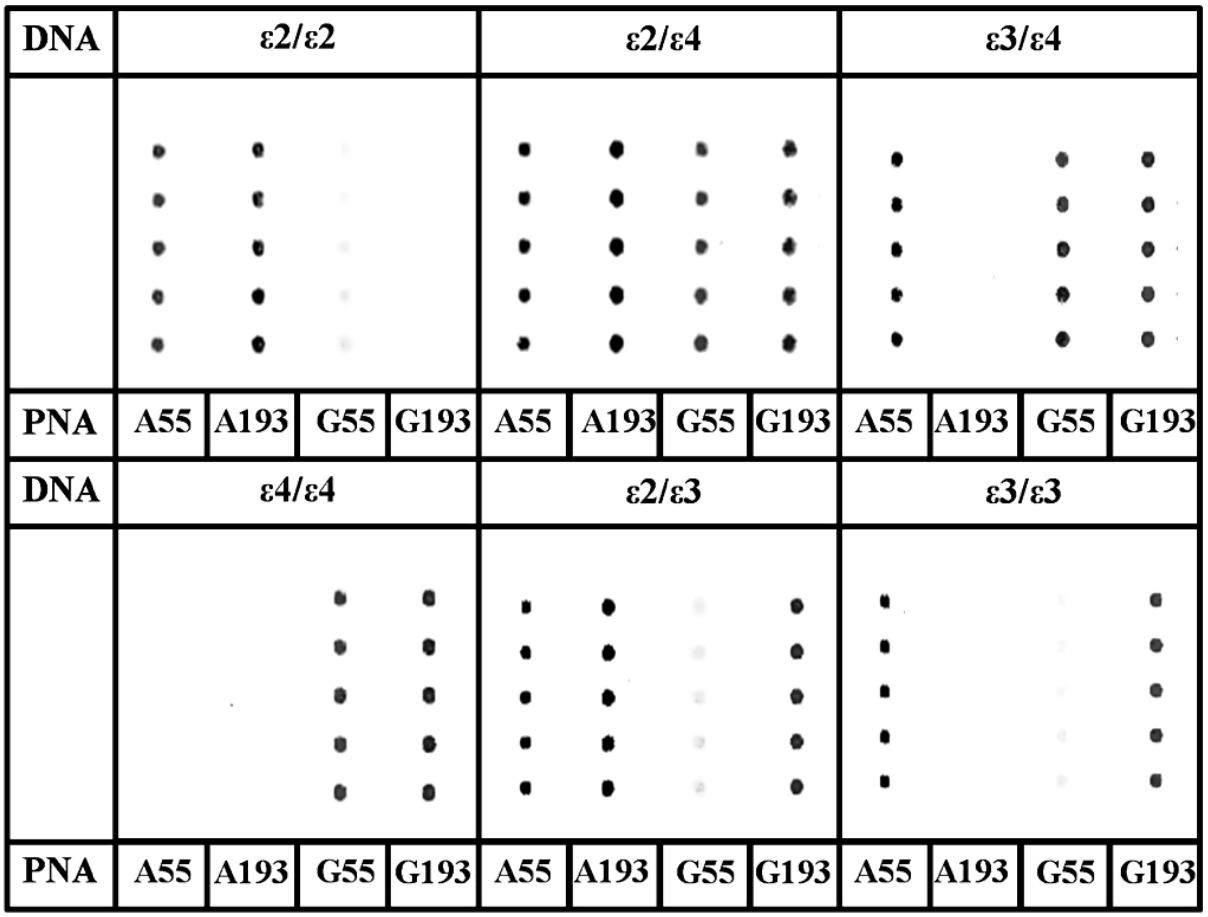


Figure 4-5: PNA microarray analysis with Cy5-oligonucletides in different combinations (see Table **4-2**) to simulate all the possible genotypes.

Six clearly different hybridization patterns were obtained, thus demonstrating that arginine-based PNAs are very efficient in discriminating single point mismatches also when deposited on the solid surface. These data demonstrate that the arginine-PNA microarrays could easily discriminate among different genotypes related to the three ApoE isoforms, thus providing a potential way to rapidly assess the genetic predisposition to the Alzheimer disease.

4.3 Conclusions

In this work we have demonstrated that the arginine-based PNAs synthesized carrying one chiral monomer with two stereogenic centers of suitable configuration in the middle of the strand, are very efficient in discriminating single point mutations both in solutions and on a solid surface. Microarrays built by using four arginine-PNAs were shown to be able to discriminate the presence or the absence of their complementary oligonucleotides also in

complex mixtures simulating the real genotype profiles. Six different hybridization patterns related to six different genotypes in the ApoE gene were clearly obtained in microarray experiments, potentially allowing for an easy genetic assessment. Work is now in progress in order to apply this diagnostic system to human DNA samples amplified by PCR.

4.4 Experimental Section

4.4.1 Materials and reagents

PNA N-Boc protected standard monomers, N-[1H-1,2,3-(benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU), N-[(dimethylamino)-1H-1,2,3-triazol[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU), dichloromethane, N–methylpyrrolidone (NMP) and N,N-dimethylformamide (DMF) were purchased from Applied Biosystem (Foster City, CA, USA). Carboxymethyl-O-benzyl-guanine was purchased by ASM (Hannover, Germany). Boc-Arg(Tos)-OH, Boc-D-Arg(Tos)-OH and m-cresol were from Fluka (Buchs, Switzerland). (4-Methylbenhydryl)amine (MBHA) PS resin was from Novabiochem (Laufelfingen, Switzerland). Diisopropylethylamine (DIPEA), N,O-Bistrimethylsilyl acetamide (BSA), trifluoromethane sulfonic acid (TFMSA), trifluoroacetic acid (TFA), 3-hydroxy-1, 2,3-benzotriazin-4-(3h)-one (DhBtOH) and diisopropylcarbodiimide (DIC) were from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides (guaranteed oligos grade) were purchased from ThermoFisher Scientific (Ulm, Germany) and used without further purification.

4.4.2 PNA synthesis

The synthesis of chiral (2D,5L)-Arg-based PNAs was performed as described previously for similar PNAs[30] by using manual synthesis for the chiral residues and automated Boc-SPPS protocols on a ABI 433A synthesizer, following the procedures provided from the company, for the achiral residues. The chiral submonomers were introduced by using HATU/DIEA coupling protocol, Fmoc group was deprotected by piperidine/NMP (20%) and carboxymethyl-Z-adenine[31] or carboxymethyl-O-benzyl-guanine (commercialy available)

were linked to the submonomers on resin by using DIC/DhBtOH coupling protocol. Free PNAs were cleaved from the resin by using a 1:3 TFMSA/TFA mixture (10% thioanisole + 10% m-cresol) and precipitated by Et_2O. HPLC analysis for all PNAs was carried out by LC-MS by using an XTerra anlytical C18 column (3x250mm, 5µm, flow 0.5ml/min), gradient elution from 100% H_2O (0.2% HCOOH, eluent A) to 60% H_2O and 40% CH_3CN (0.2%HCOOH, eluent B) in 30 min. MS detector set in the positive ion mode, capillary voltage 3kV, cone voltage 30V, full scan acquisition from 150 to 1500 m/z. HPLC purification for all PNAs was carried out on a semipreparative Jupiter (Phenomenex) C18 column (10x300mm, 5µm, flow 4ml/min); eluent A: 100% H_2O (0.1% TFA), eluent B: 60% H_2O and 40% CH_3CN (0.1%TFA). Specific preparative elution conditions for every PNA are given below.

PNA 1:H-$(AEEA)_2$GCCGC**A$_{(2D,5L\text{-}Arg)}$**CACGT-NH_2. The synthesis was performed on 25mg of a preloaded Boc-T-MBHA-PS resin (loading 0.2mmol/g). Crude yield: 82%. The crude products were purified by semipreparative RP-HPLC, in gradient elution: from 100% A to 100% B in 25 min ESI-MS: calcd m/z: 691.5 (MH_5^{5+}), 576.4 (MH_6^{6+}), 494.2 (MH_7^{7+}) found m/z 691.8, 576.5, 494.4. **PNA 2** H-$(AEEA)_2$CAGGC**A$_{(2D,5L\text{-}Arg)}$**CTTCT-NH_2. Crude yield: 80%. The crude product was purified by RP-HPLC in gradient elution: from 100% A to 100%B in 30 minutes. ESI-MS calcd m/z: 689.5 (MH_5^{5+}), 574.7 (MH_6^{6+}), 492.8 (MH_7^{7+}) found m/z: 689.7, 574.7, 492.6. **PNA 3**. H-$(AEEA)_2$GCCGC**G$_{(2D,5L\text{-}Arg)}$**CACGT-NH_2. The manual synthesis was performed on 25mg of a preloaded Boc-T-MBHA-PS resin (loading 0.2mmol/g). Crude yield: 80%. The crude product was purified by RP-HPLC in gradient elution : from 100% A to 100% B in 25 min. ESI calcd m/z: 694.7, 579.1, 496.5 found m/z: : 694.7, 579.0, 496.5. **PNA 4** H-$(AEEA)_2$CAGGC**G$_{(2D,5L\text{-}Arg)}$**CTTCT-NH_2 Crude yield: 85%. The crude product was purified by RP-HPLC in gradient elution: from 100% A to 100%B in 30 minutes. ESI-MS calcd m/z: 1153.8 (MH_3^{3+}) 865.6 (MH_4^{4+}), 692.7 (MH_5^{5+}), 577.4 (MH_6^{6+}) found m/z: 1154.0, 865.8, 692.8, 577.5.

4.4.3 Melting temperature measurements

Stock solutions of PNA **1**, **2**, **3**, **4** and of oligonucleotides were prepared in doubly distilled water and their actual concentration calculated by UV absorbance using the following ε260 (M^{-1} cm^{-1}) for the nucleobases: T 8600, C 6600, A 13700, G 11700. By using these concentrations, hybrid solutions containing 5µM PNA-DNA duplexes were prepared. All the

hybridisation experiments were carried out in 10mM phosphate buffer, 100mM NaCl, 0.1mM EDTA, pH= 7. All the hybrid samples reported were first incubated at 90°C for 5 minutes, then slowly cooled to room temperature. The samples were heated (1°C/min) and the UV signal variation at 260nm was recorded. Melting temperatures were measured as the maximum of the first derivatives of the melting curves.

4.4.4 PNA spotting on microarray slides

Each PNA was dissolved in a solution containing carbonate buffer (0.1M, pH= 9), SDS 0.001% at a concentration of 50 μM and spotted by using a pin system spotter SpoArray 24 (Perkin-Elmer, Waltham, USA) onto commercial slides (CodeLink, Amersham Bioscences, New Jersey, USA). Then the following washing steps were performed: washing solution 1: carbonate 0.1M and SDS 0.2%; washing solution 2: carbonate 0.1M and SDS 1%; washing solution 3: carbonate 0.1M and SDS 0.001%. Fixing was performed overnight at a controlled moisture (~ 75%). Capping of the unreacted sites was done by using a solution of Tris 0.1M, ethanolamine 50mM, pH=9, at 50°C for 1h. Conditioning of the slides was performed at 50°C with SSC buffer (0.6M NaCl, 0.06M sodium citrate, pH= 7) and SDS 1% solution for 1h. Final washings were carried out with doubly distilled water for 5 minutes.

4.4.5 Oligonucleotide hybridization on microarray slides

Each Cy5 labeled oligonucletide was dissolved in a solution containing SSC buffer (0.3M NaCl, 0.03M sodium citrate, pH= 7), SDS 0.1% at a concentration of 100nM. Before hybridization the slides were hydrated with a solution of SSC buffer (0.3M NaCl, 0.03M sodium citrate, pH= 7), SDS 0.1% at 40°C for 30 minutes. Then, oligonucleotide solutions were deposited on the slides by using a hybridization chamber and left at 50°c for 2 hours. After hybridization, washing steps were performed at 50°C for 5 minutes with a solution of SSC buffer (0.3M NaCl, 0.03M sodium citrate, pH= 7), SDS 0.1%. Final washing was carried out for 1 minute at room temperature with a two different SSC buffer solutions: 0.03M NaCl, 0.003M sodium citrate, pH= 7 and 0.01M NaCl, 0.001M sodium citrate, pH= 7.

4.4.6 Microarrays analysis

Pictures of the PNA microarrays were recorded by using a scanner ScanArray Express 20 (Perkin Elmer, Waltham, USA). Laser was set at excitation wavelenght of 646nm and emission at 664nm, for Cy5. Pictures were acquired with laser power at 100% and photomultiplier at 40%.

4.5 References

[1] A.J. Brookes, *Gene*, **1999**, 234, 177

[2] G. Ultermann; U. Langebeck; U. Beisiegel; W. Weber, Am., *J. Hum. Genet.*, **1980**, 32; 921

[3] K. Kontula, K. Aalto-Setälă, T. Kuusi, L. Hămălăinen, A. Syvănen, *Clin. Chem.*, **1990**, 36. 12, 2087

[4] Corder E. H., Saunders A. M., Risch N. J. et al., *Nature Genetics*, **1994**, 7, 180

[5] A. Borenstein, R., C.I. Copenhaver, J.A. Mortimer, *Alzheimer Dis.Assoc.Disord.*, **2006**, 20, 2006, 63

[6] A.M. Saunders, C. Hulette, K.A. WelshBohmer, D.E. Schmechel, B. Crain B, J.R. Burke, M.J. Alberts, W.J. Strittmatter, J.C.S. Breitner, C. Rosenberg, S.V. Scott, P.C. Gaskell, M.A. PericakVance, A.D. Roses, *The Lancet*, **1996**, 348, 90

[7] K. Sobin, M. Ashish, *Annu. Rev. Biomed.,* **2007**, 9, 289

[8] J. Kian-Kon, Wen-Tso Liu*, Anal. Bioanal. Chem*., **2006**, 386, 427

[9] A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.*, 2008, **108**, 109

[10] M. B. Miller, Yi-Wei Tang, Clinical Microbiology Reviews, 2009, 22, 4, 611

[11] E.M. Southern, *Electrophoresis*, **1995**, 16, 1539

[12] J. Weiler, H. Gausepohl, N. Hauser, O. N. Jensen, J. D. Hoheisel, *Nucleic Acids Res.*, **1997**, 25, 2792

[13] P.E. Nielsen, M., Egholm, R.H. Berg, O. Buchardt, *Science*, **1991**, 254, 1497

[14] O. Brandt, J. D. Hoheisel, *Trends Biotechnol.*, **2004**, 22, 617

[15] A. Germini, A. Mezzelani, F. Lesignoli, R. Corradini, R. Marchelli, R. Bordoni, C. Consolandi, G. De Bellis, *J. Agric. Food Chem*., **2004**, 52, 4535

[16] Z. C. Liu, D.S. Shin, M. Shokouhimehr, K. N. Lee, B. W. Yoo, Y. K. Kim, Y. S. Lee, *Biosens. Bioelectron*., **2007**, 22, 12, 2891

[17] F. R. Raymond, H. Hoang-Ann, R. Peytavi, L. Bissonnette, M. Boissinot, F. J. Picard, M. Leclerc and M. Bergeon, *BMC Biotechnology*, **2005**, 5, 10

[18] J. K. Pokorski, J. M. Nam, R. A. Vega, C. A. Mirkin, D. H. Appella, *Chem. Comm*, **2005**, 16, 2101

[19] J. Choi, C. Kim, H. Park, *Journal of Clinical Microbiology*, **2009**, 47, 6, 1785

[20] O. Brandt, J. Feldner, A. Stephan, *Nucleic Acids Research*, **2003**, 31, 19,1

[21] O. Brandt, J. Feldner, S. Hellweg et al., *Applied Surface Science*, **2006**, 252, 6935

[22] C. Sun, B. S Gaylord, J. W Hong, B. Liu, G. C Bazan, *Nature Protocols*, **2007**, 2, 9, 2149

[23] F. He, F. Feng, S. Wang, Trends in Biotechnology, **2007**, 26, 2, 57

[24] J. K. Pokorski, J. Nam, R. A. Vega, C. A. Mirkin, D. H. Appella, *Chem. Commun.*, **2005**, 2101

[25] R. Corradini, S. Sforza, T. Tedeschi, F. Totsingan, R. Marchelli, *Curr. Top. Med. Chem.*, **2007**, 7, 681

[26] S. Sforza, T. Tedeschi, R. Corradini, R. Marchelli, *Eur. J. Org. Chem*., **2007**, 35, 5879.

[27] A. Germini, S. Rossi, A. Zanetti, R. Corradini, C. Fogher, R. Marchelli, *J. Agric. Food Chem*., **2005**, 53, 3958

[28] S. Sforza, T. Tedeschi, R. Corradini, D. Ciavardelli, A. Dossena, R. Marchelli, *Eur. J. Org. Chem.*, **2003**, 6, 1056

[29] C. Sun, B.S. Gaylord, J. W. Hong, B.Liu, G.C. Bazan, *Nature Protocols*, **2007**, 2, 2148

[30] T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Lett.*, **2005**, 46, 8395

[31] S.A. Thomson, J.A. Josey, R. Cadilla, M.D. Gaul, C.F. Hassman, M.J. Luzzio, A.J. Pipe, K.I. Reed, D.J. Ricca, R.W. Wiethe, S.A. Noble, *Tetrahedron*, **1995**, 51, 6179

Chapter 5

Peptide Nucleic Acid microcontact printing for the fabrication of microarrays

In this chapter an advanced technique for surface derivatization is reported. Microcontact Printing is a widely used technique to pattern surfaces with micrometer features, allowing to obtain well-defined and homogeneous patterns in a shorter time than standard methods. Surface patterning with PNAs and characterization of the surface is shown in the first part. Hybridization studies with complementary and mismatch TAMRA labeled oligonucleotides is shown in the second part. Finally the system is combined with a commercial arrayer in order to create a PNA-microarray by microcontact printing, for the fabrication of arrays that are able to recognize DNA on surface with high selectivity. The work reported in this section has been carried out at the University of Twente, The Netherlands, in the group of Molecular Nanofabrication (MNF).

5.1 Introduction

The increasing requirements for methods able to selectively recognize nucleic acid sequences in a short time and for routine analysis has strongly pushed science and technology towards the development of a wide range of devices. The systems used for this purpose should ideally have a high selectivity towards complementary sequences, good sensitivity, and the possibility to perform high throughput analysis in a short time[1]. The fabrication of such devices should also be easy, less time consuming and reproducible. As previously described, the analysis by microarray-based techniques fulfills most of these requirements[2,3]. These platforms are obtained by linking the probes through covalent or non covalent interactions to a great variety of solid supports such as glass, silicon, gold, carbon etc[4]. Array techniques can exploit different properties in order to have a hybridization readout signal, and among these, glass surfaces can be used in fluorescence assays and are the most common. Although a lot of improvements have been introduced lately, it is clear that some features can still be further improved, especially in the fabrication, where the coupling with advanced printing techniques can show good performances[5].

The use of modified DNA analogues having improved performance in DNA or RNA recognition is one of the strategies used to develop better performing techniques. Among all the modification inserted in DNA structure[6], PNAs present some of the best performances[7] in terms of affinity, selectivity and chemical and enzymatic resistance, as described in previous chapters.

PNAs have been extensively applied in many devices for DNA analysis either in solution or on surface. The use of PNAs for surface-based recognition has been carried out on a wide range of materials (glass, gold, silicon), obtaining devices based on the read out of many chemical or physical signals. Among the various systems developed, some examples are quite interesting, such as sensors based on Electrochemical or electrical signal on surface or nanowires [8,9,10,11], Surface Plasmon Resonance[12,13], IR absorbance[14], Mass Spectrometry[15], Surface Enhanced Raman Scattering[16] or Fluorescence. The chemistry usually adopted for the immobilization depends on the material to which the probe should be linked. When the probe has to be linked to gold, a cysteine is usually linked at the N-terminus of the PNA in order to exploit its SH group[17,18]. Thiol groups have been used also to link probes to silicon through bifunctional molecules bearing a maleimido group able to react with the thiol and an N-hydroxysuccinimidyl group that should react with an amino or hydroxyl group linked to the

surface[19]. The immobilization on glass or silicon can be done by activating the surface through reactive monolayers such as isothiocyanate[20], N-hydroxysuccinimidyl[21,22] or aldehydic[23] groups used to link the amino-end; click chemistry has been exploited, as well, in a reaction between an acetylene-modified surface and an azido-coumarin modified-PNA[24]. Moreover coupling of PNAs to nanoparticles has been studied as well; these systems have been developed by coupling the PNA directly through its amino group to the nanoparticle by EDC mediated coupling[25], or by click chemistry[26] and their aggregation has been studied with complementary DNA sequences.

Although the combination of PNA probes with the microarray technology can show good performances, the fabrication methods are still time consuming and good performances can usually be achieved only under strictly controlled moisture and temperature conditions which are not always reproducible[24]. One way to improve the fabrication of these devices on surface, is to derivatize the surface by microcontact printing (μCP).

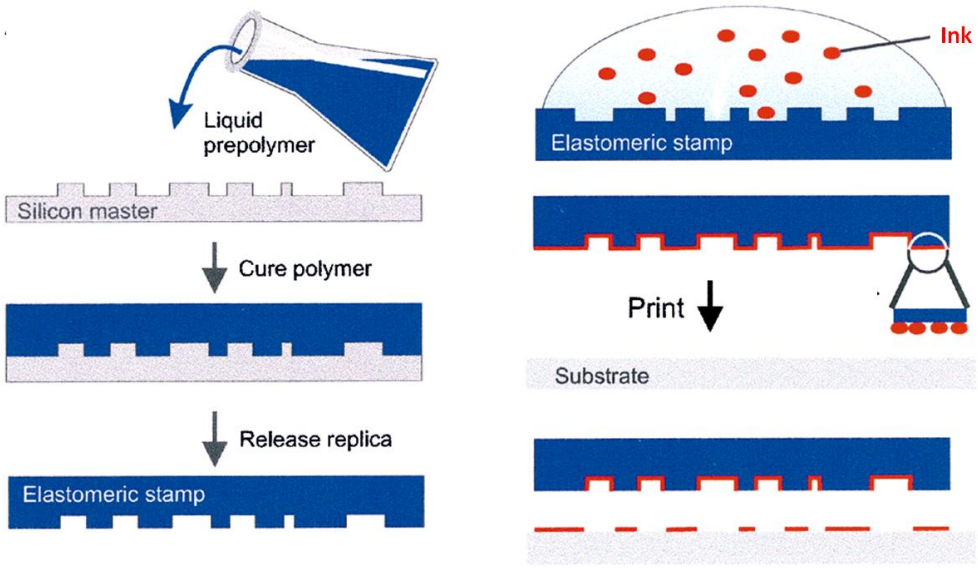


Figure 5-1: Schematic representation of microcontact printing (A. Bernard, J. P. Renault et al., Adv. Mater., 2000, 12, 14, 1069)

Microcontact printing is a well-known powerful tool to functionalize substrates with spatially patterned molecular monolayers[27]. The technique is based on the use of a stamp made of a cross-linked elastomer bearing the desired features on the surface exploited for the printing. The stamps are obtained using a silicon master containing the pattern to be printed and silanized to make it hydrophobic. The elastomer (usually polydimethtylsiloxane, PDMS) is poured on the master and the crosslinking reaction is carried out at 60°C for at least 4 hours. The solid stamp is then removed from the master and inked with a solution of the molecule to

be printed, in order to make this molecule adsorbed on the surface. The inked stamp is afterwards printed on the surface, resulting in the transfer of the molecules to the surface in a pattern exactly reproducing that of the stamp (Figure 5-1).

μCP was first used to print monolayers of alkylthiols on gold surfaces[28]. It has been shown that also silanes, lipids, proteins, DNA, synthetic polymers, nanoparticles, and even metal nanofilms can be printed by μCP upon modification of the stamps[29]. A striking feature of μCP is the short contact time necessary to form a dense monolayer of ink on the substrate. Contact times are typically around one minute, but μCP has also been performed with millisecond contact of stamp and substrate[29]. Another feature of this technique, when used to pattern surfaces, is the possibility to perform reactions that usually would not take place in standard conditions, such as formation of amide bonds by printing of amino acids over an amino monolayer[30] or the Huisgen cycloaddition of alkynes on azides monolayer ("click chemistry") without using Cu(I) as catalyst[31]. Although the general mechanism of microcontact chemistry remains uncertain, it is reasonable to assume that any reaction would benefit from a concentration effect due the micrometer confinement of the reagents. In addition, preorganisation effects on the substrate, due to alignment caused by the self assembled monolayer formation, are also probably present. Moreover, also the pressure used for stamping the surface probably can help the reaction[29].

In this work we show that the printing of chiral Arginine-PNAs (Figure 5-2)[21] on glass, quartz and silicon surfaces can be achieved by μCP. Afterwards, the surfaces and the hybridization properties of the linked PNAs are studied, demonstrating the good probe performances in terms of selectivity. Finally, we show that this strategy can be coupled with a commercial arrayer for the fabrication of PNA-microarrays, which demonstrate improved features as compared to standard arrays in terms of the time required for the reaction and the quality of the surfaces obtained. The work has been carried out in the laboratories of the group of Molecular Nanofabrication (MNF) at the University of Twente (The Netherlands), under the supervision of Dr. Pascal Jonkheijm and Prof. Jurriaan Huskens,

5.2 Results and Discussion

5.2.1 PNA Design and synthesis

The probes used for the fabrication of PNA these devices were chiral Arginine-PNAs containing an arginine side chain in position two or two and five[21] as shown in Figure 5-2.

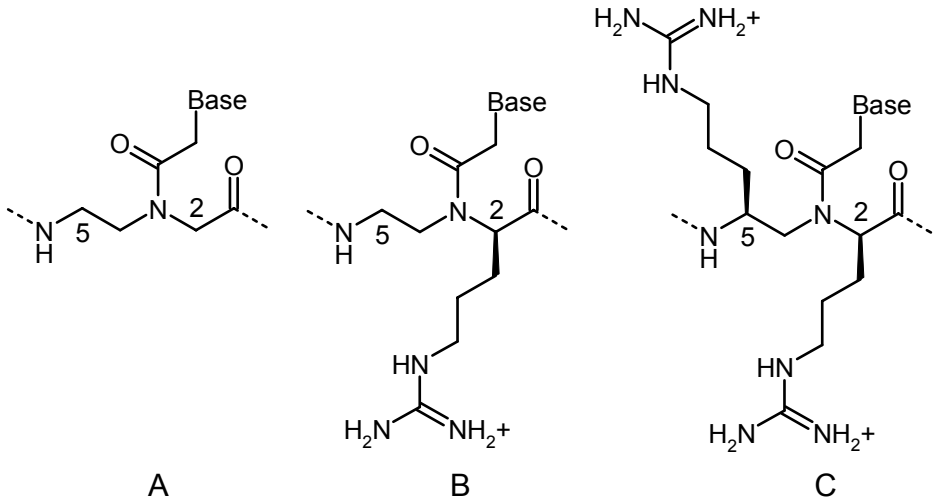


Figure 5-2: Structure of achiral (A), 2D-Arginine (B), and 2D,5L Arginine PNA monomers inserted in the probes used in this work

Chiral PNA synthesis was performed by using the submonomeric strategy as described in chapter 3: a submonomer was synthesized with a Boc group on the primary amino group and a Fmoc on the secondary amine, then it was coupled to the growing PNA chain, Fmoc group was removed, and the carboxymethyl base was linked on the resin. 2D-Arg submonomer and PNA 2 have been synthesized in a previous work[32]. The synthesis of PNAs 3, 4, 5 and 6 was reported in chapter 4. The PNA sequences used in this work are shown in Table 5-1.

Table 5-1. PNA sequences and melting temperatures of the PNA:DNA antiparallel fullmatch/mismatch duplexes in phospate buffer (pH=7) at a 5μm concentration for each strand

PNA	Sequence[a]	Tm (°C) PNA/DNA Fullmatch[b]	Tm (°C) PNA/DNA Mismatch[b]	ΔTm (°C)
1	H-(AEEA)$_2$TTACTC**T**$_{(2D\text{-}Arg)}$TTCACC-Lys(Fluorescein)-NH$_2$	48 (with **c1**)	37 (with **c2**)	11
2	H-(AEEA)$_2$TTACTC**A**$_{(2D\text{-}Arg)}$TTCACC-**N**H$_2$	55 (with **c2**)	35 (with **c1**)	20
3	H(AEEA)$_2$GCCGC**A**$_{(2D,5L\text{-}Arg)}$CACGT-NH$_2$	78 (with **c3**)	61 (with **c5**)	17
4	H-(AEEA)$_2$CAGGC**A**$_{(2D,5L\text{-}Arg)}$CTTCT-NH$_2$	69 (with **c4**)	50 (with **c6**)	19
5	H(AEEA)$_2$GCCGC**G**$_{(2D,5L\text{-}Arg)}$CACGT- NH$_2$	85 (with **c5**)	69 (with **c3**)	16
6	H-(AEEA)$_2$CAGGC**G**$_{(2D,5L\text{-}Arg)}$CTTCT-NH$_2$	75 (with **c6**)	60 (with **c4**)	15

[a] The chiral monomer corresponding to the SNP position is underlined in bold

[b] [b] Oligonucleotide sequences: **c1**: 5'-GGTGAAAGAGTAA-3', **c2**: 5'-GGTGAATGAGTAA-3' **c3**: 5'-ACGTGTGCGGC-3', **c4**: 5'-AGAAGTGCCTG-3', **c5**: 5'-ACGTGCGCGGC-3', **c6**: 5'-AGAAGCGCCTG-3'

PNA 1 was first used in order to study the patterning on the surface by a fluorescent label. For this reason, a lysine unit protected on the side chain with a Dde group was introduced at the C-end of the PNA, and, when the synthesis was complete, it was removed by hydrazine and a carboxyfluorescein molecule was coupled to the primary amino group of the lysine side chain, with the PNA still on resin. This was necessary because the N-terminal primary amino group was to be used for linking the probe to the surface. Then, the PNA was cleaved from the resin; characterization of PNA 1 is shown in figure 5-3.

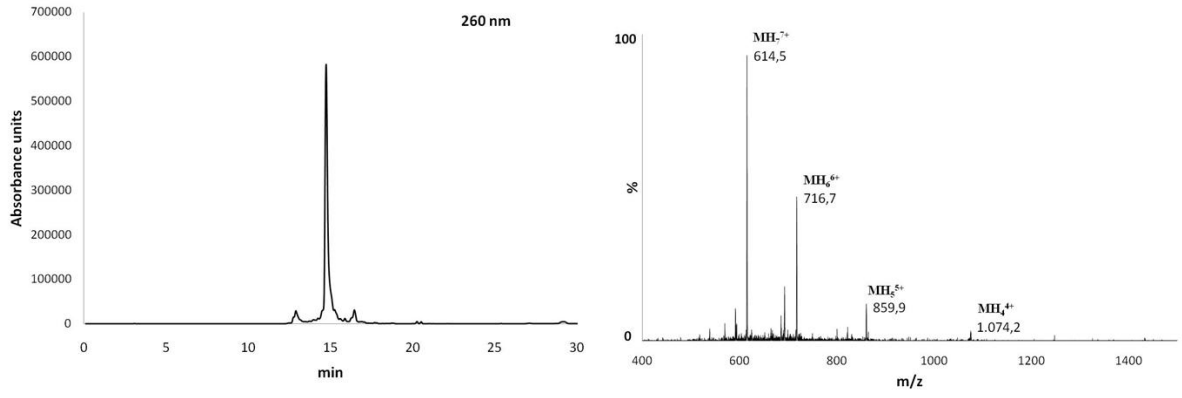


Figure 5-3: HPLC profile and mass spectrum of PNA 1

5.2.2 PNA microcontact printing

The chemical strategy adopted for the fabrication of PNA monolayers was based on the reaction between the PNA primary amino group and an aldehyde monolayer on the surface, obtaining a Schiff base that could be reduced to secondary amine by $NaBH_4$[15]. The microcontact printing strategy was used to print PNA full layers or to pattern them in well defined structures. Since the material used to make the stamp, polydimethylsyloxane (PDMS), is hydrophobic, it had, first to be modified by oxygen plasma oxidization, in order to be compatible with more polar molecules such as PNA. Then, the oxidized PDMS stamps were inked with a 10µM solution of PNA 1 (Table 5-1) in CH_3CN for 5 minutes, then dried and printed over an aldehyde-derivatized substrate for 15 min applying a pressure of 15g. The samples thus obtained were reduced with a solution of $NaBH_4$, sonicated for two minutes in a solution of sodium carbonate 0.1M and SDS 2% (pH=9) in order to remove non reacted molecules, and finally washed with a 1mM solution of sodium carbonate. The samples were imaged using a fluorescence microscope.

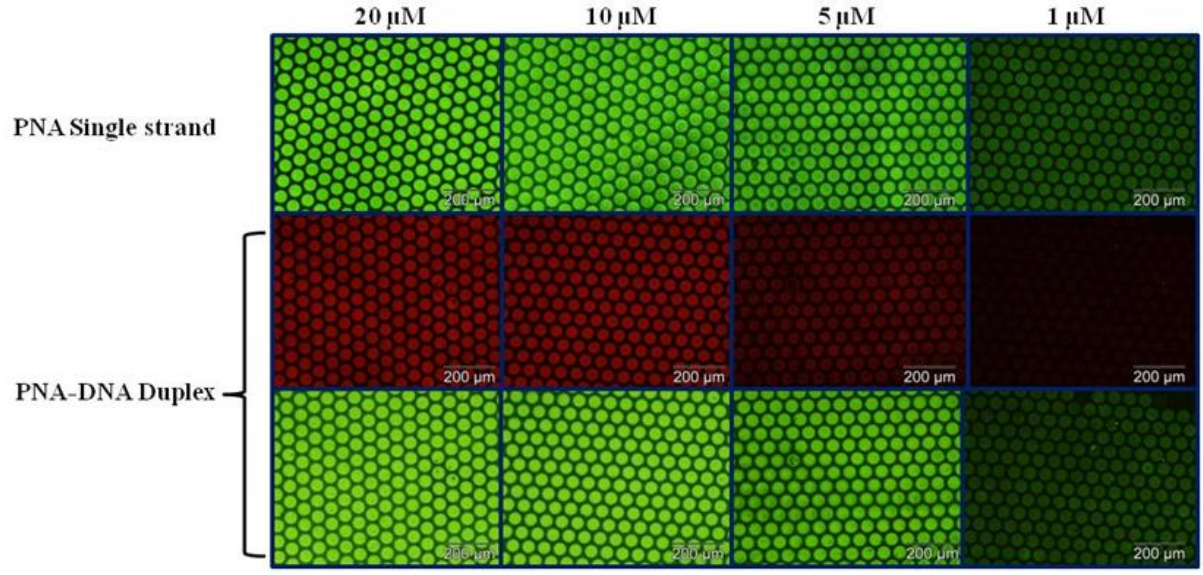


Figure 5-4: Pattern obtained printing increasing concentration of PNA 1 and hybridizing with DNA c1 labeled with TAMRA. Samples were imaged using a green filter (fluorescein) and a red one (TAMRA)

In figure 5-4 (first row) a printing test performed using different concentration of PNA is reported. The figure shows the quality of the patterning process, which allowed to obtain well defined structures with high fluorescence intensity. In order to verify the specificity of the bond between PNA and the aldehyde group of the surface, a blank experiment was performed

printing the PNA on a glass slide not derivatized with the aldehyde monolayer (Figure 5-5). The lack of any significant pattern confirmed that the patterning took place through chemical reaction and not by physisorption.

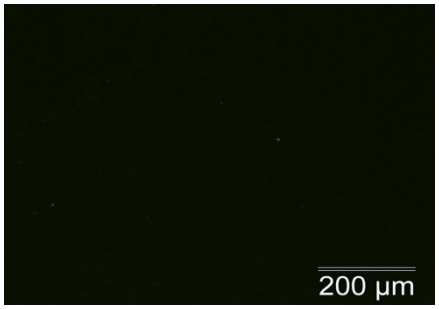


Figure 5-5: Blank sample obtained printing PNA1 on a non derivatized glass slide

The same samples were hybridized with the complementary DNA oligonucleotides labeled with TAMRA, in order to demonstrate that the probes attached to surface presented the possibility to recognize complementary DNA sequences via Watson-Crick hydrogen bonds. The hybridization was done by using a 1 µM solution of DNA in a buffer saline sodium citrate 300 mM of NaCl and 34 mM of citrate (SSC 2x), containing sodium dodecyl sulfate (SDS) 0.1%; this solution was incubated on the PNA-slide for 2h at room temperature and briefly washed with a solution of SSC 2x and SSC 0.2x at room temperature. Figure 5-4 shows the hybridization experiment with the same concentration of DNA on different concentrations of PNA used for deposition (second row). Looking at the first row, it is clear that the optimal concentration for the printing was 10 to 20 µM, because the fluorescence reached its maximum, whereas the second row, where fluorescence is read at the emission wavelength of TAMRA, demonstrates that oligonucleotides could be recognized and complexed by the PNAs on the surface. Comparing the upper row (PNA only) with last one (PNA + complementary DNA at the emission wavelength of fluorescein), it can be seen that no PNA loss occurred during the hybridization process, because the green fluorescence did not change before and after the hybridization. The increase in red fluorescence of TAMRA labeled oligonucleotide with the increase of PNA concentration suggests that higher probe density did not disturb the hybridization process.

In order to further confirm that PNA monolayers can be printed on solid surfaces, a full layer of the molecule was printed on quartz slides using flat stamps (no pattern), and UV-Vis absorption spectra were registered (Figure 5-6). The spectra of the printed slides show the typical peak at 260 nm deriving from the nucleobases absorbance, absent in non printed slides.

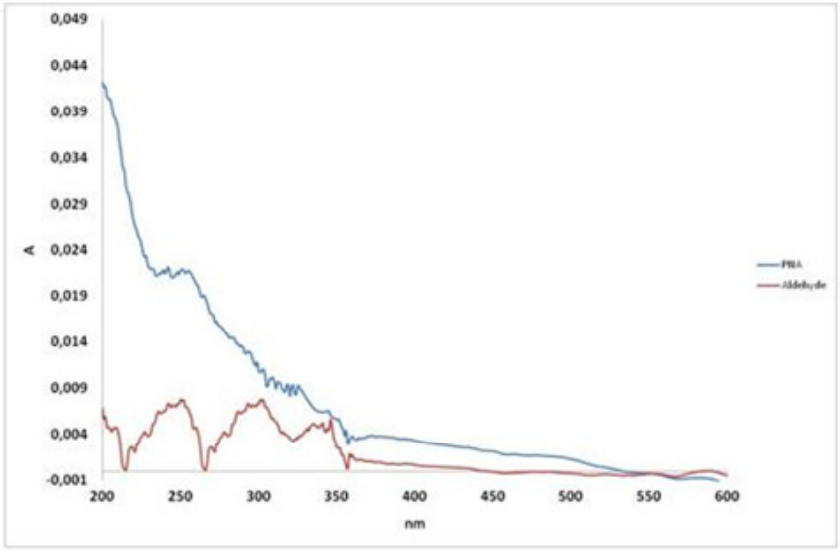


Figure 5-6: UV-vis absorption spectra of aldehyde (red) and PNA (blue)monolayers obtained derivatizing quartz slides

5.2.3 PNA-DNA binding properties on surface

The recognition properties of the PNAs linked to the surface were then throughly studied, in order to determine their affinity for the complementary DNA sequences and the specificity of the recognition. The possibility to obtain well defined patterns in the range of micrometers and to image them with microscopy was applied to study the hybridization properties of the PNA onto the surface, studying the affinity and the selectivity of the binding by evaluating the melting curve of the duplex formed with fluorescent DNA oligonucleotides on surface. Patterns of non labelled PNAs were prepared by printing PNA 2 (containing a 2D-Arginine modified monomer) on glass slides, and hybridizing it with the complementary and mismatched oligonucleotides labeled with TAMRA (DNA c2 and c1) at room temperature for 90 min, followed by a short wash with SSC 2x solution. In order to simulate the conditions in which hybridization usually takes place in solution, the slides were immersed in 1 ml solution of DNA 10 nM in SSC 2x SDS 0.1% pH=7 buffer and imaged using a laser scanner confocal microscope during a temperature increase from 20°C to 40°C. The reason for using a confocal microscope lies in the necessity to have a high resolution, which is necessary in order to separate the fluorescence contribution of the solution from that of the pattern. The pictures obtained showed a very intense and well defined fluorescent pattern at room temperature, whose intensity and definition decreased by increasing the temperature of the solution. At the end of the experiment the pattern was hardly detectable (Figure 5-7).

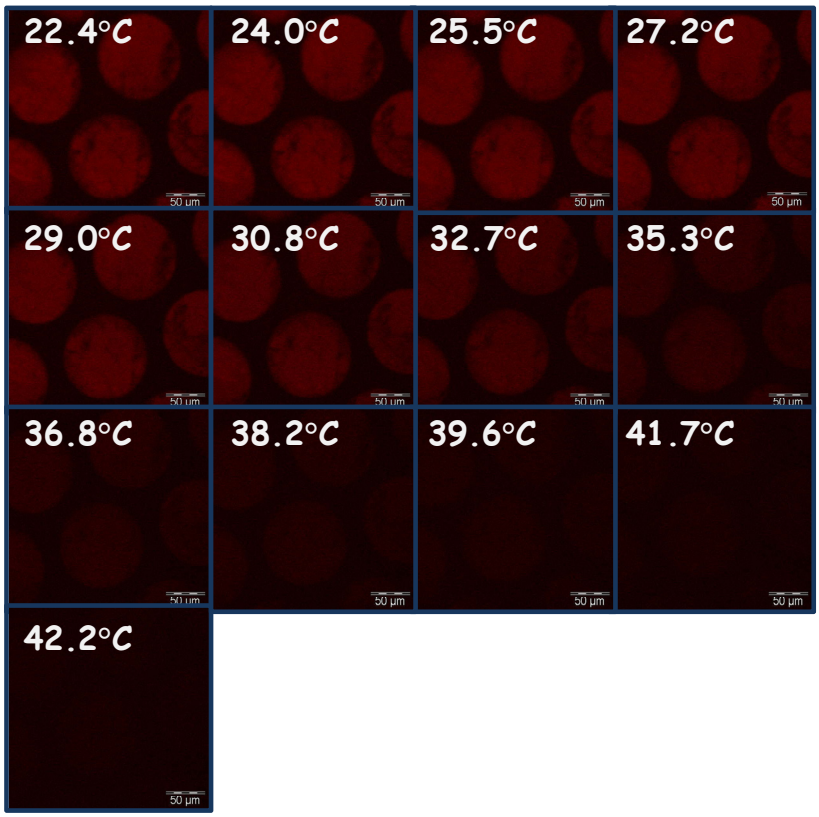


Figure 5-7: Images of fullmatch TAMRA-labeled PNA-DNA duplex at different temperatures. Images were taken using a laser scanning confocal microscope

The fluorescence intensity was measured in three different areas of the picture, and then averaged; at the same time, the same was done for the background, which was subtracted from the fluorescence intensity. These averaged and normalized values were plot against the temperature, resulting in a sigmoid curve typical of the cooperative behavior shown by PNA-DNA duplexes during their melting (Figure 5-8). The possibility to fit the points with a sigmoidal curve allowed to calculate the flex point, obtaining the melting temperatures for these hybrids directly on surface. In table 5-2 the values obtained on surface are compared with the melting temperatures in solution, either measured in PBS or in SSC 2x SDS 0.1%, order to have the same buffer used for the hybridization on surface. It is possible to notice that a significative destabilization both of the fullmatch and of the mismatch PNA-DNA duplexes in solution was caused by the presence of SDS; indeed this surfactant, being negatively charged, apparently interferes with the positively charged PNAs in DNA binding, decreasing the stability of the duplexes of about 10°C (Tm from 55°C to 45°C for the fullmatch duplex and from 35° to about 25°C for the mismatch duplex). The data obtained on the surface show that, for fullmatch PNA-DNA duplexes, a further destabilization was introduced when the binding took place between molecules in different phases, with the Tm decreasing from 45°C

to 35°C going from solution to surface recognition. The fullmatch destabilization can be explained by the interaction of the probe with the surface that may disturb the hybridization in terms of steric hindrance, since it reduces the access of the DNA in solution to the unbound PNA. The value obtained for the mismatch complex (which appear to be less destabilized) can be rationalized in terms of aspecific interaction of the labeled DNA with the surface, which cannot be detected when more stable and specific duplexes are formed, but becomes relevant when specific duplex formation is not possible. It is, anyway, clear that a temperature range exists where mismatch discrimination is possible also on the surface, as in solution.

In order to test the performance of the PNA molecules in a SNP recognition experiment, a set of chiral 2D,5L-Arg PNA (PNA 3-6) was used. These PNAs were the same previously designed and synthesized for obtaining a microarray system for the recognition of two single nucleotide polimorphysms (SNPs) related to the mutation of ApoE lipoprotein (see chapter 4)[21]. Three of these probes (PNA 3, 5, 6) were printed on the same glass slide by μCP using

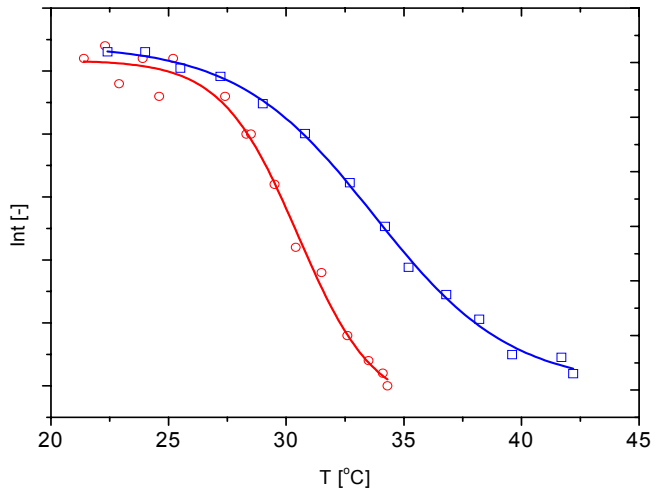


Table 2: melting temperatures of the PNA:DNA antiparallel fullmatch/mismatch duplexes of **PNA 2** in phospate buffer (pH=7) or in SSC 2x SDS o.1% at a 5μM concentration for each strand

Hybrid	**Solution** (PBS)	**Solution** (SSC 2X SDS 0.1%)	**Surface** (SSC 2X SDS 0.1%)
Fullmatch	55°C	45°C	35°C
Mismatch	35°C	~25°C	30°C

Figure 5-8: Melting curves obtained by plotting the fluorescence of the images taken in the melting experiment. Blue line corresponds to the fullmatch hybrid, while red line is obtained for the mismatch hybrid

the same method optimized with the fluorescent probe. The slides were hybridized with different combinations of TAMRA labeled DNA oligonucleotides, both fullmatched and mismatched (DNA c3 and c5). The hybridization was done using DNA solutions 1 μM in a SSC buffer and SDS 1% at 50°C for 4h. The slides were then washed with a solution of SSC 2X, SDS 0.1% for 5 minutes at 50°C and then shortly rinsed with a solution of SSC 0.2X at room temperature.

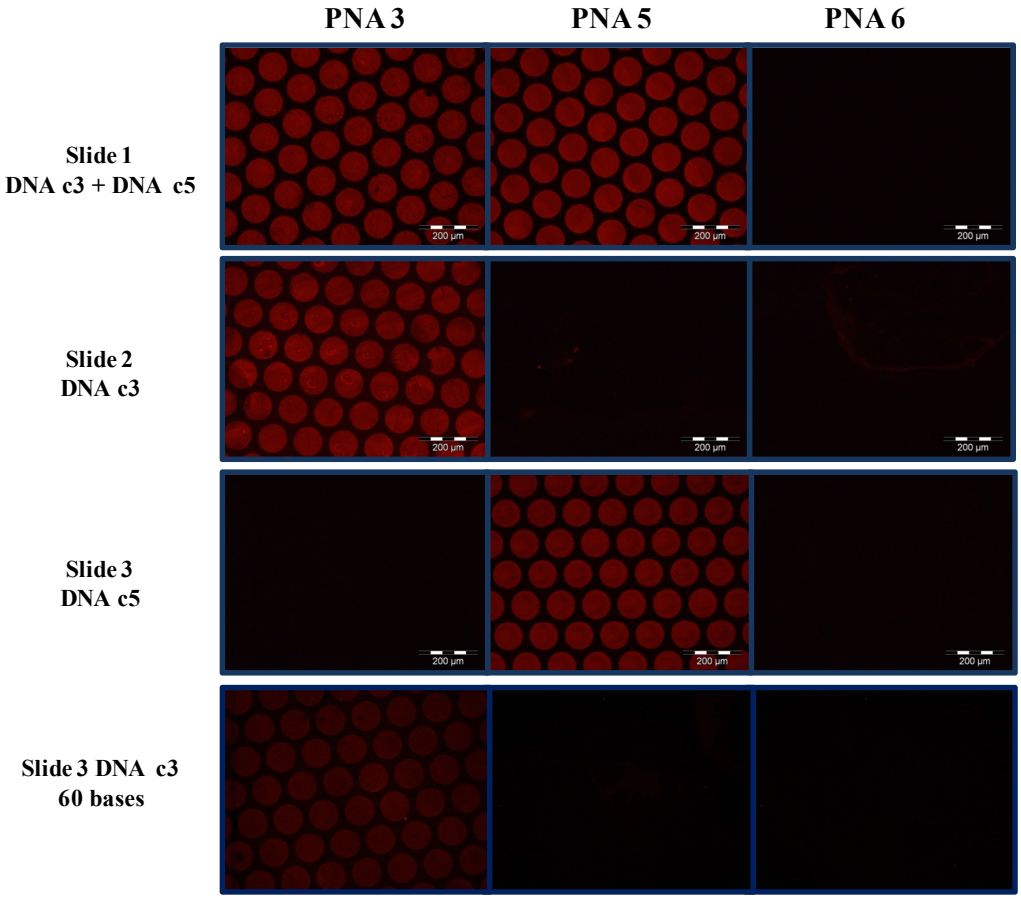


Figure 5-9: Fluorescence images of PNA slides hybridized with full-matched and mismatched fluorescent oligonucleotides

The fluorescence pictures (Figure 5-9) shows that these devices fabricated as previously reported have excellent selectivity. PNA 6 has a sequence totally unrelated to DNA c3 and c5, and never binds to any of these oligonucleotides (figure 5-9, last picture of every row): this PNA is used to demonstrate the selectivity towards different sequences. PNAs 3 and 5 recognize their complementary sequence (Figure 5-9, first row, first and second pictures; second row, first picture; third row, second picture). Moreover, the figure demonstrates that point mutations can be discriminated with a good selectivity (no signal was visible when mismatched DNA was used, Figure 5-9, second row, second picture; third row, first picture). The last row of figure 5-9 shows the images obtained upon hybridization with a long synthetic oligonucleotides (60mer) including the c1 sequence, in order to simulate a real PCR product. As it can be seen, the system recognized quite efficiently also this molecule. A signal, in fact, is produced only where the fullmatch hybrid is formed, while no recognition takes place in the case of PNA 5 which only differs for one nucleobase, demonstrating that point mutation discrimination is totally feasible with this system. However, it should be noted that in this

case the signals are weaker, and this may be due to the steric hindrance of the surface, which might be an important factor for long DNA sequences and could be more relevant if even longer sequences are used.

5.2.4 PNA Microarrays fabrication by microcontact printing

The good results obtained by derivatizing surfaces using this technique, the short time necessary to fabricate the devices, and the quality of the recognition process when PNAs were linked to surface, led us to explore the possibility to couple μCP with an existing technology in order to fabricate microarrays.

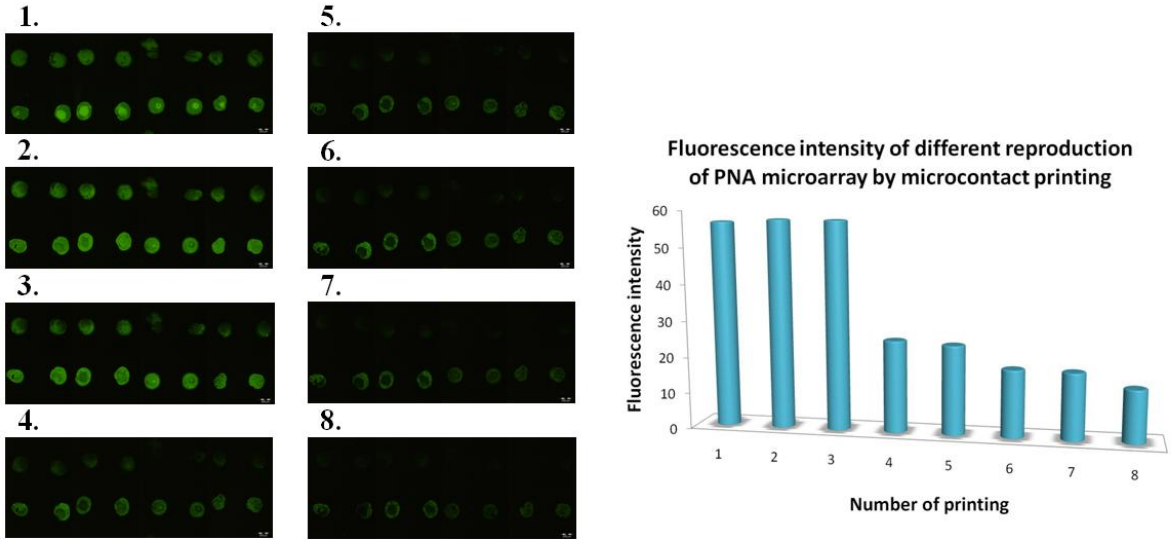


Figure 5-10: Fluorescence image (fluorescein dye) of PNA microarray fabricated via printing with a flat PDMS stamp onto which a PNA array was spotted; aside the fluorescence intensity after 8 printing is reported.

As previously reported[5], in fact, the application of microcontact printing in the fabrication of microarray devices can make the process cheaper, more reproducible and less time consuming. The system used to fabricate these devices is a commercial piezoelectric arrayer; the fabrication of the device took place producing PDMS stamps having the same size of a microarray slide without any pattern on the surface and gluing the oxidized stamp onto glass slides in order to fit in the arrayer system. The stamps so modified were used as substrate for PNA spotting. The stamp was inked with a PNA solution, dried for 15 minutes and printed over glass slides derivatized with the aldehyde monolayer using 3 weights of 15gr each. The slides were printed for 15 minutes and the same stamp was used to repeat the printing on other slides without any further modification of the PDMS. The printing was repeated eight

times. The substrates were, then, treated using the same procedure shown before and imaged using a fluorescence microscope. The array was done printing water solution having different concentrations of PNA 1 (10, 20, 50, 100 μM) and PNA 3-4-5 (1, 5, 10, 20, 50, 100 μM) to test the selectivity performance of the fabricated platform. PNA 1 was visualized using a fluorescence microscope; since the array was too big to fit in one picture, the overall image was obtained pasting together many pictures (Figure 5-10). The results show that μCP technique can improve standard microarray fabrication, since a good homogeneity between the spots and within each spot was obtained. The fluorescence intensity of the first three replicates was almost the same, while after the fourth a decrease was observed, demonstrating the good reproducibility of the process.

The possibility to apply the slides fabricated for DNA recognition assays was tested using two of them in hybridization experiments with fullmatch and mismatch oligonucleotides. TAMRA labeled DNA oligomer c3 and c5 100nM in SSC2x SDS 0.1% were used to incubate the slides at 55°C for 3 hours. The slides were then imaged using a laser scanner (Figure 5-11); the results showed the good performance of the platform in terms of selective recognition, since signals were seen only where expected.

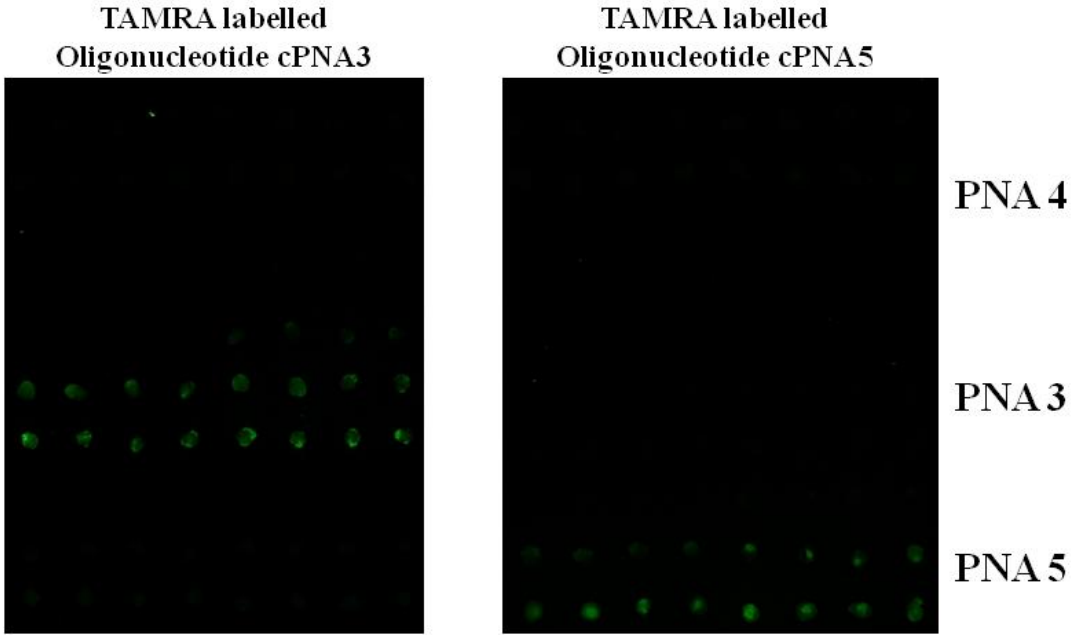


Figure 5-11: Hybridization of the devices fabricated as previously described with complementary and mismatched oligonucleotides. Image is taken using a laser scanner fluorescence system

5.3 Conclusions

In this work the possibility to use microcontact printing techniques to derivatize different kind of surfaces with PNAs is demonstrated. In particular, chiral Arg-PNAs have been tested in patterning of surfaces and imaged by a fluorescein dye inserted in the molecule. The molecules linked on surfaces have been studied in order to evaluate their recognition properties, measuring for the first time a melting temperature of a PNA-DNA duplex directly on surface. All the techniques developed in the first part of the work have been exploited for preparing PNA microarrays by microcontact printing, demonstrating several improvements in comparison with standard microarrays. Further optimization of the method may allow to develop a commercial method for preparing PNA microarrays based on microcontact printing, reducing fabrication cost, time, and improving the quality.

5.4 Experimental Section

5.4.1 Materials and reagents

PNA N-Boc protected standard monomers, N-[1H-1,2,3-(benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU), N-[(dimethylamino)-1H-1,2,3-triazol[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU), dichloromethane, N–methylpyrrolidone (NMP) and N,N-dimethylformamide (DMF) were purchased from Applied Biosystem (Foster City, CA, USA). Carboxymethyl-O-benzyl-guanine was purchased by ASM (Hannover, Germany). Boc-Arg(Tos)-OH, Boc-D-Arg(Tos)-OH and m-cresol were from Fluka (Buchs, Switzerland). (4-Methylbenhydryl)amine (MBHA) PS resin was from Novabiochem (Laufelfingen, Switzerland). Diisopropylethylamine (DIPEA), N,O-Bistrimethylsilyl acetamide (BSA), trifluoromethane sulfonic acid (TFMSA), trifluoroacetic acid (TFA), 3-hydroxy-1, 2,3-benzotriazin-4-(3h)-one (DhBtOH) and diisopropylcarbodiimide (DIC) were from Sigma-Aldrich (St. Louis, MO, USA). Oligonucleotides were purchased from Sigma or MWG eurofins. All nucleotides were purified by HPLC and modified by the manufacturer. All buffers and immobilization solutions were prepared with 18 MΩ·cm distilled water (MilliQ). The following materials and

chemicals were used as received: poly(dimethylsiloxane) (PDMS) (Dow Corning), $NaBH_4$ (Aldrich), trimethoxysilylalkylaldehyde (Fluorochem). All solvents were HPLC grade, and all other reagents were analytical grade. Other solvents or reagents were purchased from either Aldrich or Sigma.

5.4.2 PNA synthesis

The synthesis of chiral 2D-Arg-based PNAs was performed as described previously for similar PNAs[33] by using manual synthesis for the chiral residues and automated Boc-SPPS protocols on a ABI 433A synthesizer, following the procedures provided from the company, for the achiral residues. The chiral submonomers were introduced by using HBTU/DIEA coupling protocol, Fmoc group was deprotected by piperidine/NMP (20%) and carboxymethyl-Z-adenine[34] or carboxymethyl-O-benzyl-guanine (commercialy available) were linked to the submonomers on resin by using DIC/DhBtOH coupling protocol, for PNA 1 Dde group was deprotected by hydrazine/DMF 2% and carboxyfluorescein was linked by DIC/DhBtOH coupling protocol. Free PNAs were cleaved from the resin by using a 1:3 TFMSA/TFA mixture (10% thioanisole + 10% m-cresol) and precipitated by Et_2O. HPLC analysis for all PNAs was carried out by LC-MS by using an XTerra anlytical C18 column (3x250mm, 5µm, flow 0.5ml/min), gradient elution from 100% H_2O (0.2% HCOOH, eluent A) to 60% H_2O and 40% CH_3CN (0.2%HCOOH, eluent B) in 30 min. MS detector set in the positive ion mode, capillary voltage 3kV, cone voltage 30V, full scan acquisition from 150 to 1500 m/z. HPLC purification for all PNAs was carried out on a semipreparative Jupiter (Phenomenex) C18 column (10x300mm, 5µm, flow 4ml/min); eluent A: 100% H_2O (0.1% TFA), eluent B: 60% H_2O and 40% CH_3CN (0.1%TFA). Specific preparative elution conditions for every PNA are given below.

PNA 1: H-$(AEEA)_2$TTACTC**T$_{(2D\text{-}Arg)}$**TTCACC-Lys(Fluorescein)-NH_2. The synthesis was performed on 25mg of a preloaded Fmoc-Lys(Dde)-MBHA-PS resin (loading 0.2mmol/g). Crude yield: 70%. The crude products were purified by semipreparative RP-HPLC, in gradient elution: from 100% A(H_2O + TFA 0.1%) to 100% B(CH_3CN + TFA 0.1%) in 30 min ESI-MS: calcd m/z: 1075.1 (MH_4^{4+}), 860.6 (MH_5^{5+}), 717.3 (MH_6^{6+}), 615.0 (MH_7^{7+}) found m/z 1074.2, 859.9, 716.7, 614.5**. PNA 2** H-$(AEEA)_2$TTACTC**A$_{(2D\text{-}Arg)}$**TTCACC-NH_2. Crude yield: 88%. The crude product was purified by RP-HPLC in gradient elution: from 100% A to

100%B in 30 minutes. ESI-MS calcd m/z: 956.0 (MH_4^{4+}), 764.9 (MH_5^{5+}), 637.6 (MH_6^{6+}), 546.7 (MH_7^{7+}) found m/z: 955.8, 764.8, 637.8, 546.7.
The synthesis and characterization of chiral (2D,5L)-Arg submonomer chiral (2D,5L)-Arg-based PNAs (**PNA 3 to 6**) were described in chapter 4.

5.4.3 Melting temperature measurements

Stock solutions of PNA **1**, **2**, **3**, **4** and of oligonucleotides were prepared in doubly distilled water and their actual concentration calculated by UV absorbance using the following ε260 (M^{-1} cm^{-1}) for the nucleobases: T 8600, C 6600, A 13700, G 11700. By using these concentrations, hybrid solutions containing 5μM PNA-DNA duplexes were prepared. All the hybridisation experiments were carried out in 10mM phosphate buffer, 100mM NaCl, 0.1mM EDTA, pH= 7. All the hybrid samples reported were first incubated at 90°C for 5 minutes, then slowly cooled to room temperature. The samples were heated (1°C/min) and the UV signal variation at 260nm was recorded. Melting temperatures were measured as the maximum of the first derivatives of the melting curves.

5.4.4 Modification of substrate.

Clean microscope cover glass (Paul Marienfeld Gmbh & Co. KG, Germany), quartz slides were activated with piranha solution for 30 min (concentrated H_2SO_4 and 33% aqueous H_2O_2 in a 3:1 ratio) (Warning! Piranha solution should be handled with caution: it has been reported to detonate unexpectedly!), rinsed with water (MilliQ), and put under vacuum with 0.1 ml of trimethoxysilylalkylaldehyde overnight; the substrates were rinsed with toluene to remove any excess of silanes and subsequently dried in N_2.

5.4.5 Fabrication of Stamps.

Silicon wafer-based masters with etched structures were prepared by UV photolithography. The master surface was fluorinated using fluorosilanes. PDMS stamps were fabricated by curing Sylgard 184 on the surface of the master at 60°C overnight.

5.4.6 Microcontact Printing of PNA.

PDMS stamps were first oxidized in UV/plasma reactor (Ultra-Violet Products, modelPR-100) for 30 min at a distance of about 2 cm from the plasma source. This reactor contains a low-pressure mercury UV light operating with UV emissions at 185 nm (1.5 mW cm^{-2}) and 254 nm (15 mW cm^{-2}). The stamps thus obtained were stored in MilliQ water. Subsequently the hydrophilic stamps were dried with nitrogen and incubated with a drop of 10 μM PNA solution in acetonitrile for 5 minutes at room temperature. The stamp was dried with nitrogen and brought into conformal contact with the aldehyde-terminated glass slide for 15 min using a 15 gr weight. After printing the glass slides were immersed in a solution containing 100 mg $NaBH_4$ in 40 mL PBS with 10 mL EtOH for 5 min. After reaction time the substrate was rinsed in MilliQ water, sonicated in a carbonate buffer 0.1 M pH=9 with SDS 2% for 2 minutes, rinsed in a 1 mM carbonate buffer and dried with nitrogen.

5.4.7 Hybridization on the Substrate Surface.

For hybridization oligonucleotides were diluted to 1 μM in 4X SSC containing 0.1% SDS and applied to the surface of the modified glass using disposable hybridization chamber (Gene Frame, Thermo Scientific, UK). The temperature was tuned depending on the affinity of the probe for the complementary sequence, and the time was 4 hours. Non hybridized molecules were removed washing with SSC 2X, SDS 0.1% at hybridization temperature for 5 min. and with SSC 0.2X at room temperature for 1 min. After washing, the glass slides were dried with nitrogen

5.4.8 Fluorescence Microscopy

Fluorescence microscope images were taken using an Olympus inverted research microscope IX71equipped with a mercury burner U-RFL-T as light source and a digital Olympus DR70 camera. For image acquisition blue excitation (450nm $\leq \lambda ex \leq$ 480 nm), green emission ($\lambda em \geq$ 515nm), green excitation (510nm $\leq \lambda ex \leq$550nm) and red emission light ($\lambda em \geq$ 590nm) were filtered using a U-MWB Olympus filter cube. All fluorescence microscopy images were acquired in air and analyzed on a fluorescence microscope (Olympus inverted research microscope IX71).

5.4.9 Surface Melting experiments.

The samples for the melting experiments were prepared printing the PNA, hybridizing it with a 1 μM solution of TAMRA labeled oligonucleotide and washing with SSC 2X at room temperature. The samples, thus prepared were immersed in a 10 nM solution of the same oligonucleotide in SSC 2X SDS 0.1% buffer and imaged using a laser scanning confocal microscope during temperature increase the temperature.

5.4.10 Laser scanning confocal microscopy

Confocal microscopy images of the microcontact-printed substrates were taken on a Carl Zeiss LSM 510 microscope. The light was focused on the substrate using a 40X LD Acroplan objective. TAMRA derivatised features were excited at 543 nm, using a Helium-Neon laser. The emitted fluorescence was collected on. The images were acquired in a 10nM solution of TAMRA labeled oligonucleotides in a SSC (2x) SDS (0.1 %) buffer pH= 7. Temperature was regulated using a Temperature Controller TC202A Harvard Apparatus with a heating stage. Real temperature was checked using a thermocouple dipped in the solution. Fluorescence intensity was calculated in three different areas of the image and averaged; the value was normalized subtracting the background intensity calculated in the same way.

5.4.11 Contact Printing by Robotic Systems.

The spots of oligonucleotides were fabricated using Scienion S3 spotter (Scienion AG, Berlin, GER). Spots of 350 pL were dispensed using the piezo non-contact printing system in a 16x8 array. Flat, 2-3 mm thick stamps were glued to a glass microscope glass, oxidized and used as substrates for PNAs spotting. The PNAs used for this fabrication were prepared in water at concentration from 1 to 50 μM. After 15 minutes from the spotting, the stamps were used for multiple microcontact printing. Samples were hybridized as described before and scanned using a fluorescence scanner.

5.5 References

[1] A. L. Beaudet, J. W. Belmont, *Annu. Rev. Med.,* **2008**, 59, 113

[2] J. Kian-Kon, Wen-Tso Liu, *Anal. Bioanal. Chem.*, **2006**, 386, 427

[3] A. Sassolas, B. D. Leca-Bouvier, L. J. Blum, *Chem. Rev.*, **2008**, 108

[4] F. Lucarelli, S. Tombelli, M. Minunni, G. Marazza, M. Mascini, *Analytica Chinica Acta,* **2008**, 609, 139

[5] D. I. Rozkiewicz, W. Brugman, R. M. Kerkhoven, B. J. Ravoo, and D. N. Reinhoudt, *J. Am. Chem. Soc.*,**2007**,129, 11593

[6] S. Karkare, D. Bhatnagar, *Appl. Microbiol. Biotechnol.,* **2006**, 71, 575

[7] P.E. Nielsen, M., Egholm, R.H. Berg, O. Buchardt *Science* **1991**, 254, 1497

[8] A. Cattani-Scholtz, D. Pedone et al., *ACS Nano* **2008**, 2, 8, 1653

[9] G. Zhang, J. H. Chua, R. Chee, A. Agarwal, S. M. Wong, *Biosensors and Bioelectronics,* **2009**, 24, 2504

[10] A X. Luo, T. Ming-Hung Lee, and I-M. Hsing, *Anal. Chem.,* **2008**, 80, 7341

[11] Y. Fan, X. Chen, J. Kong, C. Tung, and Z. Gao, *Angew. Chem. Int. Ed.*,**2007**,46, 2051

[12] R. D'Agata, R. Corradini, G. Grasso, R. Marchelli and G. Spoto, *ChemBioChem,* **2008**, 9, 2067

[13] J. Liu, L. Tiefenauer, S. Tian, P. E. Nielsen, W. Knoll, *Anal. Chem.*, **2006**, 78, 470

[14] E. Mateo-Martì, C. Briones, C. M. Pradier, and J. A. Martín-Gago, *Biosensors and Bioelectronics,* **2007**, 22, 1926

[15] H.F. Arlinghaus, M. Schroder, J.C. Feldner, O. Brandt, J.D. Hoheisel, D. Lipinsky, H.F. Arlinghaus, *Applied Surface Science*, **2004** 231–232 392

[16] C. Fang, A. Agarwal, K. D. Buddharaju et al., *Biosensors and Bioelectronics,* **2008**, 24, 216

[17] E. Mateo-Martì, C. Briones, E. Román, E. Briand, C. M. Pradier, and J. A. Martín-Gago, *Langmuir,* **2005**, 21, 9510

[18] A. Paul, S. Bezer, R. Venkatramani et al., *J. Am. Chem. Soc.*, **2009**, 131, 6498

[19] A. Cattani-Scholtz, D. Pedone et al., *Biomacromolecules,* **2009**, 10, 489

[20] C. Sun, B. S. Gaylord, J. W. Hong, B. Liu, G. C. Bazan, *Nature Protocols*, **2007**, 2, 2148

[21] A. Calabretta, T. Tedeschi, G. Di Cola, R. Corradini, S. Sforza, and R. Marchelli, *Molecular Biosystems,* **2009**, 5, 1323

[22] Afanassiev, V.; Hanemann, V.; Wölfl, S. *Nucleic Acids Res*. **2000**, 28, e66

[23] C. Rogero, B. T. Chaffey et al., *J. Phys. Chem. C.,* **2008**, 112, 9308

[24] S. Yoon Lim, W. Chung, H. Kyu Lee, M. Soon Park, H. Gyu Park, *Biochemical and Biophysical Research Communication,* **2008**, 376, 633

[25] J. L. Turner, M. L. Becker, X. Li et al., *Soft Matter.*, **2005**, 1, 69

[26] G. Prencipe, S. Maiorana, P. Verderio, M. Colombo, P. Fermo, E. Caneva, D. Prosperi, E. Licandro, *Chem. Comm*., **2009**, 6017

[27] A. Perl, D. N. Reinhoudt, J. Huskens, *Advanced Materials,* **2009**, 21, 2257.

[28] Y. Xia, G. M. Whitesides, *Angew. Chem. Int. Ed.*, **1998**, 37, 550

[29] B. J. Ravoo, *J. Mater. Chem.*, **2009**, 19, 8902

[30] T. P. Sullivan, M. L. van Poll, P. Y. W. Dankers, W. T. S. Huck, *Angew. Chem. Int. Ed*., **2004**, 43, 4190

[31] D. I. Rozkiewicz, D. Jancewski, W. Verboom, B. J. Ravoo, D. N. Reinhoudt, *Angew. Chem. Int. Ed.*, **2006**, 45, 5292.

[32] A. Calabretta, *Master Thesis*, **2006**

[33] T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Lett.*, **2005**, 46, 8395

[34] S.A. Thomson, J.A. Josey, R. Cadilla, M.D. Gaul, C.F. Hassman, M.J. Luzzio, A.J. Pipe, K.I. Reed, D.J. Ricca, R.W. Wiethe, S.A. Noble, *Tetrahedron*, **1995**, 51, 6179

Chapter 6

The double nature of Peptide Nucleic Acids: design and synthesis of a PNA embedding a Nuclear Localization Signal pseudopeptide sequence in the backbone able to behave as a peptide mimic

In this chapter it is shown how the introduction of amino acid-derived functional groups within the backbone of a PNA can introduce new properties, affecting not only the recognition and discrimination of complementary nucleic acid sequences, but also giving the PNA peptide-like properties, enabling them to behave as nucleic acids and peptides at the same time. The design and synthesis of a short PNA model embedding in the backbone the amino acid side chains which mimics a Nuclear Localization Signal peptide will be described. Its ability to interact with proteins involved in the cell nuclear uptake, exactly like the natural peptide, will be demonstrated by nuclear internalization experiments.

6.1 Introduction

The use of PNAs in diagnostic applications has been presented in the previous chapters. Although PNAs are useful tools in diagnostics, this is not the only field in which these molecules can be usefully applied. As reported in the first chapter, application of PNAs in gene therapy is a very hot topic, since they have features suited to most requirements for selectively targeting and blocking a DNA or RNA sequence[1]. Enzymatic and chemical resistance, in fact, make these molecules suitable for in-vivo applications, since these features increase their life in a biological system[2], whereas selectivity and affinity for the complementary sequences allow specific and strong binding of the sequences of interest. Nevertheless, some drawbacks still limits their extensive application; the low solubility due to the apolar structure is a problem, since it limits the concentrations to be used in therapy, and also cellular and nuclear uptake[3] might be a limiting factor, since, in order to have a significant effect, these molecules have to selectively concentrate in the cell region where the targeting is required (the cytoplasm if the target is RNA, the nucleus when the target is DNA). Although it has been demonstrated that, in some cases, unmodified PNAs are able to enter some types of cell without any modification or transfection approach[4], usually there is the necessity to develop a strategy aimed at increasing cellular and/or nuclear uptake of these molecules. Some approaches involved the techniques developed for DNA uptake[4], such as electroporation[5] or co-transfection with lyposomes[6]. Nevertheless, the necessity to develop more general and efficient delivery methods led to the design of modified PNAs for enhanced uptake. The most exploited strategy has been the conjugation of PNAs with molecules able to cross cellular or nuclear membrane, thus carrying the linked probe; a great variety of moieties have been linked to PNAs for this purpose. The conjugation of PNAs to terpyridine units promoted the cellular and nuclear delivery of such molecules. The possibility for terpyridine to chelate the zinc ion, turned out in a preferential uptake by zinc-rich tissues[7]. The synthesis of a PNA linked to a neamine unit allowed to obtain a molecule able to cross the cellular membrane and hydrolyze RNA within the cytoplasm[8]. Lipophilic units such as adamantane or triphenylphosphonium cation have been bound as well to PNAs in order to improve the uptake, but this approach showed always good results in terms of uptake and targeting efficience only in some cases[4,9]. Other groups linked to PNAs aimed at interacting with specific cell membrane receptors. For example, PNAs linked to a lactose unit were selectively internalized into hepatic cells that possessed a receptor for this sugar[10]. In another experiment,

the coupling of PNAs with dihydrotestosterone caused the selective uptake of such molecules by the cells having the corresponding receptor[11].

The most explored strategy, both receptor-based and non receptor-based, to modify PNAs for cell delivery is the conjugation with a peptide able to cross the cellular membrane and/or to selectively carry the molecules inside the nucleus. The main reason for selecting this modification as the strategy of choice, beside its efficiency, is undoubtedly the easiness of obtaining peptide-PNA conjugates, given the identical chemistry used for their synthesis. An example is the linking of PNAs to trojan peptides, a class of amphiphilic cationic/hydrophobic peptides, able to transport several classes of molecules (oligonucleotides, peptides, etc.) across biological membranes in a receptor independent way[4]. The conjugation of PNAs to positively charged peptides such as spermine was studied in order to improve the solubility of the molecule and the stability of the duplex with the complementary DNA[12], obtaining an enhanced cellular uptake. The development of PNAs conjugated to cell penetrating peptides was carried out using peptides rich in positively charged amino acids, able to promote uptake through receptor-mediated active processes. One of firstly developed PNA-peptide conjugate was obtained by using penetratin, a 16-residue peptide derived from the Drosophila homeodomain transcription factor[13]. Also arginine-rich peptides derived from the Tat sequence were coupled to PNAs, allowing a good cellular and nuclear uptake[14,15]. Many other classes of peptides have been studied in order to promote PNA uptake and to trigger enhanced endosomal release in the cytoplasm, where further nuclear uptake may take place[15]. The active uptake of PNAs conjugated to cell penetrating peptides was also furtherly enhanced by linking a fatty acid[16] or a phospholipid[17] unit to a PNA-peptide conjugate, thus improving the uptake due to the possibility to form micelles. Similar cooperative effects have been exploited by using PNA-peptide conjugates able to form dendrimers, upon supramolecular interactions, that enhanced their cellular uptake[18]. PNA-peptide conjugates have also been labeled in order to study the uptake mechanism, usually by linking a fluorophore, in order to exploit fluorescence microscopy, but also , introducing a 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) group, able to coordinate a Gadolinium atom, in order to study the interaction with the cell by Magnetic Resonance Imaging (MRI)[19].

Among all the peptides used as cytoplasmatic and/or nuclear carriers, one of the most exploited is the so-called Nuclear Localization Signal peptide (NLS), that showed a high efficiency in the transport of macromolecules inside the nucleus. This peptide consists of seven amino acids (PKKKRKV), a hydrophylic sequence derived from the simian virus 40 large T-antigen, able to carry macromolecules such as proteins, plasmidic DNA and PNA within the nucleus of a cell[20]. Its conjugation with PNAs and the application for gene regulation has been deeply investigated and exploited [20]. Deeper studies into this field allowed to define at least five classes of NLS, differing in sequences and lengths, but having in common the presence of one or two basic amino acid core within the sequence[21]. These molecules are able to penetrate the nucleus, together with the linked macromolecules, through binding to specific receptors, known as importins (karyopherins). Nuclear uptake is, in fact, mediated by specific gates (nuclear pores) on the nuclear membrane, where specific receptors selectively recognize peptide-importin complexes and allow the passage through the pore. The best understood pathway of nucleocytoplasmic transport is the nuclear import pathway represented in Figure 6-1. Importin α recognizes and binds the NLS peptide in the cytoplasm, linking it to the importin β. Importin β then mediates the interaction of the trimeric complex (NLS-Importin α- Importin β) with the nuclear pore, which translocates it into the nucleus. Once the complex has reached the inner part of the nucleus, it is dissociated by Ran GTPase-activating protein (RanGTP). Binding of RanGTP to importin causes a conformational change that results in the release of the importin α-NLS complex[22]. A subsequent interaction with other factors determines the final release of the NLS peptide, with the eventual cargo molecule linked to it. An asymmetric distribution of Ran-GTP and Ran-GDP between the nucleus and the cytoplasm controls the import and export, and this gradient is maintained by various Ran associated regulatory factors[23]. Antigene strategies often made use of NLS-labeled molecules, like PNAs, allowing their nuclear uptake and improving the system efficiency[20,24].

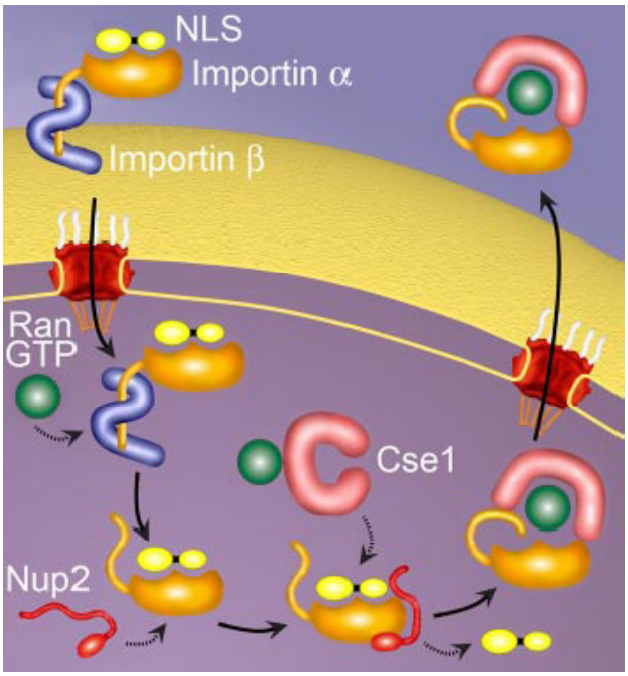


Figure 6-1: The NLS-based nuclear import cycle. (A. Lange et al., *Journal of Biological Chemistry*, 2007,282, 8, 5101)

6.2 Results and discussion

6.2.1 PNA design and synthesis

Recently, it has been demonstrated that PNAs bearing *ad hoc* modifications within the backbone have enhanced nuclear uptake properties, comparable to those linked to carrier peptides. In particular, chiral PNAs having arginine-modified monomers were demonstrated to concentrate in the nucleus upon uptake experiments (like poly arginine peptides)[25,26] due to the positive charges of the arginine side chains.

These crude preliminary experiments demonstrate that backbone derivatization of PNAs can be exploited in order to create more complex systems, characterized by new properties typical of aminoacidic sequences. In fact, a particularly fascinating, but seldom explored, aspect of PNAs, is their "double nature": PNAs are not only able to recognize through the nucleobases a complementary DNA sequence (a property typical of nucleic acids), but they also possess a pseudopeptidic backbone and therefore might potentially display properties similar to those performed by proteins. The backbone can be, indeed, considered as a long sequence of pseudoglycylglycine dipeptides, therefore a mimic of a polyGly protein. In order to express all the "proteic potential" of PNAs, the backbone should be modified in order to become a real peptide mimic, by inserting amino acid-derived side chains in positions 2 and 5 (Figure 6-2). In this way, a real "peptide" and "nucleic acid" could be obtained, fully exploiting the intrinsinc double nature of the molecule.

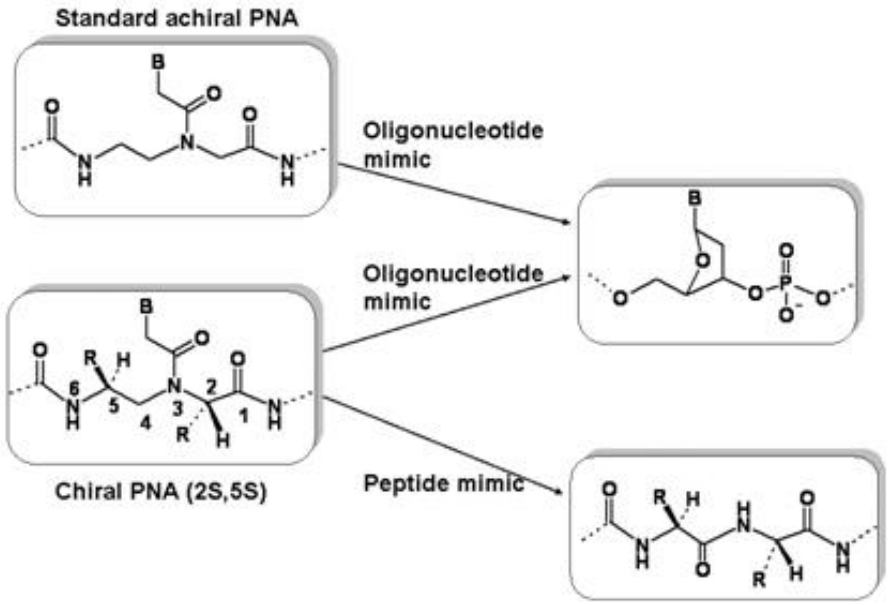


Figure 6-2: A standard PNA compared with a 2S,5S-modified PNA: both are able to perform DNA recognition, but the backbone of the latter has also the potential to mimic a peptide sequence

Although the introduction of substituents in the PNA backbone have been used in order to introduce functional groups and charges (thus modulating the properties of PNA)[27,28,29], the use of side chains mimicking an entire complex peptide consensus sequence with a specific biological function has never been reported, and represents a challenging task both conceptually and synthetically. The possibility to develop a PNA able to act as a mimic of NLS peptide, would allow the PNA to enter a cell, recognize importin α protein, enter the nucleus, and perform its antigene activity without the need to couple the molecule to any cell penetrating peptide. In this chapter the design and the synthesis of a modified model PNA embedding in its backbone a mimic of a Nuclear Localization Signal (NLS) is reported, together with preliminary studies on its ability to penetrate cell nuclei.

As reported in the introduction (chapter 1), the correct configurations for having right-handed PNAs, thus able to efficiently bind complmentary DNA, in 2,5 substituted PNAs have been found to be 2D,5L and 2L,5L. Thus, a backbone including L-amino acid-derived side chains at positions 2 and 5 (Figure 6-2) has the potential to be a good mimic of natural peptides, formed by all L-amino acids, at the same time preserving the correct handedness for DNA binding.

The sequence chosen to be embedded in the PNA backbone was the NLS peptide PKKKRKV. The structure of the NLS peptide (**1**) chosen to be embedded in the PNA backbone is represented in Figure 6-3. A model PNA consisting in only three residues was chosen for embedding the peptidic structure (**2**, Figure 6-3). The fusion of the two structures led us to design the modified PNA **3** (Figure 6-3), embedding side chains derived from natural L-amino acids. The proline residue was linked as aminoacidic residue at the N-terminus, since, bearing a secondary amine, could not be included in a PNA structure in that particular position. The modified PNA **4**, lacking the terminal proline, was also synthesized to be used as control. All compounds were synthesized with a rhodamine linked at the N-terminus, with an aminoethoxyethoxyacetyl (AEEA) spacer in between, in order to be visualized in the cell penetration studies, and with a glycine residue at the C-terminus, on account of synthesis on solid phase.

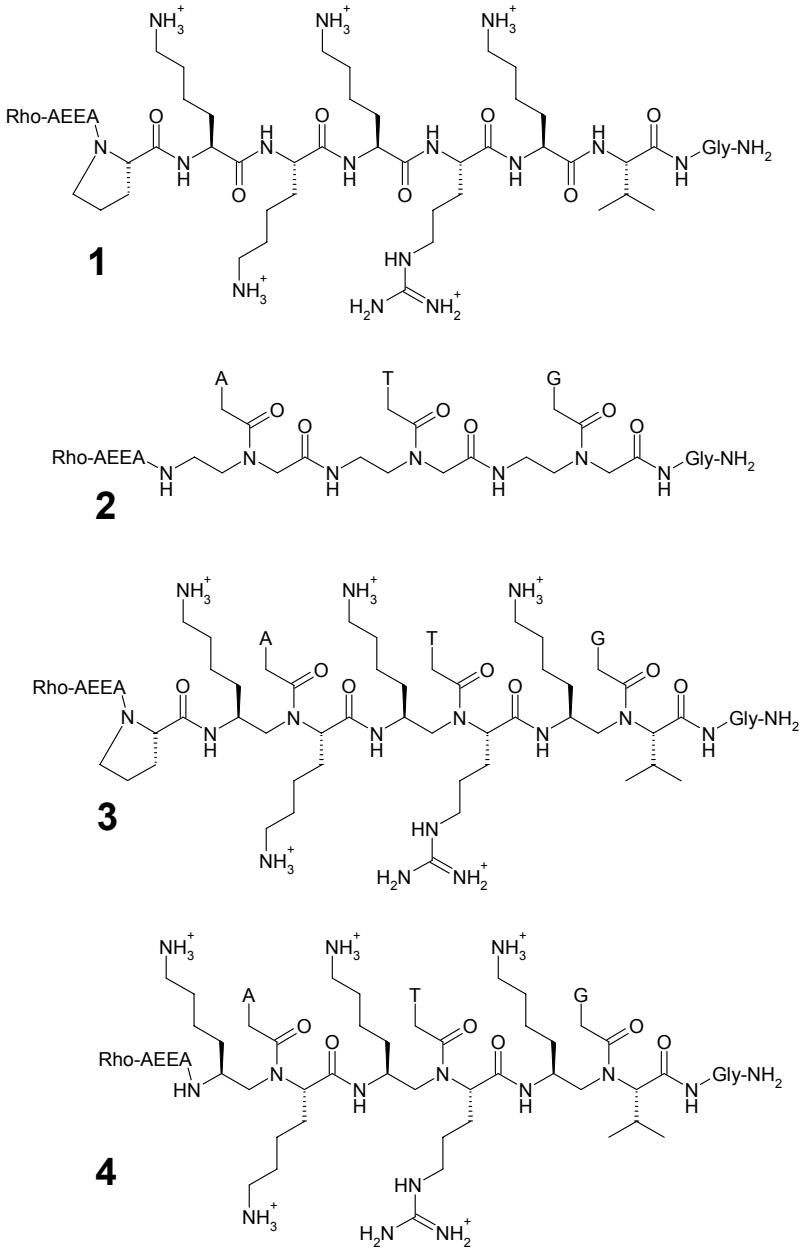


Figure 6-3: The NLS peptide PKKKRKV (1), a standard PNA trimer ATG (2), the modified PNA trimer ATG embedding the NLS sequence (3), the modified PNA trimer ATG embedding the NLS sequence without proline (4). A = adenine, T = thymine, G = guanine, Gly = glycine, Rho = Rhodamine, AEEA = 2-aminoethoxyethoxyacetyl spacer.

The syntheses of the NLS peptide **1** and of the standard PNA trimer **2** were performed on solid phase by following standard Fmoc protocols. After cleavage from the resins, the NLS

peptide **1** and the PNA trimer **2** were purified by RP-HPLC and characterized by ESI-mass spectrometry.

A Boc submonomer strategy was used for the modified PNA **3**, in order to better preserve the optical purity at the 2 position[30]. The submonomers were prepared from the corresponding commercial Boc-L-amino acids suitably protected on the side chains. The Boc-L-Lysine-(2-Cl-Z)-OH, to be used as synthon for inserting the lysine side chains in positions 5, was transformed in the corresponding Weinreb amide and then reduced to the aldehyde. Boc-L-Lys-(2-Cl-Z)-OH, Boc-L-Arg-(Tos)-OH and Boc-L-Val were transformed in the corrsponding methyl esters and Boc deprotected by using HCl/MeOH. The PNA backbones were then synthesized by reductive amination on L-amino acid methyl esters with the Boc-L-Lys-(2-Cl-Z) aldehyde. The methyl esters were hydrolyzed and the secondary amines were protected by the introduction of a Fmoc group. All the compounds were characterized by ^{1}H and ^{13}C NMR and ESI mass spectrometry.

According to the previously reported procedures30, the chiral submonomers units were inserted by manual coupling with the HATU/DIEA protocol on a MBHA-PS resin and, after Fmoc deprotection, the nucleobase residues were introduced by a double coupling with DIC/DhBTOH. After the cleavage from the resin, the PNA trimer **3** was purified by RP-HPLC and characterized by ESI-mass spectrometry (Figure 6-4).

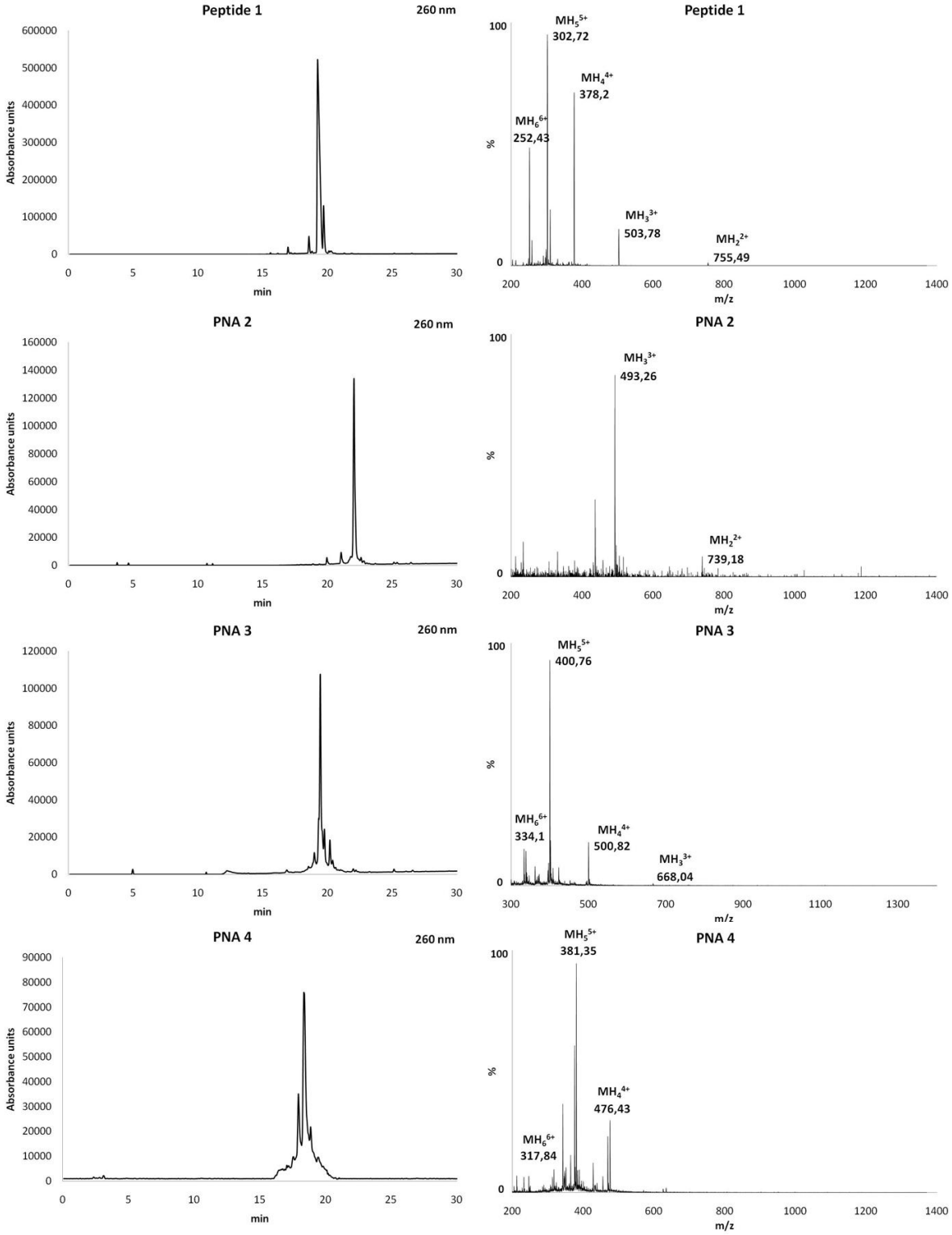


Figure 6-4: HPLC profiles and mass spectra of the synthesized PNAs

6.2.2 Uptake experiments

The ability of the different compounds to be internalized in the cell nuclei was then assessed in vivo: RH30 cells (a rabdomiosarcoma line) were incubated with 10 μmol/L of peptide **1**, standard PNA **2** or modified PNAs **3** and **4** and analyzed after 3 hours. Fluorescence microscopy images showed that after 3 hours in the RH30 cell line, high intracellular levels with main nuclear localization could be observed for the NLS peptide **1** (Figure 6-5), as expected. The unmodified PNA **2**, on the other hand, does not show any nuclear internalization, and a very low cellular uptake, confirming the lack of recognition from cargo proteins, as reported in previous data[24]. Incubation of cells with PNA **3**, instead, showed a very intense red fluorescence signal in the nucleus, with a behavior comparable to that displayed by the NLS peptide.

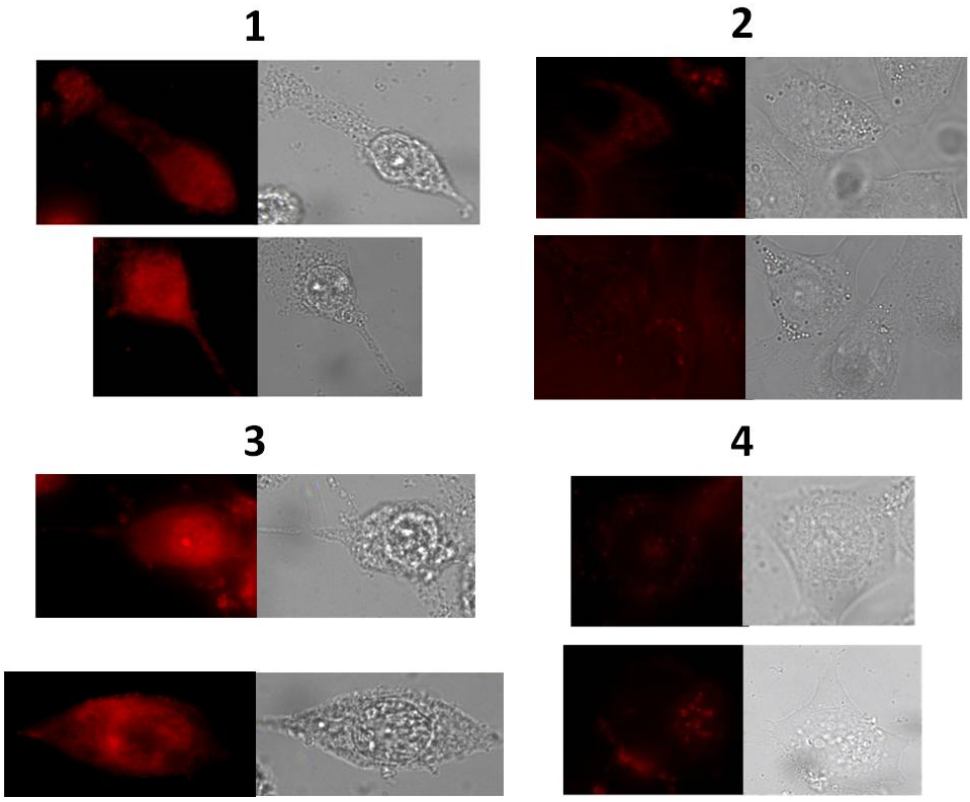


Figure 6-5: Fluorescence microscopy analysis (left) of uptake of RH30 cells treated for 3 hours with NLS peptide 1, unmodified PNA 2, modified PNA 3, and modified PNA 4 without proline. Optical images are also reported (right)

The uptake in the nuclear region and the homogeneous diffusion is consistent with a receptor-mediated internalization process, showing no difference in behavior between the NLS peptide **1**, for which the importin-mediated nuclear transport mechanism has been demonstrated, and

the modified PNA **3** (Figure 6-5). RH30 cells were then incubated with the modified PNA **4** (10 μmol/L) and analyzed after 3 hours, in order to confirm the specificity of the uptake mechanism for PNA **3.** The results obtained showed a very scarce and punctuated internalization (Figure 6-5). Although the number of positively charged side chains was the same for the PNA **3** and **4**, a different uptake is clear from the images in figure 6-5. This suggests that, while PNA **4** probably undergoes an unspecific uptake process and for this reason only a small amount is internalized, PNA **3** is selectively recognized by the cargo protein complex and carried in the nucleus through a specific uptake with the same mechanism of the NLS peptide **1**, indicating that such PNA can behave as peptide mimic.

6.3 Conclusions

We have demonstrated the chemical feasibility of synthesizing Peptide Nucleic Acids embedding in the structure amino acid side chains mimicking a complex peptide sequence. The preliminary data here reported on an NLS-mimicking PNA demonstrate that the pseudopeptide sequence encripted in the backbone of such modified PNA can behave as a fully functional peptide mimic, shedding light on a new dimension concerning the PNA properties, which may find numerous applications in bioorganic chemistry and molecular biology. For example, a PNA embedding the NLS sequence could be used for efficiently traslocate the PNA in the cell nuclei after injection in the blood stream avoiding the degradation from serum proteases, as it may happen when the NLS sequence is simply linked as normal peptide at the C-terminus. In general terms, a peptide nucleic acid embedding the functionalities of the proteins might be potentially used in order to develop PNAs endowed of catalytic functions, fully exploiting their intrinsic double nature of nucleic acid and peptide.

6.4 Experimental section

6.4.1 Materials and reagents

PNA N-Boc protected standard monomers, N-[1H-1,2,3-(benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide

(HBTU), N-[(dimethylamino)-1H-1,2,3-triazol[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU), dichloromethane, N–methylpyrrolidone (NMP) and N,N-dimethylformamide (DMF) were purchased from Applied Biosystem (Foster City, CA, USA). Carboxymethyl-O-benzyl-guanine was purchased by ASM (Hannover, Germany). Boc-Arg(Tos)-OH, Boc-Lys(2-Cl-Z)-OH, Val-OH, Boc-Pro-OH and m-cresol were from Fluka (Buchs, Switzerland). (4-Methylbenhydryl)amine (MBHA) PS resin was from Novabiochem (Laufelfingen, Switzerland). Carboxymethyl-thymine, diisopropylethylamine (DIPEA), N,O-Bistrimethylsilyl acetamide (BSA), trifluoromethane sulfonic acid (TFMSA), trifluoroacetic acid (TFA), 3-hydroxy-1, 2,3-benzotriazin-4-(3h)-one (DhBtOH) and diisopropylcarbodiimide (DIC) were from Sigma-Aldrich (St. Louis, MO, USA), Rhodamine. Oligonucleotides (guaranteed oligos grade) were purchased from ThermoFisher Scientific (Ulm, Germany) and used without further purification.

6.4.2 Submonomer syntheses

General procedure for submonomer synthesis

L-amino acid methyl esters (Val, Lys, Arg) were prepared by dissolving the N-Boc and side chain protected (Lys as 2-Cl-Z and Arg as Tosyl) amino acid derivatives, commercially available, in methanol with magnetic stirring. The mixtures were cooled at 0 °C and added of thionyl chloride dropwise up to a final concentration of 1M. The solutions were stirred overnight at room temperature. Methanol was evaporated and the residues were added of methanol and evaporated again (few times) to yield Boc-deprotected, side chain protected chloridrated methyl esters as white foams in quantitative yields. In order to synthesize the different substituted *N*-(2-aminoethyl)amino acid (PNA backbones), the corresponding methyl esters were dissolved in the minimum amount of methanol at room temperature together with diisopropylethylamine (DIPEA, 1 eq.) and the mixtures were stirred for 20 minutes. Boc-L-Lys(2-Cl-Z)-H aldehyde (1 eq.), synthesized as previously reported[31], was then added and the solutions were stirred for 30 min at room temperature, then cooled to 0°C and $NaBH_3CN$ (3 eq) and CH_3COOH (1.1 eq) were added. After 2h the methanol was evaporated. The residues were dissolved in CH_2Cl_2 and washed with saturated $KHSO_4$ (twice) and $NaHCO_3$ (twice). The combined organic phases were dried over $MgSO_4$ and filtered, and the solvent was evaporated under vacuum. The crude products were purified by flash chromatography (eluent ethyl acetate : methanol = 95:5) and obtained as colourless foams.

Yields 30-35%. Methyl ester deprotections were performed by dissolving the backbones in CH_3OH:H_2O = 1:1 (10 ml) and by adding NaOH up to a final concentration of 0.25 M. The mixtures were refluxed for 2.5 hour, then CH_3OH was evaporated and the products were precipitated as white solids at pH 5.5, filtered and dried under vacuum. Yields: 87-90%. Fmoc protections of the secondary amines were achieved by mixing the zwitterionic backbones in CH_2Cl_2, in flasks equipped with $CaCl_2$ drying valves. Bis(trimethylsilyl)acetamide (BTSA, 2 eq) and DIPEA (1.5 eq) were then added and when the solutions were nearly clear (10-15 min were usually required), Fmoc chloride (2 eq) was added and the mixtures were stirred for 2 hours at room temperature. CH_3OH (1 ml) was then carefully added and the mixtures were stirred for additional 15 min, diluted with CH_2Cl_2, washed with 1M $KHSO_4$ (3 times) and saturated NaCl (1 time), dried over $MgSO_4$ and evaporated to dryness. The residues were purified by flash chromatography (dichloromethane : methanol = 9:1) and the products were obtained as white foams (Yields 30-35%).

Compounds 2 (a- L-Val methyl ester hydrochloride, b- L-Arg-N^{ω}-Tosyl methyl ester hydrochloride)..**Compound 2 a**: Yield: Quantitative. ^{1}H NMR (300MHz ,$CDCl_3$, 25°C): δ(ppm): 8.82 (sb, 1H, NH_3^+), 3.90-4.03 (m, 1H, CHα), 3.8 (s, 3H, methyl ester), 2.41-2.53 (m, 1H, CH side chain), 1.20 (dd, 6, J=3 Hz, 7Hz, CH_3 side chain). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 169.1, 57.1, 54.4, 29.1, 18.3, 17.4. ESI-MS (CH_3OH, positive ions): calculated m/z: 131.1 (MH^+), 154.1 (MNa^+); found: 132.0, 154.0. **Compound 2 b:** ^{1}H NMR (300MHz ,[D_6]-DMSO, 25°C): δ(ppm) : 10.37 (sb, 3H, N-H amine), 8.64 (sb, 1H, N-Hη arginine side chain), 7.64 (d, 2H, J= 6 Hz aromatic tosyl group), 7.38 (sb, 1H, N-Hε arginine side chain), 7.29 (d, 2H, J= 6 Hz aromatic tosyl group), 6.79 (sb, 1H, N-Hη arginine side chain), 3.99-3.97 (m,1H, C-H α), 3.71 (s, 3H, methyl ester), 3.1-3.05(m, 2H, CH_2 arginine side chain), 2.34 (s, 3H, tosyl methyl group), 1.77-1.72 (m, 2H, CH_2 arginine side chain), 1.49-1.44 (m, 2H, CH_2 arginine side chain). ^{13}C NMR (75 MHz, $CDCl_3$):δ(ppm) 169.6,156.6, 141.3, 141.0,129.0, 125.6, 52.6, 51.4, 48.4, 27.0, 24.4, 20.8. ESI-MS (CH_3OH, positive ions): calculated m/z: 343.1 (MH^+) found: 343.3. HRMS (ESI^+): calcd m/z for $C_{14}H_{23}N_4O_4S$ (MH^+): 343.1440, found: 343.1443

Compound 3 (N^{α}-Boc-L-Lys-N^{ω}-2-Cl-Z-N,O-dimethylhydroxyamide). Yeld: 92%. ^{1}H NMR (300MHz , CDCl3): δ (ppm) 7.43-7.20 (m, 4H, CH aromatic 2-Cl-Z group), 5.19 (sb, 2H, CH_2 2-Cl-Z group), 4.99 (bs, 1H, N-Hω lysine side chain), 4.65 (sb, 1H N-Hα), 3.75 (s, 4H, N-metoxy group + CHα), 3.23-3.11 (m, 5H, N-methyl group + CH_2 lysine side chain),

1.41 (s, 9H, Boc methyl groups), 1.78-1.35 (m, 6H, CH_2 lysine side chain). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 172.9, 156.1, 155.6, 134.3, 133.4, 129.6, 129.4, 129.2, 126.7, 79.6, 63.7, 61.5, 49.9, 40.7, 32.5, 32.0, 29.1, 28.3, 22.3. ESI-MS (CH_3OH, positive ions): calculated m/z: 480.5 (MNa^+), found: 480.1

Compound 4 (N^{α}-Boc-L-Lys-N^{ω}-2-Cl-Z-aldehyde). Yield: 78%. ^1H NMR (300MHz , $CDCl_3$): δ (ppm) 9.54 (s, 1H, CH aldehyde), 7.43-7.20 (m, 4H, CH aromatic 2-Cl-Z group), 5.18 (s, 2H, CH_2 2-Cl-Z group), 5.09 (bs, 1H, N^{ω}-H lysine side chain), 4.20 (sb, 1H N^{α}-H), 3.74 (s, 1H, CHα), 3.26-3.09 (m, 2H, CH_2 lysine side chain), 1.65-1.47 (m, 2H, CH_2 lysine side chain), 1.47-1.30 (m, 4H, CH_2 lysine side chain) 1.42 (s, 9H, Boc methyl groups). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 199.9, 156.3, 155.7, 134.2, 133.5, 129.7, 129.3, 128.5, 126.9, 80.1, 63.8, 59.5, 40.4, 32.5, 29.4, 28.3, 22.1. ESI-MS (CH_3OH, positive ions): calculated m/z: 453.89, found: 453.3

Compound 5 (a- N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Z)-L-Val methyl ester, b- N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Z)-L-Arg-(N^{ω}-Tosyl) methyl ester). Compound 5 a: Yield: 31%. ^1H NMR (300MHz, $CDCl_3$): δ (ppm) 7.46-7.19 (m, 4H, CH aromatic 2-Cl-Z group), 5.20 (s, 2H, CH_2 2-Cl-Z group), 4.93 (bs, 1H, N^{ω}-H lysine side chain), 4.58 (sb, 1H, N^{α}-H), 3.70 (s, 3H, CH_3 methyl ester), 3.54 (bs, 1H, C(2)Hα), 3.24-3.10 (m, 2H, CH_2 lysine side chain), 2.98-2.87 (m, 1H, C(5)Hα), 2.72-2.28 (m, 2H, Lysψ CH_2), 1.60-1.21 (7H, m, CH_2 lysine side chain, CH valine side chain), 1.42 (s, 9H, CH_3 Boc group), 0.91 (d, 6H, J(H,H) = 6.6 Hz, CH_3 valine side chain) ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 175.3, 156.3, 156.2, 138.3, 129.6, 129.2, 128.6, 126.9, 125.7, 79.5, 64.2, 62.5, 52.3, 51.5, 50.6, 40.7, 32.5, 31.4, 29.8, 28.3, 22.8, 19.1, 18.6; ESI-MS (CH_3OH, positive ions): calculated m/z: 515.0 (MH^+), 537.0 (MNa^+), 553.0 (MK^+), found: 514.4, 536.4, 552.4. **Compound 5 b**: Yield: 30%. ^1H NMR (300MHz, $CDCl_3$): δ (ppm) 7.74 (d, 2H, J(H,H) = 8.1 Hz, CH aromatic tosyl group), 7.42-7.20 (m, 4H, CH aromatic 2-Cl-Z group), 7.20 (d, 2H, J(H,H) = 8.1 Hz, CH aromatic tosyl group), 6.46 (bs, 2H, N-Hη arginine side chain), 5.11 (bs, 2H, CH_2 2-Cl-Z group), 3.68 (s, 3H, CH_3 methyl ester), 3.51 (bs, 1H, C(2)Hα), 3.25-3.10 (m, 5H, CH_2 arginine side chain + lysine side chain + C(5)H α), 2.77-2.31 (m, 2H, Lysψ CH_2), 2.36 (s, 3H, CH_3 tosyl group), 1.89-1.21 (m, 10H, CH_2 arginine side chain, CH_2 lysine side chain), 1.38 (s, 9H, CH_3 Boc group.^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 175.1, 156.7, 156.4, 156.2, 141.7, 140.9, 134.2, 133.1, 129.4, 129.3, 129.1, 129.0, 126.7, 125.8, 79.2, 63.7, 60.8, 51.8, 51.6, 50.8, 40.7, 40.5, 32.2, 29.6, 29.4, 28.3, 25.5, 22.6, 21.3. ESI-MS (CH_3OH, positive ions): calculated m/z: 725.3 (MH^+), 747.3 (MNa^+); found: 725.4, 747.4

Compound 6 (a- N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Zl)-L-Val-OH, b- N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Z)-L-Arg-(N^{ω}-Tosyl)-OH). Compound 5 a Yield: 87% ^{1}H NMR (300MHz, DMSO): δ (ppm) 7.50-7.32 (m, 4H, CH aromatic 2-Cl-Z group), 5.07 (s, 2H, CH_2 2-Cl-Z group), 3.45 (bs, 1H, C(2)Hα), 3.02-2.91 (m, 2H, CH_2 lysine side chain), 2.91-2.80 (m, 1H, C(5)Hα), 2.65-2.38 (m, 2H, Lysψ CH_2), 1.50-1.15 (7H, m, CH_2 lysine side chain, CH_2 valine side chain), 1.37 (s, 9H, CH_3 Boc group), 0.88 (d, 6H, J(H,H) = 6.6 Hz, CH_3 valine side chain). ^{13}C NMR (75 MHz, DMSO): δ (ppm) 173.3, 155.6, 155.4,134.5, 132.1, 129.5, 129.1, 127.1, 79.3, 67.0, 62.3, 51.7, 49.5, 40.1, 31.5, 30.0, 29.1, 28.1, 22.6, 18.9, 18.4. ESI-MS (CH_3OH, positive ions): calculated m/z: 500.0 (MH^+), 522.0 (MNa^+), 552.0 (MK^+), found: 500.4, 522.3, 552.3. **Compound 6 b** Yield: Quantitative. ^{1}H NMR (300MHz , DMSO): δ (ppm) 7.62 (d, 4H, J(H,H)= 8.0 Hz, CH aromatic tosyl group), 7.51-7.32 (m, 4H, m, 4H, CH aromatic 2-Cl-Z group), 7.27 (d, 4H, J(H,H) = 8.0 Hz, CH aromatic tosyl group), 6.72 (d, 1H, J(H,H) = 8.31 Hz, N-Hη arginine side chain), 5.07 (s, 2H, CH_2 2-Cl-Z group), 3.52 (bs, 1H, C(2)Hα), 3.11-2.90 (m, 5H, CH_2 arginine side chain + CH_2 lysine side chain + C(5)Hα), 2.78-2.55 (m, 2H, Lysψ CH_2), 2.33 (s, 3H, CH_3 tosyl group), 1.63-1.12 (m, 10H, CH_2 arginine side chain, CH_2 lysine side chain), 1.36 (s, 9H, CH_3 Boc group). ^{13}C NMR (75 MHz, DMSO): δ (ppm) 172.8, 156.7, 155.6, 155.3, 141.6, 140.8, 134.5, 132.1, 129.5, 129.1, 128.9, 127.1, 125.4, 79.2, 62.4, 61.6, 50.4, 48.7, 40.1, 38.3 31.7, 29.1, 28.3, 28.1, 25.4, 22.5, 20.7. ESI-MS (CH_3OH, negative ions): calculated m/z: 709.3 ($[M-H]^-$), 745.6. ($[MCl]^-$); found: 709.3, 709.3

N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Zl)-L-Val-(N^{α}-Fmoc)-OH (L-Lys-L-Val submonomer). ^{1}H-NMR (300MHz, d_6-DMSO): δ(ppm) 0.84 (d, J=6 Hz, 6H, CH_3 Val), 1.32 (s, 9H, CH_3 Boc), 1.0-1.6 (m, 6H, -CH_2-CH_2-CH_2- Lys), 2.4-2.5 (1H, m, CH Val) 2.8-3.6 (m, 6H, CH_2-N Lys + CH_2-N backbone + CHα Val + CHα Lys), 4.1-4.7 (m, 3H, CH-CH_2 Fmoc), 5.07 (s, 2H, CH_2 Z group), 7.2-7.5 (m, 8H, aromatic CH Fmoc + CH Z group), 7.64 (d, J=7Hz, 2H, CH aromatic Fmoc group), 7.86 (d, J=7Hz, 2H, CH aromatic Fmoc group). ^{13}C-NMR (75 MHz, d_6-DMSO): δ(ppm) 19.1, 22.5, 27.6, 28.4, 29.2, 31.9, 40.6, 46.6, 50.0, 51.0, 52.0, 62.4, 66.3, 77.3, 119.9, 124.7, 126.9, 127.1, 127.4, 129.1, 129.5, 132.1, 134.5, 140.7, 143.6, 143.7, 155.1, 155.6, 156.5, 172.0. HRMS (ESI-MS, positive ions): calcd m/z for $C_{39}H_{48}ClN_3O_8Na$ (MNa^+): 744.30277; found m/z: 744.30066.

N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Z)-L-Arg-(N^{α}-Fmoc)-(N^{ω}-Tosyl)-OH (L-Lys-L-Arg submonomer).Yield: 18% ^{1}H-NMR (300MHz, $CDCl_3$): δ(ppm) 1.33 (s, 9H, CH_3 Boc), 1.1-1.8 (m, 10H, -CH_2-CH_2-CH_2- Lys + -CH_2-CH_2- Arg side chains), 2.31 (s, 3H, CH_3 tosyl group), 2.8-3.5 (m, 6H, CH_2-N Arg + CH_2-N Lys + CH_2-N backbone), 3.5-3.8 (m, 2H, CHα

Arg + CHα Lys), 4.0-4.6 (m, 3H, CH-CH_2 Fmoc), 5.16 (s, 2H, CH_2 Z group), 6.40 (sb, 2H, NH), 7.2-7.4 (m, 9H, aromatic CH tosyl + CH Fmoc + CH Z group), 7.4-7.6 (m, 2H, CH aromatic Fmoc group), 7.7-7.8 (m, 5H, aromatic CH tosyl + CH Fmoc + CH Z group). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 21.3, 22.6, 22.9, 26.6, 28.4, 29.4, 32.4, 40.3, 40.7, 47.1, 50.3, 51.5, 63.7, 67.0, 77.1, 79.7, 119.2, 124.6, 126.0, 126.8, 127.1, 127.3, 127.5, 127.7, 129.1, 129.3, 133.3, 134.3, 140.8, 141.2, 141.9, 143.7, 156.4, 156.6, 156.7, 156.8, 172.0. HRMS (ESI-MS, positive ions): calcd m/z for $C_{47}H_{58}ClN_6O_{10}S$ (MH^+): 933.36237; found m/z: 933.36298.

N^{α}-Boc-L-Lysψ-(N^{ω}-2-Cl-Z)-L-Lys-(N^{α}-Fmoc)-(N^{ω}-2-Cl-Z)-OH (L-Lys-L-Lys submonomer). The synthesis and characterization of the submonomer of has already been already reported[31]. HRMS (ESI-MS, positive ions): calcd m/z for $C_{48}H_{56}Cl_2N_4O_{10}Na$ (MNa^+): 941.32712; found m/z: 941.32703.

6.4.3 PNA and peptide synthesis

The NLS peptide 1 and the achiral PNA 2 were synthesized by standard Boc-SPPS protocols on a ABI 433A synthesizer, following the procedures provided from the company, whereas modified PNA 3 (with or without the N-terminal proline) was synthesized by submonomeric strategy[31]. The syntheses were performed on 25 mg of a preloaded Boc-Gly-MBHA-PS resin (loading 0.2 mmol/g). The synthetic cycle for the synthesis of modified PNA 3 were the following: Boc deprotection by neat TFA (added of 5% m-cresol), coupling of the desired chiral submonomer by using HATU/DIEA, Fmoc removal by piperidine/NMP (20%) and carboxymethyl-Z-adenine, carboxymethyl-*O*-benzyl-guanine or carboxymethyl-thymine coupling by using DIC/DhBtOH. In all cases an aminoethoxyethoxyacetyl (AEEA) spacer and rhodamine B were coupled to the growing chains before the cleavage. The peptide and the PNAs were cleaved from the resin by using a 1:3 TFMSA/TFA mixture (10% thioanisole + 10% m-cresol) and precipitated by Et_2O. HPLC analyses of the crude products were carried out by LC/MS by using an analytical C18 column (3x250 mm, 5 μm, flow 0.5 ml/min), gradient elution from 100% H_2O (0.2% HCOOH, eluent A) to 100% CH_3CN (0.2%HCOOH, eluent B) in 30 min. MS acquisition in the positive ion mode, capillary voltage 3kV, cone voltage 30V, full scan acquisition from 150 to 1500 m/z. HPLC purifications of the crude products were carried out on a semipreparative Jupiter (Phenomenex) C18 column (10x300mm, 5 μm, flow 4 ml/min), gradient elution from 100% H_2O (0.1% TFA, eluent A) to

100% CH_3CN (0.1% TFA, eluent B) in 30 min. Final purity was checked by analytical HPLC and was always higher than 95%.

NLS peptide 1: Rhodamine-(AEEA)-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-NH_2 Crude yield: 85%. ESI-MS, positive ions: calcd m/z 755.5 (MH_2^{2+}), 504.0 (MH_3^{3+}), 378.2 (MH_4^{4+}), 302.8 (MH_5^{5+}), 252.5 (MH_6^{6+}); found m/z 755.6, 503.8, 378.2, 302.8, 252.4.

Standard PNA 2: Rhodamine-(AEEA)-ATG-Gly-NH_2. Crude yield: 82%. ESI-MS, positive ions: calcd m/z 739.3(MH_2^{2+}), 493.2 (MH_3^{3+}), 370.2 (MH_4^{4+}); found m/z 739.1, 493.1, 370.1.

Modified PNA 3: Rhodamine-(AEEA)-Pro-$A_{(2L,5L\text{-}Lys)}T_{(2L\text{-}Arg,5L\text{-}Lys)}G_{(2L\text{-}Val,5L\text{-}Lys)}$-Gly-$NH_2$. Crude yield: 80%. ESI-MS, positive ions calcd m/z: 667.4 (MH_3^{3+}), 500.8 (MH_4^{4+}), 400.8 (MH_5^{5+}), 334.2 (MH_6^{6+}); found m/z: 667.5, 500.8, 400.8, 334.2.

Modified PNA 4 without N-terminal proline: Rhodamine-(AEEA)-$A_{(5L,\ 2L\text{-}Lys)}T_{(5L\text{-}Lys,\ 2L\text{-}Arg)}G_{(5L\text{-}Lys,\ 2L\text{-}Val)}$-Gly-$NH_2$. Crude yield: 85%. ESI-MS, positive ions calcd m/z: 635.0 (MH_3^{3+}), 476.5 (MH_4^{4+}), 381.4 (MH_5^{5+}), 318.0 (MH_6^{6+}); found m/z: 634.9, 476.5, 381.4, 318.0.

6.4.4 Fluorescence microscopy

Fluorescence microscopy analysis to evaluate the intracellular localization of NLS peptide 1, standard PNA 2, modified PNA 3 and modifed PNA 4 in RH30 cells was done by the method previously described[24].

6.5 References

[1] D. A. Braasch, D. R. Corey, *Biochemistry*, **2002**, 41, 14, 4503

[2] V.V. Demidov, V.N. Potaman, M.D. Frank-Kamenetskii et al., *Biochem. Pharmacol.*, **1994**, 48, 1310

[3] P. Wittung, J. Kajanus, K. Edwards,et al., *FEBS Lett.*, **1995**, 365, 27

[4] U. Koppelhus, P.E. Nielsen, *Advanced Drug Delivery Reviews*, **2003**, 55, 267

[5] M. A. Shammas, C. G. Simmons, D. R. Corey, R. J. Shmookler, *Oncogene*, **1999**, 18, 6191

[6] D. F. Doyle, D. A. Braasch, C. G. Simmons, B. A. Janowski, D. R. Corey, *Biochemistry*, **2001**, 40, 53

[7] A. Füssl, A. Schleifenbaum, M. Göritz, A. Riddell, C. Schultz, R. Krämer, *J. Am. Chem. Soc.*, **2006**, 128, 5986

[8] E. Riguet, S. Tripathi, B. Chaubey, J. Désiré et al., *J. Med. Chem.*, **2004**, 47, 4806

[9] M. Mehiri, G. Upert, S. Tripathi, A. Di Giorgio, R. Condom, V. N. Pandey, N. Patino, *Oligonucleotides*, **2008**, 18, 3, 245

[10] X. Zhang, C. G. Simmons, D. R. Corey, *Bioorganic & Medicinal Chemistry Letters*, **2001**, 11, 1269

[11] L. C. Boffa, S. Scarfi', M. R. Mariani et al., *Cancer Research*, **2000**, 60, 2258

[12] B. P. Gangamani, V. A. Kumar, K. N. Ganesh, *Biochemical and Biophysical research communication*, **1997**, 240, 778
[13] R, Villa, M. Folini, S. Lualdia et al., R. Villa et al., *FEBS Letters*, **2000**, 473, 241
[14] H. Nagahara, A. M. Vocero-Akbini, E. L. Snyder,A. et al., *Nat.Med.*, **1998**, 4, 1449
[15] G. D. Ivanova, A. Arzumanov, R. Abes, H. Yin, M. J. A. Wood, B. Lebleu, M. J. Gait, *Nucleic Acids Research*, **2008**, 36, 20, 6418
[16] U. Koppelhus, T. Shiraishi, V. Zachar, S. Pankratova, P. E. Nielsen, *Bioconjugate Chem.*, **2008,** *19,* 1526
[17] G. Shen, H. Fang, Y. Song, A. A. Bielska, Z. Wang, J. A. Taylor, *Bioconjugate Chem.*, **2009**, 20, 1729–1736
[18] F. S. Hassane, G. D. Ivanova et al., *Bioconjugate Chem.*, **2009**, 20, 1523
[19] R. Mishra, W. Su, R. Pohmann, J. Pfeuffer, M. G. Sauer, K. Ugurbil, J. Engelmann, *Bioconjugate Chem.*, **2009**, 20, 10, 1860
[20] G. Cutrona, E. M. Carpaneto, M. Ulivi, S. Roncella, O. Landt, M. Ferrarini, L. C. Boffa, *Nature Biotechnology*, 2000, 18, 300
[21] S. Kosugi, M. Hasebe, N. Matsumura et al., *Journal of Biological Chemistry*, **2009**, 284, 1, 478
[22] A. Lange, R. E. Mills, C. J. Lange, M. Stewart, S. E. Devine, A. H. Corbett, *Journal of Biological Chemistry*, **2007**, 282, 8, 5101
[23] M. Stewart, *Biochemical Society Transactions*, **2006**, 34, 5, 701
[24] A. Pession, R. Tonelli, R. Fronza, E. Sciamanna, R. Corradini, S. Sforza, T. Tedeschi, R. Marchelli, L. Montanaro, C. Camerin, M. Franzoni, G. Paolucci, *Int. J. Oncol.* **2004**, 24, 265
[25] P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner, D.H. Ly, *J. Am. Chem. Soc.*, **2003**, 125, 6878
[26] B. Sahu, V. Chenna, K. L. Lathrop,S. M. Thomas, G. Zon, K. J. Livak, D.H. Ly, *J. Org. Chem.*, **2009**, 74, 1509
[27] V. Menchise, G De Simone, T. Tedeschi, R. Corradini, S. Sforza, R. Marchelli, D. Capasso, M. Savino, C. Pedone, *Proc. Natl. Acad. Sci. USA*, **2003**, 100, 12021
[28] A. Dragulescu-Andrasi, S. Rapireddy, G. He, B. Bhattacharya, J.J. Hyldig-Nielsen, G. Zon, D.H. Ly, *J. Am. Chem. Soc.*, **2006**, 128, 16104
[29] V. Chenna, S. Rapireddy, B. Sahu, C. Ausin, E. Pedroso, D.H. Ly, *ChemBioChem*, **2008**, 9, 2388
[30] S. Sforza, T. Tedeschi, R. Corradini, D. Ciavardelli, A. Dossena, R. Marchelli, *Eur.J. Org. Chem.*, **2003**, 1056
[31] T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, *Tetrahedron Lett*. **2005**, 46, 8395-8399

Acknowledgements

There are several people to whom I am grateful for the help received during my PhD. First of all I would like to thank my tutors prof. Rosangela Marchelli and prof. Stefano Sforza. They gave me the possibility to work in stimulant environment and helped me when stacked with my work. Their collaboration allowed me to improve my scientific knowledge.

I would like to thank my co-workers Alessandro Accetta, Dr. Tullia Tedeschi, Dr. Andrea Faccini, Dr. Roberto Pela, Dr. Filbert Totsingan and Alex Manicardi, their daily help in the laboratory has been precious, and their company during work time and after has been always pleasant. I would like to thank also prof. Roberto Corradini, he was always ready to give suggestions when asked, and his supervision in some of the works reported in this thesis was very useful. I am grateful to all the bachelor and master students that in these years helped me, Marika Bigi, Annalisa Balducchi and Manuela Bersellini.

There are many people who collaborated with me for the realization of some of the works reported in this thesis, many of the experiments here reported would have not been possible without their help. I would like to thank prof. Roberto Tonelli and Dr. Consuelo Camerin from the Laboratory of Pediatric Oncology and Hematology "Lalla Seràgnoli" at “Sant’Orsola” hospital, Bologna, Italy. I am indebted to Prof. Jurriaan Huskens and Dr. Pascal Jonkheijm from the Molecular Nanofabrication group of the University of Twente, The Netherlands, for the period I spent in their group. They taught me all I know about surface chemistry, and hosted me in their laboratories for a long period giving me the possibility to acquire experience in a new field. I would like to thank also Shu-Han Hsu and Alberto Gomez Casado for their daily help in the laboratory. I am grateful also to all my co-workers from MNF and SMCT, for being always ready to give me suggestions and making my period in Enschede more amusing with their company.

Contributions

Publications including work reported in this thesis

R. Corradini, S. Sforza, T. Tedeschi, A. Calabretta, A. Accetta, A. Tonelli, R. Marchelli, PEPTIDE NUCLEIC ACIDS (PNAs) AND MODIFIED PNAs FOR DNA TARGETING, *Minerva Biotecnologica*, **2008**, 20, 139-147.

A. Calabretta, T. Tedeschi, G. Di Cola, R. Corradini, S. Sforza, R. Marchelli, ARGININE-BASED PNA MICROARRAYS FOR APOE GENOTYPING, *Molecular Biosystems*, **2009**, 5, 1323-1330.

A. Manicardi, A. Calabretta, M. Bencivenni, T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, AFFINITY AND SELECTIVITY OF C2- AND C5-SUBSTITUTED "CHIRAL-BOX" PNA IN SOLUTION AND ON MICROARRAYS, *Chirality*, submitted.

Oral Comunications to conferences including work reported in this thesis

S. Sforza, T. Tedeschi, A. Calabretta, R. Corradini, R. Marchelli, DESIGN AND SYNTHESIS OF A PEPTIDE NUCLEIC ACID EMBEDDING A PSEUDOPEPTIDE NUCLEAR LOCALIZATION SEQUENCE IN THE BACKBONE: A NEW PEPTIDE MIMIC, XXIII Congresso Nazionale della Società Chimica Italiana, Sorrento, July 5-10 2009, ORG-OR-24/DCSB, p281.

Poster Comunications to conferences including work reported in this thesis

Calabretta, S. Rossi, T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, PNA CHIRALI BASATI SU ARGININA (ARG-PNA) PER L'IDENTIFICAZIONE DI SNPs DI CULTIVAR DI OLIVO, XXXI Convegno Nazionale della Divisione di Chimica Organica della Società Chimica Italiana, Rende, September 10-14 2007, P40

Calabretta, T. Tedeschi, S. Sforza, R. Corradini, R. Marchelli, PNA CHIRALI BASATI SULL'ARGININA (ARG-PNA)PER LA GENOTIPIZZAZIONE RAPIDA DEL GENE APOE, XXXII Convegno Nazionale della Divisione di Chimica Organica, Taormina, July 26-30 2008, PO9, p105.

S. Sforza, T. Tedeschi, A. Calabretta, R. Corradini, R. Marchelli, ARGININE-BASED CHIRAL PNAs AND MICROARRAY TECHNOLOGY FOR FAST APOE GENOTYPING, 2nd European Chemistry Congress, Torino, Italy, September 16-20 2008, electronic proceedings on DVD.

Calabretta, P. Jonkheijm, S. Sforza, R. Marchelli, J. Huskens, PEPTIDE NUCLEIC ACID PATTERNING BY MICROCONTACT PRINTING, BIOTECH.ORG Organic Chemistry and Biotechnology: Challenges and Opportunities, Forte dei Marmi (LU), May 20-23 2009.

T. Tedeschi, S. Sforza, A. Calabretta, A. Tonelli, M. Bencivenni, R. Corradini, R. Marchelli, DNA ANALYSIS BY PNA-BASED TECHNOLOGIES FOR ASSESSING FOOD AUTHENTICITY, International Conference on FoodOmics, Cesena, May 28-29 2009, pp111-113.

T. Tedeschi, S. Sforza, A. Tonelli, A. Calabretta, R. Corradini, G. Spoto, R. Marchelli, HIGHLY SENSITIVE AND SPECIFIC PNA-BASED METHODS FOR FOOD SAFETY AND AUTHENTICATION, EuroFoodChem XV, Copenhagen, Denmark, July 5-8 2009, p102.

S. Sforza, T. Tedeschi, A. Calabretta, M. Bencivenni, F. Lambertini, G. Galaverna, A. Dossena, R. Marchelli, DNA AND PEPTIDES AS MOLECULAR MARKERS FOR FOOD AUTHENTICITY, 4th International Symposium on Recent Advances in Food Analysis, Prague (Czech Republic), November 4-6 2009, J-5, p447.