Russian Chemical Reviews **71** (1) 71 – 83 (2002) © 2002 Russian Academy of Sciences and Turpion Ltd

DOI 10.1070/RC2002v071n01ABEH000691

Peptide nucleic acids: structure, properties, applications, strategies and practice of chemical synthesis

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Contents

I.	Introduction	71
II.	Properties of peptide nucleic acids	71
III.	Applications of peptide nucleic acids	72
IV.	Basic principles of chemical synthesis of peptide nucleic acids	73
V.	Factors determining the efficiency of condensation in the synthesis of peptide nucleic acids	73
VI.	The main strategies of peptide nucleic acid synthesis	74
VII.	Some regularities of condensation reactions in the synthesis of peptide nucleic acids	75
VIII.	Conclusion	81

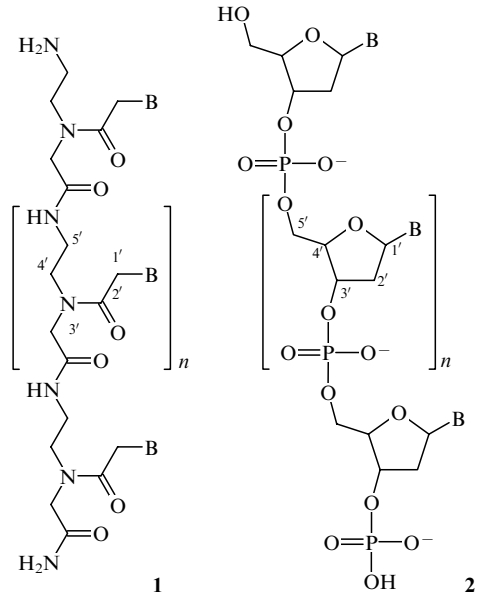
Abstract. The information on the structure and properties of peptide nucleic acids (PNA) is generalised. The use of PNA oligomers in biomolecular studies and biotechnology is exemplified. The published data on the most important methods for the chemical synthesis of PNA oligomers with the main emphasis on the efficiency of condensation reactions are considered. The methods for PNA synthesis are systematised; their advantages and disadvantages are discussed. Some recommendations for optimisation of the condensation procedure and synthesis of PNA are presented. The bibliography includes 153 references.

I. Introduction

Peptide nucleic acids (PNA, **1**) represent analogues of nucleic acids (NA, **2**),[1–4] but, in contrast to the latter, contain neither carbohydrate nor phosphate residues and have uncharged pseudopeptide backbones.[1,5–8] The monomeric unit of classical PNA comprises *N*-(2-aminoethyl)glycine and a heterocyclic base (purine or pyrimidine) bound through an acetyl linker. The monomers are linked by amide bonds. The geometry of the achiral backbone and its relative flexibility[3,9] confer on PNA an ability to mimic, with striking exactness, the spatial structure of carbohydrate – phosphate backbones of NA.[4]

From the chemical standpoint, PNA represent a hybrid of an oligonucleotide wherefrom the nucleases have been adopted and a peptide, the structure of which gave birth to the structural principle of the PNA backbone. Thus, PNA possess properties of both these classes of compounds.[8,10] This structural-and-functional duality of PNA determines their unique property.[4] Indeed, these molecules combine uniquely the strict recognising ability inherent in NA with the flexibility and stability of proteins.

It should be noted that the term 'peptide nucleic acids' is used to point to the structural similarity of these compounds to NA and to reflect the similarity of the PNA oligomeric backbone to that of peptides, although neither the term 'acid' nor 'peptide' are applicable to PNA, for in contrast to nucleic acids PNA are not polymeric acids and in contrast to peptides they do not contain amino acids. Nevertheless, the abbreviation 'PNA' has now come into general use, although it would be more correct to refer to these compounds as polyamide analogues of oligonucleotides.[10]



1 **2**

B = Ade, Gua, Thy, Cyt.

II. Properties of peptide nucleic acids

Complementary PNA molecules form specific antiparallel PNA – PNA duplexes having helical structures[4,11] similar to those of DNA and RNA duplexes but, which is even more important, they form highly stable specific (antiparallel and parallel) duplexes with complementary DNA and RNA sequences[1,5–7,9,12–14] containing Watson – Crick base pairs.[2,15,16] In all cases, antiparallel PNA – DNA duplexes are more stable than the parallel ones, *viz.*, their melting temperatures differ by ~1 °C for each base pair.[2] The circular dichroism spectra of PNA – DNA and DNA – DNA duplexes are similar,[2,17] which points to the formation of right-hand helices during the formation of

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Received 11 July 2001
Uspekhi Khimii **71** (1) 81 – 96 (2002); translated by R L Birnova

PNA – DNA duplexes despite the fact that base pairs in PNA – DNA and DNA – DNA duplexes have slightly different geometries.[17] NMR and X-ray crystallography studies of PNA – PNA and PNA – DNA complexes revealed that PNA – PNA duplexes possess broad, deep major grooves and narrow, shallow minor grooves; noteworthy, one complete turn of a helix in PNA – PNA duplexes corresponds to 18 base pairs, whereas that in PNA – DNA duplexes, to 13 base pairs.[17–20]

PNA – PNA, PNA – DNA and PNA – RNA duplexes are considerably more stable than DNA – DNA, DNA – RNA and RNA – RNA duplexes of the same compositions.[1, 2, 11–17, 21–23] Even four-membered PNA sequences produce highly stable duplexes with complementary DNAs.[17] In contrast to NA – NA duplexes, the stabilities of PNA – NA duplexes are little dependent on the solution ionic strength.[2, 24–27] PNA – DNA duplexes are formed faster than the corresponding DNA – DNA duplexes,[14, 22] but high specificity of hybridisation is preserved.[28]

The stabilities of PNA – DNA duplexes can be predicted based on a model which takes into account the interactions between only the nearest adjacent bases;[17, 29, 30] however, this model describes adequately the stabilities of short duplexes comprising no more than eight units.[17]

The most essential property of PNA is their unique sensitivity to mismatches in the structure of NA targets.[7] The difference in melting temperatures of a perfect PNA – DNA complex and a duplex containing one mismatch amounts to 20 °C and even more.[3]

Peptide nucleic acids form PNA – $(DNA)_2$ triplexes with double-stranded DNA.[31–34] These triplexes are less stable than the classical $(DNA)_3$ triplexes.[34] On the other hand, PNA forms stable $(PNA)_2$ – DNA and $(PNA)_2$ – RNA triplexes with single-stranded DNA and RNA targets, respectively.[5, 12, 14, 16, 33, 35–39] Such triplexes are often formed in the interaction of PNA with double-stranded NA.[37, 40–44] In the latter case, the formation of triplexes leads to the displacement of one of the DNA strands resulting in complete separation or P-loop formation[38] with subsequent incorporation of PNA chains.[5, 33, 37, 40–44] PNA form Watson – Crick pairs with DNA. The attachment of the second PNA chain is accompanied by the formation of Hoogsteen pairs.[35, 39] It was found that Watson – Crick chains of PNA are antiparallel to DNA chains, while Hoogsteen strands are parallel to them.[37, 39]

It is noteworthy that Hoogsteen strands stabilise Watson – Crick PNA – DNA duplexes which are formed first; the latter can be relatively unstable.[38]

Homopyrimidine PNA molecules and PNA enriched with pyrimidine residues are especially prone to triplex formation.[9, 35–39] The respective triplexes are extremely stable, *e.g.*, ten-membered $(PNA)_2$ – DNA triplexes have the melting temperatures of $\sim$70 °C.[9, 39] The ability of cytosine-containing PNA to form triplexes is pH-dependent, since cytosine can form Hoogsteen pairs with guanine residues only in the protonated state.[9] No triplexes with the composition $(PNA)_3$ have been found.[45]

Recently, a new type of PNA-containing triplexes has been discovered.[37] Both PNA chains in the $(PNA)_2$ – DNA triplex formed upon binding of the PNA oligomer 5′-$T_4G_2(TG)_2$-3′ to the oligonucleotide 5′-$A_4C_2(AC)_2$-3′ are in the antiparallel orientation relative to the DNA strand.

The binding potential of PNA with respect to NA is far from being exhausted. Studies of NA-binding properties of PNA aimed at the synthesis of novel PNA possessing improved structures and able to enhance the specific binding of PNA to complementary NA are currently under way.[37]

The melting of short-chain $(PNA)_2$ – DNA triplexes is a non-equilibrium process, *viz.*, the melting temperature depends on both the concentrations of components and heating velocity.[38] Thus, the stabilities of $(PNA)_2$ – DNA complexes were found to be kinetic.[38] The dissociation of such triplexes occurs in two steps, *viz.*, the first (limiting) step includes separation of the Hoogsteen strand of PNA, while the second step includes rapid separation of the Watson – Crick strands of PNA.[38]

Peptide nucleic acids manifest high chemical and biological stabilities.[25, 46] They are highly resistant against cell nucleases, proteases and peptidases.[25, 27, 47] PNA oligomers undergo very slow enzymatic hydrolysis in both cell extracts and *in vivo*.[25, 46] PNA molecules are distinguished by generally low toxicity and are not prone to non-specific binding to cellular proteins. Being immobilised on solid supports, PNA molecules preserve their hybridisation properties.[28]

It is known that modifications of NA backbones by replacing phosphodiester or carbohydrate units by non-charged or cationic structures may confer useful properties on NA, *e.g.*, enhanced resistance against nucleases and effective penetration through cell membranes and more specific and stronger binding to complementary target NA.[48] Attempts are being made to improve the PNA structure in order to increase the ability of PNA for nonspecific binding to NA and their transport across cellular membranes.[2, 3, 8–10, 36] Thus the addition of certain peptides,[49–53] *e.g.*, the 16-membered peptide 'transportan',[50, 51] to PNA increases considerably the rate of intracellular transport of PNA.[9, 23, 49–53] α-Helical PNA (αPNA) have been obtained in which the role of the backbones is played by α-helical peptide structures.[54–56] Such PNA analogues form highly specific stable Watson – Crick duplexes with complementary NA[54, 55] and manifest very high biological stabilities.[56] PNA analogues with chiral backbones with positively and negatively charged groups, PNA – DNA chimeras, *etc.*, have been synthesised.[36]

Comparative studies of structurally different PNA analogues have shown that classical PNA with *N*-(2-aminoethyl)glycine backbones first synthesised by Peter E Nielsen ten years ago[1] manifest optimum NA-binding properties.[4] Therefore, the main attention in this review will be given to methods of synthesis of classical PNA molecules based on *N*-(2-aminoethyl)glycine residues.[1]

III. Applications of peptide nucleic acids

By virtue of their unique properties,[13, 37] PNA have found wide use in molecular-biological, biochemical, genetic engineering and clinical investigations.[2, 3, 14, 26, 27, 36, 57–65] They represent attractive candidates for new-generation genetic therapeutic drugs which interfere selectively with gene expression.[9, 14, 26, 36, 57, 62–71] The use of PNA oligomers in antisense[9, 26, 27, 36, 57, 67, 69, 72–74] and antigen[27, 36, 57, 67, 68, 72, 75, 76] biotechnologies is of considerable promise.

Antisense PNA selectively inhibit the expression of brain proteins.[47] The design of anticancer and antiviral drugs based on PNA seems to be a very promising approach.[26, 57, 65] Some PNA derivatives manifest antibacterial[36] and antisense activities[9, 47] towards eukaryotic cells and animal organisms.[9, 47]

There is evidence that PNA interfere with all the key stages of gene expression.[9, 46, 57, 77] Used in nanomolar concentrations, PNA cause a practically complete specific arrest of transcription of DNA templates.[46, 78] The usefulness of PNA oligomers in gene-oriented technologies has been demonstrated with a PNA-dependent arrest of transcription elongation of RNA polymerase as an example.[27, 46, 67, 68, 72] By virtue of their ability to induce effective blocking of transcription, PNA oligomers represent potential inhibitors of cell growth, which makes them a useful tool in the design of antitumour drugs.[78]

PNA duplexes and especially triplexes with mRNAs, *e.g.*, PNA – RNA and $(PNA)_2$ – RNA, effectively inhibit the translation of mRNA.[67, 73, 74] Their potent antisense effects *in vitro* are due to high specificities and stabilities of triplex $(PNA)_2$ – RNA.[38] The arrest of translation elongation of mRNA occurs even upon addition of six-membered complementary homopyrimidine PNA.[73]

The antisense efficiencies of duplex-forming PNA are lower than those of triplex-forming ones; in this case, no less than 20-

membered PNA are required for the inhibition of translation elongation of mRNA (however, the translation initiation can be arrested due to formation of even short-chain PNA – RNA duplexes).[73, 74] Nevertheless, RNA molecules in hybrid PNA – RNA duplexes are not cleaved by RNase H.[67, 73, 79–81] The antisense effect of duplex-forming PNA is largely due to steric hindrances upon formation of stable PNA – RNA complexes[9] which hinder the translation of mRNA. PNA-induced degradation of mRNA, which is unrelated to the effect of RNase H, should not be ruled out either.[9]

Duplex-forming PNA can inhibit translation *in vitro*, being specifically directed against the binding sites of ribosomes, whereas triplex-forming PNA are more specific against polypurine sites located 'below' the translation initiation point.[9]

Peptide nucleic acids are used for mapping of RNA molecules in molecular biological studies, particularly for detection of RNA domains responsible for binding to other RNAs and peptides.[80] Their applications open up new possibilities for elaboration of novel approaches to the study of RNA – RNA and RNA – protein interactions and such processes involving non-translatable RNA molecules as splicing. There is evidence that PNA molecules behave as effective 'traps' for some DNA-binding proteins.[81] PNA – DNA chimeras are convenient primers for DNA polymerases.[82] In recent years, PNA have extensively been used as biomolecular tools for the studies of various intracellular processes.[2, 3, 8, 10, 27, 57, 61–64, 80–82]

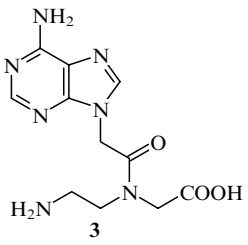
The use of PNA in the design of efficient procedures for the detection of hybridisation, which are extremely sensitive to mismatches in NA targets, is a promising approach.[28, 83] Fluorescently labelled PNA are used as diagnostic probes for detecting specific NA sequences and for the study of penetration of PNA oligomers through cellular membranes and their intracellular distribution.[84–87] The use of PNA in combination with ion-exchange HPLC (the detection limit is 150 pmol),[88] MALDI TOF mass spectrometry,[89] capillary electrophoresis[90] and other advanced analytical techniques[88] allows reliable identification of specific genetic sequences in various test samples.

The use of PNA in the design of electrochemical biosensors[28, 91–93] opens up new possibilities for fast screening of primary NA structures and helps overcome many problems of modern biotechnology.[2–4, 8, 10, 61] These compounds can be used as an outstanding basis for the construction of new generations of highly efficient diagnostic tools, *e.g.*, biochips.[83]

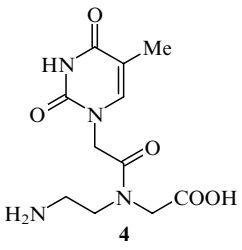
In this context, the development and optimisation of versatile techniques for the synthesis of PNA oligomers are becoming currently central tasks. The condensation of PNA monomers and oligomers with formation of amide bonds induced by various activating reagents is the key step in PNA synthesis. The combination of protective groups, deprotection and capping conditions as well as post-synthetic work-up of synthetic PNA oligomers strongly depend on the condensation method used.

IV. Basic principles of chemical synthesis of peptide nucleic acids

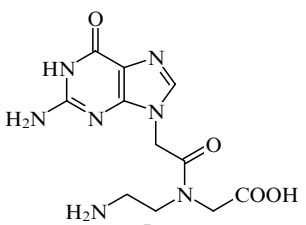
The chemical synthesis of PNA molecules consists essentially in the oligomerisation of the monomers **3**–**6** comprising *N*-(2-aminoethyl)glycine backbones and acetic acid residues (acetyl linkers), each containing one of four nucleobases as a substituent.[14] At present, a broad range of methods for the chemical



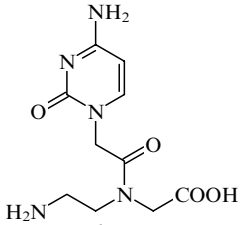
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4



5



6

synthesis of PNA are available; the most efficient of them have become especially popular in the past decade.[14, 94–97]

First of all, a solid-phase methodology is applied for the synthesis of PNA. The synthesis of oligomeric molecules on the surface of polymeric supports was first developed by Merrifield for the synthesis of peptides and proteins in 1962. The strikingly simple idea to immobilise growing oligomeric chains on solid supports has brought biooligomer synthesis to a qualitatively new level. At present, the principle proposed by Merrifield[98] is widely used in the synthesis of peptides and proteins as well as of DNA and RNA fragments (oligonucleotides). In the overwhelming majority of cases, PNA oligomers are also synthesised on solid polymeric supports. In this review, the main emphasis will be laid on the problems related to the efficiency of solid-phase synthesis of PNA.

Although PNA oligomers can be synthesised by classical methods commonly employed in peptide synthesis,[14] specially designed condensation procedures should be preferred, taking into account peculiarities of chemical structures of PNA monomers. In fact, of the different methods for the activation of carboxy groups based on the use of activated esters, symmetrical anhydrides, acid halides and *in situ* activating reagents, the *in situ* activation has become the most promising approach, which is currently especially popular.

This method envisages the use of reagents based on uronium and phosphonium salts which effect fast (within several seconds) activation of carboxy groups of PNA monomers. Owing to high activation rates, mixing of PNA monomers with an activating reagent can be performed directly in a column with a polymeric support to which the growing oligomeric chain is attached (it is this procedure that represents *in situ* activation) or immediately before the addition of the monomer to the reaction column. This makes it possible to conduct PNA synthesis in an automated regime.

The solid-phase procedure for PNA synthesis involving *in situ* activation will be considered below; its advantages have been corroborated by chemical practice. The data on the efficiencies of other activation techniques can also be useful and proper consideration will be given to them.

V. Factors determining the efficiency of condensation in the synthesis of peptide nucleic acids

The yields of condensation products in the synthesis of PNA using the *in situ* activation procedures depend critically on a number of factors. The most essential of them are as follows:

— the nature and concentration of the activating reagent;

— the nature and concentration of the PNA monomer;

— the nature of a nucleobase component of the PNA monomer and the nature of the nucleobase incorporated into the PNA oligomer in the preceding step;

— the nature and the concentration of a base (as a rule, tertiary amines);

— the nature of the solvent(s);

— the presence or absence of catalysts, *e.g.*, 1-hydroxybenzotriazole;

— the experimental procedure (*e.g.*, preactivation of the PNA monomer or mixing of the PNA monomer with the condensation reagent *in situ*);

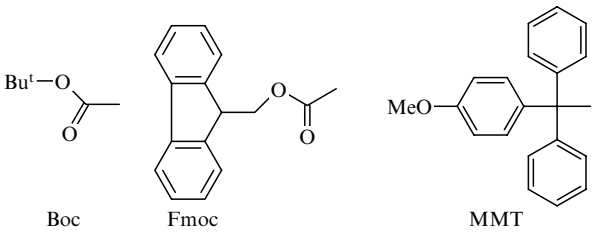
— condensation conditions (reaction time and temperature);
— other conditions (*e.g.*, the quality of reagents, dryness of solvents, inertness of the reaction atmosphere, *etc.*).

It should be noted that information concerning the dependence of the yields of the condensation products on the nature of the heterocyclic bases of PNA monomers is practically absent. This problem demands special investigation. The majority of literature sources cite average yields of PNA condensation products calculated per coupling cycle of a hypothetical monomeric fragment (irrespective of the nature of the monomer) or the total yields of the PNA oligomer.

It seems reasonable to consider all the factors mentioned above. Hence, it is expedient to discuss in detail the main aspects related to the efficiencies of PNA condensations inherent in particular synthetic strategies.

VI. The main strategies of peptide nucleic acid synthesis

Usually, PNA synthesis utilises conventional solid-phase peptide synthesis protocols.[14] Three synthetic strategies are currently especially popular which produce PNA in high yields and purity. These strategies differ in the nature of protective groups blocking 5′-terminal primary aliphatic amino groups in PNA monomers. *tert*-Butoxycarbonyl (*t*Boc or Boc), 9-fluorenylmethoxycarbonyl (Fmoc) and 4-methoxyphenyldiphenylmethyl (monomethoxytrityl, MMT) groups are generally used as protective groups, and Boc,[12, 94, 99, 100] Fmoc,[77, 97, 101–106] and MMT strategies [96, 107–112] of PNA synthesis are distinguished, correspondingly.



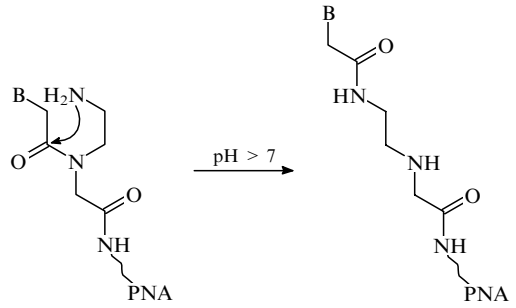
The chemical nature of these groups and the differences in the conditions for their removal determine the choice of optimum condensation reagents and condensation conditions for each particular strategy, although the main principles of PNA synthesis are the same.

The Boc strategy [12, 94, 99, 100] was the first to be used for the PNA synthesis. In this case, exocyclic amino groups of heterocyclic bases are usually protected by the benzyloxycarbonyl (Z, Cbz) group (the Boc/Z version),[94] and *O*-benzyl groups are sometimes used as additional protective groups for guanine.[113] The Boc/acyl version of the Boc strategy, where exocyclic amino groups of nucleobases are protected by acyl groups, has also been described.[95] One of the well-known disadvantages of the Boc strategy is the necessity to use strong acids for the removal of Boc groups (trifluoroacetic acid) and the cleavage of PNA oligomers from the polymeric supports (hydrofluoric acid, trifluoromethanesulfonic acid, *etc.*). Such drastic conditions limit the range of PNA synthesised according to the Boc protocol.

A search for milder conditions has led to the development of the Fmoc strategy of PNA synthesis.[14, 77, 96, 97, 101–106, 114] Here, the Fmoc groups are removed by mild treatment with piperidine.[14] The benzyloxycarbonyl (Z),[97] benzhydryloxycarbonyl (Bhoc) [106] or MMT groups [12, 96, 114] are used to protect amino groups of nucleobases. The advantage of a Fmoc/acyl version of this strategy [77, 102–105] is the possibility of selective removal of the Fmoc groups without affecting the acyl protective groups of the heterocyclic bases.[102, 103] The use of this strategy ensures higher yields of PNA in comparison with the Fmoc/Z strategy, while the conditions for PNA synthesis are compatible with those of peptide and oligonucleotide syntheses.[104, 115] This opens up new opportunities for the synthesis of hybrid PNA–DNA and PNA–peptide molecules.

In addition to Boc and Fmoc protection, it was proposed to use MMT groups for protection of 5′-terminal primary amino groups.[96, 107–112, 116] Although the MMT group, like the Boc group, is acid-labile, this can be cleaved under considerably milder conditions than the Boc groups (the MMT groups are split off by treatment with 3% trichloroacetic acid).[107] In the MMT strategy, the amino groups of heterocyclic bases of the PNA monomers are usually protected by acyl groups (*e.g.*, acetyl, isobutyryl, anisoyl, benzoyl, *tert*-butylbenzoyl) (MMT/acyl version of the MMT strategy).[96, 107, 110, 116] The reaction conditions are mild, which makes it possible to perform automated synthesis of PNA oligomers using the oligonucleotide synthesisers, while their compatibility with oligonucleotide synthesis protocols allows one to obtain PNA–DNA chimeras.[107–109]

The efficient condensation requires that the 5′-terminal amino groups were not protonated, since in the form of cations they do not manifest nucleophilic properties. However, under basic conditions where the amino group is not protonated and hence is active, the undesirable transfer of the *N*-terminal acetyl group with the attached heterocyclic base to 5′-terminal primary aliphatic amino group may take place.[94, 97, 115, 117]

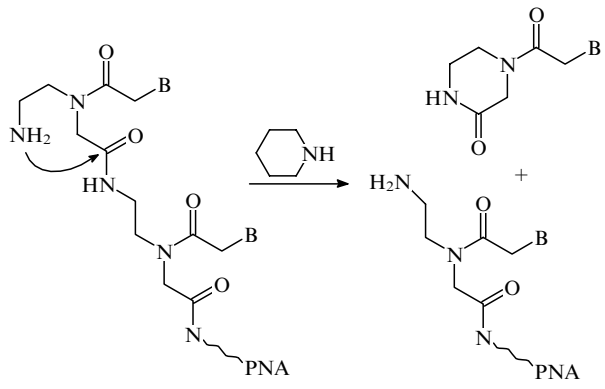


PNA — C-terminal fragment of the PNA oligomer.

This reaction can also occur under neutral conditions [94] resulting in the break of growing PNA chains and accumulation of short-chain oligomers. This affords a mixture of products and the isolation of target PNA presents a serious problem.

The formation of isomeric structures can be avoided provided the condensation is very fast. In this case, side products cannot be formed, since the *N*-acyl transfer is a rather slow process.[94, 97, 100, 117] In the presence of reagents based on uronium and phosphonium salts, the condensation occurs so fast that even *in situ* activation of carboxy groups of PNA monomers is possible.

Splitting of the *N*-terminal monomeric unit [12] under the action of piperidine used for removal of Fmoc groups is yet another side reaction.



It should be stressed that the problem of side reactions and the efficiency of PNA synthesis on the whole depend critically on the combination of protective groups used. Indeed, the Boc and Fmoc strategies differ essentially in the conditions of deprotection of 5′-amino groups of the last added monomer. As mentioned above, the removal of Boc groups requires rather drastic acidic treatment, which leads to the protonation of 5′-amino groups. Therefore, this group should be neutralised before the addition of the next monomeric fragment, which is not required in the case of the Fmoc strategy.

On the other hand, the removal of Boc groups is always quantitative and rather fast, whereas splitting of Fmoc groups by basic treatment proceeds slowly and not always completely,[118, 119] which negatively affects the efficiency of condensation.

Aggregation of growing oligomeric chains is an additional obstacle to efficient condensation. Interchain aggregation makes the deprotected terminal amino group only partly accessible for subsequent condensation, which decreases the efficiency of the process on the whole.[120, 121] It should be noted that aggregation is only possible in the case of the non-protonated amino group.[120, 121] Apparently, repulsion of positively charged amino groups prevents the aggregation of oligomers. Thus, the interchain aggregation never takes place in acidic media and terminal amino groups are more accessible to condensation. However, the amino group cannot efficiently react with the activated monomer, since its nucleophilicity is suppressed.

Deprotonation of terminal amino groups of PNA with simultaneous condensation (neutralisation *in situ*) is the most elegant approach to solving this problem. This methodology was first employed for peptide synthesis in 1987.[122] The use of neutralisation *in situ* in the synthesis of peptides using Boc and Fmoc protocols results in a significant increase in the condensation rate.[123–125] This effect was especially spectacular in the synthesis of 'problematic' sequences which are especially prone to undergo interchain aggregation. Their syntheses by conventional methods involving preliminary neutralisation of terminal amino groups face the most serious problems.[126, 127]

At present, neutralisation *in situ* has become very popular for the synthesis of PNA along with conventional methods where deprotonation of 5′-amino groups of PNA oligomers precedes condensation. In some cases, the use of neutralisation *in situ* helps solve the problem of side reactions of PNA isomerisation and increases the yields of condensation products.

VII. Some regularities of condensation reactions in the synthesis of peptide nucleic acids

A detailed knowledge of condensation reactions associated with PNA synthesis and a search for efficient procedures for its optimisation demand that the data available should be interpreted with due regard to the nature of reagents used for the activation of PNA monomers. The methods for the synthesis of PNA–DNA chimeras are described separately, since in this case condensation has a number of specific features.

1. Activated esters and carbodiimide activation

For the first time, the method of activated esters has been successfully employed for the synthesis of thymidine PNA oligomers.[1, 5] The synthesis of thymidine PNA using Boc-protected pentafluorophenyl (PFP) ester of a thymidine PNA monomer as a monomer was carried out in 1992.[5] When the monomer concentration was 0.1 mol litre^{-1}, the yield of the condensation product was > 99%. However, an attempt to apply this method to the synthesis of cytidine PNA oligomers was without success: the yield of the target product did not exceed 50% under identical conditions.[6]

There are some examples of successful applications of the method of activated esters for the synthesis of heterogeneous PNA. Thus the synthesis of PNA oligomers from Fmoc/Z-protected monomers by ester activation has been described.[97] This was one of the first examples of the implementation of the Fmoc protocol to the synthesis of PNA. In this case, the use of the activated esters strategy ensured high efficiency of condensation.

The yields of condensation products in the synthesis of PNA oligomers with the chain lengths of up to 20 residues varied from 95% to 99%.[97] The average yields in the synthesis of PNA containing all the four types of nucleobases were 97% over each step, which corresponds to the 70% yield of target oligomers. With allowance for subsequent isolation, the yield of PNA oligomers was 43%.

It is of note that the choice of strategies for the synthesis of PNA is often determined by the necessity to obtain high yields of condensation products at low expenditures of expensive PNA monomers where the use of manyfold (fourfold and higher) monomer excess is undesirable. A combined use of the Fmoc protocol and PFP activation allows the use of as little as a twofold excess of PNA in a single condensation.[97] The use of threefold (or greater) excesses of PNA monomers did not result in further increase in the yields of condensation products. The dimethyl sulfoxide (DMSO)–*N*-methyl-2-pyrrolidinone (MP) mixture (1 : 4) appeared to be the solvent system of choice.[97] Apparently, this system favours rapid access of the reagents to the growing PNA oligomers.[128] However, for other activation procedures, other solvent systems were more efficient.

The use of the activated esters approach to PNA synthesis sometimes gives reasonable results;[12] however, in the majority of cases, higher (94%–99%) yields of condensation products were obtained using the *in situ* activating reagents.[1, 5] In principle, the PNA synthesis can successfully be performed using the so-called carbodiimide activation involving, *e.g.*, dicyclohexylcarbodiimide (DCC) or *N*,*N*′-diisopropylcarbodiimide,[12] which sometimes affords high (up to 98%–99%) yields of condensation products.[6, 7] Low rates of PNA condensation is the main disadvantage of this activation procedure.[99]

Carbodiimide activation by DCC made it possible to obtain addition products of thymidine and cytidine PNA monomers in quantitative yields. Purine monomers are only partly incorporated into PNA oligomers; repeated condensation does not result in quantitative yields of the addition products.

The use of *N*,*N*′-diisopropylcarbodiimide as the activating reagent has made it possible to obtain nearly quantitative yields even for purine monomers.[99] However, this required the use of a fourfold excess of the monomers and the activating reagent, and the condensation lasted no less than 60 min.[99] In addition, the introduction of the adenine and the guanine monomers into PNA oligomers required two and three condensation cycles, respectively.

The carbodiimide activation is a convenient procedure for obtaining PNA adducts with other molecules. Thus the synthesis of hybrid PNA peptides containing biotin residues, which confer on PNA the ability to penetrate cell membranes efficiently, has been described.[129] The peptide fragment of the chimeric molecule was prepared using a standard peptide synthesis protocol,[130] while the synthesis of the PNA fragment was carried out manually, using the Boc strategy based on the methods described in the classical work by Nielsen.[94] The activation with DCC was performed in the presence of 1-hydroxybenzotriazole, using a fivefold excess of a PNA monomer. The condensation was performed at an elevated (37 °C) temperature to increase the yield of the target product.[129]

In other examples of the synthesis of hybrid PNA–peptide molecules,[49] the Boc protocol was combined with carbodiimide activation.[94, 129] Such an approach to the preparation of chimeras is justified, since it ensures complete compatibility of syntheses of both the peptide and PNA fragments of the hybrid molecules[49] and overall yields of target products of no less than 50%.[49]

A combined use of carbodiimide activation with the Boc/acyl protocol common in peptide synthesis,[130] allows one to obtain both classical PNA molecules with *N*-(2-aminoethyl)glycine backbones and molecules with non-canonical backbones containing

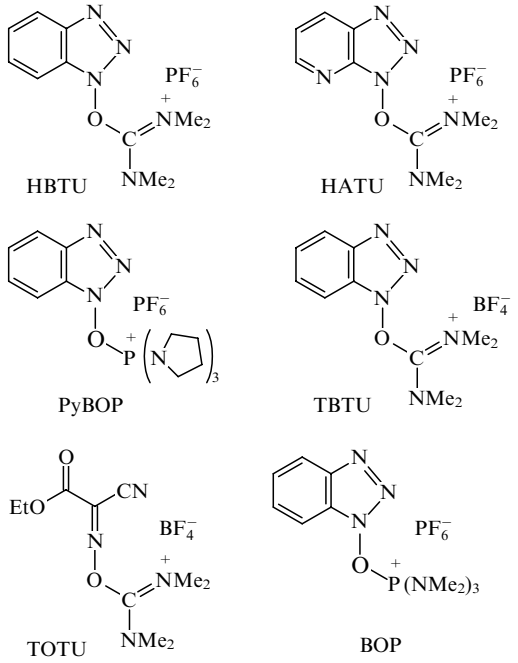
optically active monomeric fragments where the glycine residues are substituted by other amino acids, *e.g.*, lysine, serine, isoleucine and glutamic acid.[131] Incorporation of D-lysine-based monomers into PNA oligomers increases the stabilities of PNA – DNA and PNA – RNA duplexes. With other amino acids, the stabilities of these types of duplexes are usually low.[131]

In some cases, such as in the synthesis of PNA – peptide chimeras and thymidine PNA oligomers, the activated esters and carbodiimide methodologies are employed. Sometimes, the yields of condensation products prepared by the activated esters (PFP) method even exceed those obtained by *in situ* activation.[97]

Nonetheless, it is generally acknowledged [12] that PNA synthesis based on the use of *in situ* activation reagents, *viz.*, uronium and phosphonium salts, is the most reliable approach, since it ensures higher yields of the condensation products. This approach has considerably been developed in the recent years and it is this procedure that offers the broadest opportunities for the synthesis of PNA and their derivatives.

2. *In situ* activation by reagents based on uronium and phosphonium salts

The most popular activating reagents for PNA synthesis are *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), *O*-[(ethoxycarbonyl)cyanomethylideneamino]-1,1,3,3-tetramethyluronium tetrafluoroborate (TOTU) and (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), although other reagents are also known.



Since condensation strongly depends on the strategy used for PNA synthesis, in the first place, on the combination of protective groups, it seems expedient to classify the data on the *in situ* activation into three groups corresponding to Boc, Fmoc and MMT strategies.

a. The Boc strategy

The Boc/Z-modification of the Boc strategy is a classical approach to PNA synthesis. The dependence of the condensation efficiency of the Boc/Z strategy on different factors has been studied in sufficiently great detail.[94] This strategy makes it possible to obtain high yields of PNA containing > 15 units with all the four types of heterocyclic bases. The use of the carbodiimide activation procedure for the synthesis of such oligomers is usually less efficient.[94]

Studies of the effects of various factors, such as the nature of activating reagents, solvents, monomer concentrations, the nature of the organic base (tertiary amine), catalysts, *etc.*, on the efficiency of condensation in the *in situ* activation revealed that all of them are important for the optimisation of condensation conditions.[94]

Comparison of the efficiency of condensations under the action of some uronium activating reagents, *viz.*, the most popular reagents HBTU and TBTU and the relatively new reagents HATU and *O*-(1,2-dihydro-2-oxo-1-pyridyl)-*N*,*N*,*N*′,*N*′-bis-(tetramethylene)uronium hexafluorophosphate (HDPU), revealed that the condensation was efficient in all the cases under study, but the yields of addition products were the highest with HBTU.[94, 100]

It should be noted that these results are valid exclusively for a DMF – pyridine solvent system and for the concentrations of the PNA monomer and the base [diethyl(cyclohexyl)amine, DECHA] of 0.05 and 0.1 mol litre^{-1}, respectively. Under these conditions, the average yields of the condensation products were 92.2% – 97.1% irrespective of the nature of PNA monomers. However, conditions can be selected where other activating reagents will be more efficient than HBTU, *e.g.*, in other solvent systems or in the presence of other bases. This suggests that condensation conditions are to be chosen for each activating reagent.

Comparison of the efficiencies of HBTU and PyBOP has demonstrated the former to be superior under identical conditions. The overall yields of PNA oligomers using HBTU and PyBOP activation were 61% and 40%, respectively, which corresponds to average yields of 97.1% and 94.7% in each step.[94, 100] It is of note that this comparative study was carried out under conditions optimum for HBTU.

An important role in efficient condensation is played by the solvent system used. Thus in a DMF – pyridine mixture, the overall yield of the condensation product was 61%, whereas those in DMF or DMF – DMSO were 55% and 22%, respectively.[94] However, it was not indicated which monomers determined the lowest yields of PNA. Noteworthy, virtually none of the works cited in this review provide these data.

Studies of the dependence of the yield of a PNA oligomer on the nature of an organic base (tertiary amine) have shown that 4-dimethylaminopyridine (DMAP), DECHA, (dicyclohexyl)methylamine and (dicyclohexyl) ethylamine were as efficient as (diisopropyl)ethylamine (DIPEA) which is widely used in peptide synthesis.[94]

The nature of the tertiary amine only slightly affected the yields of the condensation product (93.7% – 95.3%). The condensation in the presence of DIPEA in the DMF – pyridine system is not optimum, since this amine reacts with the monomers to give insoluble salts. Better conditions for this reaction can be found. The condensation in the presence of DIPEA is efficient in MP – DMSO.

Studies of relationships between the yields of condensation products and concentrations of PNA monomers revealed that acceptable yields can be obtained when the monomers are used at concentrations no less than 0.1 mol litre^{-1}. At lower concentrations (0.05 mol litre^{-1}), the condensation proceeds as a rule too slowly resulting in the accumulation of side products from competing reactions.[94]

Addition of catalytic amounts of DMAP and 1-hydroxybenzotriazole to the reaction mixture may have a negative effect on the efficiency of condensation, although it is known that their addition sometimes favours the formation of amide bonds.[94]

The loading† of the polymeric support should not exceed 0.1 – 0.2 mol-equiv. g^{-1}, which is essential for the maximum yield

† Here, loading is expressed as the number of functional groups per unit surface area.

of the PNA oligomer. With a higher degree of loading, the efficiency of PNA synthesis is lower.[94, 100]

The use of the Boc/Z strategy ensures high (99.4%) yields of target products with *in situ* activation (HATU) in the presence of DIPEA.[95] In this case, the reaction mixture must contain a large (*e.g.*, sevenfold) excess of the PNA monomer with respect to loading of the polymeric carrier.[95] The amount of the activating reagent is usually reduced by 10%, while the tertiary amine is taken in a twofold excess with respect to the PNA monomer.[88]

Acceptable yields are obtained with a fourfold excess of the PNA monomer and by activation with HATU (the amount of HATU is 0.9 mol-equiv. with respect to a monomer) in the presence of DIPEA and lutidine.[47] The use of a fivefold excess of PNA monomers and a tenfold excess of DIPEA makes it possible to increase the overall yields of PNA oligomers to 92%.[17]

Very often, the yields can be increased upon preactivation of PNA monomers. To this end, the PNA monomer is mixed and incubated with the activating reagent for several seconds before being loaded onto the column.[95, 96, 109, 110, 113, 115, 116] Strictly speaking, here we do not deal with the *in situ* activation, however, the term '*in situ* activation' is conventionally related to the nature of the activating reagent rather than to the order of mixing of the reagents. It is of note that the methodology of preactivation does not imply the isolation of activated monomers. Usually, the amount of the activating reagent is 5% – 10% smaller than that of the monomer.

Preactivation of PNA monomers by incubating them with the activating reagent for 2 min and condensation in an MP – pyridine mixture make it possible to obtain PNA oligomers in overall yields of 90%. Depending on the length and composition of sequences, the yields of PNA vary from 68% to 90%.[88]

In a search for the most rational technique for PNA synthesis, an investigator has to select between a rapid and cheap synthesis with the use of a small excess of PNA monomers and low (but acceptable) yields and lengthy syntheses with large expenditures of expensive monomers and activating reagents, but giving nearly quantitative yields.

Attempts to specify the conditions for effective synthesis of PNA have been undertaken time and again.[95] Primary attention in the optimisation of PNA synthesis is usually given to such factors as the low cost and ease of synthesis of PNA monomers, nearly quantitative yields of condensation products, the simplicity and efficiency of the procedure for isolation of PNA oligomers from the reaction mixture after completion of the synthesis and the possibility to obtain chimeric DNA – PNA duplexes and ligand-containing PNA molecules.

Such a choice of optimisation parameters seems to be justified, however, other factors, such as reaction rate and economy, are no less important. Thus the rate of condensation should be high enough to minimise side reactions. The synthetic strategy should first of all be efficient and allow for economic expenditure of expensive PNA monomers and activating reagents. The latter factor is of crucial importance in large-scale syntheses of PNA.

While comparing the Boc/Z and the Boc/acyl strategies of PNA synthesis, preference is given to the latter, since it is thought to be more promising. Although the average yields of oligomers calculated per one condensation cycle are high in both cases, *viz.*, no less than 98% (Boc/acyl) and about 99% (Boc/Z), the yields of PNA oligomers in the case of the Boc/acyl synthetic strategy and conventional isolation procedure do not exceed 65% (relative to the support loading), and are as low as 20% according to the Boc/Z protocol.[95] In addition, the Boc/acyl strategy allows one to synthesise PNA – DNA chimeras and to prepare addition products of acid-labile ligands to PNA, which is inattainable in the case of the Boc/Z strategy.[95]

The synthesis of thymidine PNA oligomers makes use exclusively of Boc-protection of 5′-terminal amino groups, since the thymidine PNA monomer does not require protection of the heterocyclic base.[77, 79] Such oligomers are conveniently synthesised by manual techniques.[79] In this case, by analogy with previously developed procedures,[94, 131, 132] the Boc strategy is combined with HBTU activation in the presence of DECHA. This approach affords high yields of condensation products, but requires the use of a fourfold excess of PNA monomers.[79]

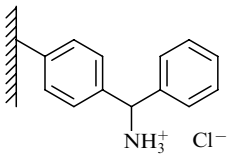
The Boc strategy is also used in the synthesis of PNA oligomers containing modified units.[131, 133 – 135] Thus PNA molecules may incorporate units containing anthraquinone and acridine residues.[133] Such oligomers are used to study the melting behaviour of hairpin-shaped PNA of high-molecular-mass PNA – PNA duplexes by fluorescence quenching (the so-called 'molecular beacon' method). High yields of condensation products are obtained. Its repeated condensation is used for the attachment of a modified PNA monomer followed by a non-modified monomer to PNA oligomers.[133]

The Boc/Z method combined with HBTU activation [94, 134] allows synthesis of PNA molecules with fluorescent labels at their N-termini.[134] Such PNA derivatives can be used for detecting specific NA sequences. The fluorescence of the label may increase more than 50-fold upon hybridisation of PNA with the complementary NA target in comparison with that of the free PNA.[134]

The classical Boc/Z strategy is also used for the synthesis of PNA chimeras containing clusters of modified fragments, *e.g.*, those with positively charged, chiral backbones built up of D-lysine residues.[135] The presence of such fragments in PNA confers useful properties on the latter.[131, 135] In contrast with ordinary PNA, chimeric PNA form exclusively antiparallel duplexes with DNA; their stability depends critically on the presence of mismatches. Even one mismatch decreases sharply the stability of hybrid duplexes. If a mismatch is located in the middle of clusters containing three modified residues, ten-membered PNA – DNA duplexes are unstable even at 15 °C (ΔT_m = 28 °C). Such PNA can serve as the basis for the development of genetic diagnostic tools, particularly, for the detection of point mutations.

Synthesis of these PNA derivatives by the Boc strategy combined with HBTU activation in the presence of DECHA,[131, 136] affords chiral oligomers in overall yields of 80% – 90%,[135] and optical purity of no less than 90%.

In the majority of cases, the Boc protocol includes the use of standard polymeric supports containing (4-methylbenzhydryl)-amino groups (MBHA).[11, 131, 132, 135, 136]



MBHA

PNA oligomers are cleaved from the solid phase by treatment with $CF_3COOH - CF_3SO_3H$.[11, 131, 136]

The Boc strategy allows the use of various activating reagents including BOP.[137] The use of BOP and DIPEA as a base affords high yields of condensation products in various solvent systems, *e.g.*, DMF – CH_2Cl_2 and DMF – DMSO. In this case, the use of a twofold excess of PNA monomers is sufficient, but each condensation reaction should be repeated in order to provide more efficient binding of the monomers.[137]

PNA can also be synthesised using the Boc/Z strategy combined with the *in situ* activation with TBTU in DMF – pyridine [113] as described in the classical work of Nielsen.[94] However, it is more expedient to carry out the condensation in DMF in the presence of a twofold excess of DECHA as a base with respect to the monomer. This is associated with the good solubility of PNA monomer salts formed in DMF.[113] The use of a threefold excess of PNA monomers is optimum. This approach allows one to use 2-amino-6-benzyloxypurine with the non-protected amino group as a guanine precursor.[113] The side reaction (capping) of the oligomeric chain can be avoided if the amount of TBTU is 10%

less than that of the PNA monomer [113] or if the PNA monomer is subjected to 5-min preactivation.[96]

Capping of oligomeric chains occurs both in PNA and peptide synthesis [123] provided the free activating reagent is present in the reaction mixture. This is possible owing to the fact that the terminal amino group of the peptide following deprotection reacts with both the activated amino acid derivative and the activating reagent.[123] Thus in the presence of an excess of HBTU, the tetramethylguanidine derivative was formed, which did not undergo subsequent elongation of the peptide chain.[138] This side reaction can be avoided if the amount of the activating reagent is smaller (by 5% – 20%) with respect to the monomer.[113]

With TBTU as the activating reagent, another side reaction, *viz*., the *N*-acyl transfer, can take place.[94, 113, 123] To avoid this, neutralisation *in situ* is used.[123] In this case, condensation is carried out in the presence of a base without preliminary neutralisation of the 5′-terminal primary amino group of the oligomer.[113] The efficiency of condensation is monitored by HPLC analysis of aliquots obtained by appropriate treatment of a small portion of a polymeric carrier (3 – 5 mg) following attachment of the next monomeric unit.[113]

In the synthesis of thymidine PNA oligomers based on the use of the Boc strategy and TBTU activation, a decrease in the yields of condensation products is observed sometimes after addition of the first 3 – 4 monomeric fragments.[113] This leads to the accumulation of short chains, whereas the overall yield of PNA does not exceed 30% (in the case of a 10-membered oligomer). The same problem sometimes arises with HATU activation, presumably, due to aggregation of growing thymidine PNA oligomers. This can partly be overcome through the attachment of lysine residues at the C-ends of PNA chains, which prevents the interchain aggregation of the oligomers formed.

It is noteworthy that the synthesis of heterogeneous PNA oligomers containing monomeric fragments of all the four types is not accompanied by significant reduction of product yields in the PNA synthesis.[113] In this case, the yield over each condensation step reaches 97%, which corresponds to 66% overall yield of the target 16-membered oligomer.

Similar problems, *viz*., aggregation of PNA chains, may arise during the removal of protective Z groups after completion of PNA synthesis resulting in a significant decrease in the yields of PNA.[11, 113] A crucial role in this process belongs to complementary interchain and intramolecular coupling of PNA oligomers. Even four-membered PNA – PNA duplexes significantly hinder post-synthetic work-up of PNA oligomers.[113] Therefore, oligomeric PNA sequences should be analysed for the possibility of intramolecular hairpin and intermolecular cluster formation prior to the synthesis. It was found that the overall yields of PNA can be increased to 75% and even more through incorporation of a lysine residue into PNA heterooligomer, since the charged ε-amino group of lysine partly prevents the aggregation of PNA chains.

These data suggest that the efficiency of synthesis of PNA oligomers strongly depends on the properties of PNA sequences to be prepared irrespective of the synthetic procedure used.[139] Theoretically, PNA may contain any combination of monomers, while the synthesis of certain sequences in quantitative yields faces difficulties. Thus the attachment of a purine monomer to an oligomeric PNA sequence containing a 5′-terminal purine base is problematic. Therefore, if PNA containing purine clusters are to be synthesised, the synthetic protocol should include repeated condensations.[139] The lowest yields were obtained in the synthesis of PNA oligomers containing several consecutive guanine residues.

In conclusion, it may be said that the Boc strategy can be implemented in both manual and automated variants.[139] Although the former seems to be rather efficient and inexpensive,[140] automated synthesis using peptide and oligonucleotide synthesisers holds especially great promise.[141] The reason is that the manual synthesis of PNA is not only lengthy and laborious, but also needs large-scale synthesis (not less than 5 mmol) in order to obtain acceptable yields of condensation products.

b. The Fmoc strategy

It is generally recognised that the Fmoc strategy of PNA synthesis[14, 77, 96, 100 – 106, 114] requires milder conditions than the Boc strategy.[12] In particular, no treatment of PNA oligomers with strong acids before and after synthesis is necessary. This allows the use of other protective groups for exocyclic amino groups of heterocyclic bases of the PNA monomers. Correspondingly, the Fmoc strategy affords higher degrees of purity of reaction mixtures and higher overall yields of PNA oligomers. The Fmoc/acyl strategy of PNA synthesis is especially promising,[77, 104, 105] since it can also be used for the synthesis of PNA – DNA and PNA – peptide chimeras.

Various versions of the Fmoc strategy are currently known. Thus Bhoc protection of heterocyclic bases of the PNA monomers and HATU activation allows automated synthesis on oligonucleotide synthesisers.[106] The use of a DIPEA – lutidine mixture seems to be more effective than the use of only one base, since it affords higher yields of the condensation products.[106]

DMF is a suitable solvent for the Fmoc condensation, and HATU is one of the most potent activating reagents.[141] However, in this case, too, the synthesis of individual PNA oligomers may face problems related to non-efficient condensation.[141] Thus the synthesis of PNA oligomers with sequences containing several identical consecutive heterocyclic bases (not necessarily purines) yields short-chain products. This problem can partly be overcome through the use of repeated condensations.[141]

Two condensation cycles are carried out in the following cases: in the synthesis of sequences containing four or more identical consecutive residues after attachment of two or three identical PNA monomers to the oligomer, in the synthesis of PNA oligomers containing purine clusters and in the synthesis of 18-membered and more extended PNA oligomers independent of the composition of the sequence after the attachment of the 17th residue.[141]

The yields of PNA obtained using the Fmoc strategy and HATU activation vary from 26% to 38%.[141] In some cases, the overall yields of PNA oligomers do not depend on the sequence lengths but are determined by the efficiency of the attachment of the first PNA monomer to the polymeric support, since the yield of the first condensation product can be lower than the yields of the products obtained in subsequent condensations.[141]

In the majority of cases, reversed phase HPLC is used for the analysis of reaction mixtures and isolation of target products under conditions for the separation of peptides rather than oligonucleotides.[141] The condensation efficiency of the Fmoc strategy can also be estimated spectrophotometrically by measuring UV absorption of a product formed upon removal of Fmoc groups by piperidine in the range of 256 – 301 nm. This method is especially convenient for stepwise monitoring of PNA oligomerisation.[97]

Thus, the Fmoc strategy can be used for the synthesis of PNA oligomers unattainable by other methods. This procedure allows modifications to the synthetic protocol aimed at increasing the yields of condensation products in separate synthetic cycles.[141]

The low efficiency of the condensation encountered in the Fmoc strategy of PNA synthesis seems to have the same reasons and solutions as those in peptide synthesis.[142] It is known that many problems of automated solid-phase peptide synthesis are related to the nature of the sequence to be synthesised.[142] Low condensation yields are due to the formation of bulky spatial peptide structures, which may interfere with the formation of amide bonds.

Those peptide chain fragments which are susceptible to interchain aggregation, can also decrease the accessibility of the amino group and thus prevent further elongation of the chain.[120]

Spatial hindrances appear in the course of PNA synthesis which decelerate the removal of protective Fmoc groups from the 5′-termini of PNA oligomers and decrease the efficiency of subsequent condensation.[143]

The synthesis of purine-rich PNA presents a special problem. If a PNA sequence contains more than two consecutive purine residues, the efficient attachment of the next purine monomer requires a longer reaction time and repetition of the condensation procedure three or four times. The attachment of the guanine monomer to the 5′-terminal guanine unit proceeds at a very slow rate.

Aggregation of PNA chains is the main reason for low condensation efficiency inherent in both Fmoc and Boc strategies. The synthesis of partly or fully self-complementary sequences may produce problems relevant to intrachain association of PNA oligomers. In addition, the solid-phase synthesis conditions favour intermolecular aggregation of growing oligomers. Thus purine-rich PNA and thymidine PNA oligomers (both four-membered and more extended ones) are prone to aggregation.

The condensation efficiency strongly depends on such factors as the loading of the polymeric support and the composition of the solvents. Thus, low densities of oligomers 'growing' on solid supports favour solution of the problem of intermolecular aggregation of PNA chains, whereas certain solvents prevent inter- and intramolecular aggregation of PNA molecules (*e.g.*, the formation of hairpin structures) and ensure effective diffusion of reagents to the N-termini of the growing PNA chains.

Studies aimed at optimisation of conditions for PNA synthesis are currently under way, since no ideal method for PNA synthesis has been developed so far. The Fmoc/acyl strategy of PNA synthesis seems to be the most advanced one, since it permits one to obtain oligomers of virtually any composition in high yields.[104, 115]

Thus the synthesis of thymidine PNA oligomers by the Fmoc/acyl method combined with HATU activation requires only small (twofold) excess of the monomer [115] and a 20% deficiency of the activating reagent. It is more expedient to use MP as a solvent and the DIPEA – lutidine mixture as a base. Preactivation of PNA monomers for 2 min is yet beneficial. The yield of the condensation product in each step is 85% – 90%, the condensation time is 30 min and the overall yields of the 7-membered oligomer is no less than 50%.[115] The efficiency of the reaction is most conveniently monitored spectrophotometrically.

Attempts have been made to optimise condensation conditions in solution using mixtures of PNA monomers and amino acid esters as model systems.[115] Direct application of the data obtained in these model systems to solid-phase PNA synthesis is questionable, since the optimum conditions for the synthesis in solution and on polymeric supports may differ in principle. This circumstance should be taken into consideration when selecting model systems. On the other hand, these studies sometimes give valuable results.

The yields of condensation products in the reaction of PNA monomers with L-phenylalanine *tert*-butyl ester were not lower than 95% irrespective of the nature of the activating reagent (HATU or HBTU in the presence of 1-hydroxybenzotriazole), of the tertiary amine (*N*-methylmorpholine, lutidine or the DIPEA – lutidine mixture) and of the solvent (DMF or MP). The nature of the base and the activating reagent did not influence the efficiency of the reaction. The best result was obtained in the case of HATU activation in the presence of lutidine in DMF.[115]

As mentioned above, the overall yields of PNA oligomers may depend on the efficiency of attachment of the first monomer to the polymeric support. Thus the yield of the attachment product of the first cytosine monomer to the polymeric support (Tentagel) in the synthesis of heterogeneous PNA by the Fmoc/acyl strategy combined with conventional HBTU activation in the presence of 1-hydroxybenzotriazole and lutidine did not exceed 50%,[115] whereas those obtained in subsequent condensation steps were no less than 80%. The efficiency of attachment of the first PNA monomer can be increased to 80% and even higher using repeated condensation. In this case, the time for each condensation can be reduced.[115] Low yields of the attachment products of the first monomeric unit to a polymeric support have also been reported.[141]

The best conditions for the PNA synthesis by the Fmoc/acyl strategy include the use of a twofold monomer excess (0.125 mol litre^{-1}), preactivation with HATU (0.8 mol-equiv.), DIPEA and lutidine (1 – 2 min), condensation (20 min) and repetition of the condensation procedure in each (with the exception of the first) reaction cycle. This provides an overall yield of heterogeneous PNA of 43% (for 16-membered oligomers), which corresponds to the average yield of the condensation product of 95% (in this case, the attachment of the first monomeric fragment occurs with high yield). The main disadvantage of this method is the necessity to repeat the condensation procedure in each cycle.[115] The expedience of this approach is doubtful despite its obvious advantages, *viz.*, good reproducibility of experimental results.

c. The MMT strategy

This strategy of PNA synthesis is rather promising [38, 96, 107 – 112, 116, 144, 145] particularly its MMT/acyl version,[96, 107, 110, 116] which is fully compatible with oligonucleotide synthesis protocols. It allows the use of automatic DNA synthesisers without modification of their design or software, which is especially convenient for conducting the syntheses of PNA – DNA chimeras.

The MMT strategy allows the use of a broad range of activating reagents,[38, 112, 144, 145] including mesitylenesulfonyloxybenzotriazole (TMSOBt) [112] and 3,4,6-triisopropylbenzenesulfonyloxybenzotriazole (TPSOBt).[144] The latter gives higher yields of the condensation products (the average yield in one cycle reaches 96%) than the commercially available activating reagent PyBOP.[112, 144] Depending on the nature of the PNA monomer and the 5′-terminal oligomeric fragment, the yield in each condensation step varies from 91% to 99%.[144]

The efficiency of condensation carried out by the MMT strategy can be estimated spectrophotometrically by measuring UV absorption spectra of the MMT^+ cation formed upon removal of the protective MMT group from the 5′-terminal amino group of a PNA oligomer. The MMT strategy combined with TPSOBt activation is compatible with oligonucleotide synthesisers.[112] This approach was first used in the synthesis of PNA molecules containing uracil residues.[112] The 'manual' variant of the MMT strategy is also effective, *e.g.*, in the synthesis of thymidine PNA oligomers.[38]

The use of PyBOP as an activating reagent allows one to reach 95% – 99% yields in each step of the monomeric fragment coupling.[96] However, in this case, the use of concentrated (0.3 M) solutions of PNA monomers and PyBOP is necessary. It is more expedient to use *N*-ethylmorpholine as a base and to perform preactivation of PNA monomers.[96]

The MMT strategy is used for the synthesis of phosphonate analogues of PNA (pPNA).[110, 116, 146, 147] The presence of negatively charged groups in the pPNA backbone makes PNA analogues readily soluble in aqueous solutions in comparison with classical PNA oligomers. These molecules bind specifically to complementary fragments in DNA and RNA, although the melting temperatures of pPNA – NA complexes are somewhat lower than those of the corresponding PNA – NA complexes.

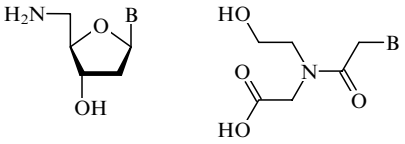
A combination of the MMT/acyl protocol with triisopropylbenzenesulfonylnitrotriazole activation is efficient in the synthesis of pPNA.[110, 116, 146, 147] The condensation in the presence of *N*-methylimidazole as a nucleophilic catalyst permits one to obtain the average yields of 95% in the condensation step with a reaction time of 10 min.[110, 116] However, the quantitative overall yields of condensation products are not achieved, although the use of dilute solutions of PNA monomers (0.05 M) and the activating reagent (0.06 M) together with preactivation (mixing of the PNA monomer with the activating reagent and *N*-methylimidazole), makes this procedure attractive.[110, 116]

3. Some peculiarities of the synthesis of PNA – DNA chimeras

The interest in PNA – DNA chimeras has arisen in the past decade, which gave a strong impetus to the development of methods for their synthesis.[77, 109, 111, 144, 148 – 150] The use of classical PNA in biochemical studies is limited due to their poor solubilities in aqueous solutions, proneness to self-aggregation[4, 12, 34] and low penetrability through cell membranes.[4, 9, 84, 151] The latter is the main obstacle for the use of canonical PNA oligomers as antisense agents *in vivo*.[9]

Chimeric PNA – DNA molecules are devoid of most of these drawbacks. They possess all the advantages of PNA together with valuable properties inherent in NA. Indeed, the PNA – DNA chimeras synthesised so far combine high biological stabilities, high affinities and selectivities of binding to NA targets typical of PNA with perfect solubilities and the ability to activate hydrolysis of RNA targets by RNAse H, which are characteristic of DNA.[9, 34] Chimeric PNA – NA molecules have various applications, *viz*., they are promising therapeutic (including antisense) drugs[9] and can be used as a basis for highly effective diagnostic tools and highly sensitive biomolecular probes.[77, 96, 107 – 109, 144, 148, 149] Synthesis of hybrid PNA – NA molecules manifests specific features, which necessitates a consideration in a separate section.

The correct choice of linkers between PNA and DNA fragments of the chimeric molecules is one of the most important problems. Depending on whether the 5′-terminal fragment of the hybrid molecule belongs to PNA or DNA, the linker used is represented either by modified nucleosides (*e.g*., 5′-amino-2′,5′-dideoxynucleosides[77, 149]) or modified PNA monomers [*e.g*., *N*-(2-hydroxyethyl)glycine derivatives].[148]



Syntheses of both types of PNA – DNA hybrids, *viz*., 5′-PNA – DNA-3′ and 5′-DNA – PNA-3′, have been described. Owing to the charged backbones of their DNA fragments, PNA – DNA chimeras are perfectly soluble in aqueous solutions, which makes possible their isolation and analysis by standard methods, such as polyacrylamide gel electrophoresis and ion-exchange reversed phase HPLC.[108]

In the case of 5′-terminal PNA fragments, PNA and DNA fragments are linked by the amide bond and 5′-amino-2′,5′-dideoxynucleosides are used as linkers.[77] Such hybrid molecules are synthesised by various methods. Thus 5′-PNA – DNA-3′ hybrids are prepared according to Boc/Z protocols commonly used in the synthesis of PNA-peptide conjugates.[152] However, the use of this technique for the synthesis of PNA – DNA chimeras containing purine nucleotide residues may result in acid-catalysed apurinisation of the DNA fragment during deprotection of heterocyclic bases of PNA.[77] Therefore, this approach is used exclusively for the synthesis of PNA – DNA hybrids the DNA fragments of which contain more stable pyrimidine nucleotides, whereas 5′-PNA – DNA-3′ chimeras are more efficiently synthesised using the Fmoc/acyl strategy.[77]

If hybrid molecules contain 5′-terminal DNA fragments, the PNA and DNA parts can be linked by phosphoramide bonds without any linkers. In this case, the synthesis of PNA fragments is carried out using the Fmoc/acyl protocol. With the thymidine monomer, Boc-protection of the 5′-terminal amino group is possible. The activation is performed with HATU in the presence of DIPEA and DMAP; the condensation is carried out in DMF. In this case, the use of acid-labile groups for protection of heterocyclic bases of PNA monomers is inadmissible because of easy acid hydrolysis of phosphoramide bonds.[77]

The synthesis of PNA fragments of such chimeric oligomers usually employs the MMT/acyl strategy. In this case, the conditions of PNA synthesis are compatible with those of oligonucleotide synthesis;[36, 149] PNA fragments can be synthesised by manual techniques. The condensation is performed in the DMF – pyridine mixture with 2-[2-oxo-1(2*H*)-pyridyl]-1,1,3,3-bis(pentamethylene)uronium tetrafluoroborate (TOPPipU) as the activating reagent and DECHA as the base.[153] Under these conditions, the condensation of thymidine and cytidine PNA monomers proceeds smoothly, purine monomers are attached inefficiently to the growing PNA chain.[149] If HATU is used as the activating reagent, DIPEA as the base and acetonitrile as the solvent in the presence of a fivefold excess of PNA monomers (necessary to attain high yields of condensation products), the reaction time is no less than 15 min.[34] This method was used for the synthesis of chimeric molecules containing 5-bromouracil and 5-methylcytosine residues.[34] The incorporation of 5-methylcytosine residues into the PNA chains of chimeric molecules increases the stabilities of their duplexes and triplexes with complementary DNA.[34]

The modified thymidine PNA monomer based on *N*-(2-hydroxyethyl)glycine[34, 107, 148] is often used as a linker between DNA and PNA fragments of chimeric molecules of the 5′-DNA – PNA-3′ type; the latter can be synthesised using both Boc[148] and MMT strategies.[34, 107] If the Boc/Z strategy is used, the PNA synthesis is carried out on a solid phase; the linkers are attached under the same conditions.[148] Subsequent synthesis of DNA fragments of hybrid molecules should also be performed on the solid phase. It is inadmissible to perform the synthesis of DNA fragments in solution, since the solubility of PNA oligomers devoid of protective groups in organic solvents is insufficient to provide efficient condensation with phosphoroamidite derivatives of nucleosides.[148]

The solid-phase MMT method is more suitable for the synthesis of the 3′-PNA fragments; after completion of PNA synthesis, solid-phase synthesis of the DNA fragment is continued without detachment of the oligomer from the support. This prevents the use of PNA monomers the heterocyclic bases of which are protected by acid-labile groups, since the DNA fragments will not withstand acid treatment used to remove protective groups. This synthetic procedure is inapplicable to chimeric molecules containing PNA monomers of all the four types, but can be used for the synthesis of hybrid molecules with the pyrimidine monomers constituting the PNA fragments.[148]

The MMT/acyl modification of this method is devoid of these disadvantages and allows the synthesis of PNA – DNA hybrids containing all the four types of nucleobases in both DNA and PNA fragments.[108, 109, 150]

The MMT/acyl strategy allows the application of the fully automated protocol on oligonucleotide synthesisers. The DNA fragments of hybrid molecules are usually synthesised according to a conventional phosphoroamidite protocol, which makes use of commercial 2′-deoxynucleoside phosphoroamidites.[108] This method of synthesis of PNA – DNA chimeras has practically no drawbacks.[108, 150]

The synthesis of PNA fragments of 5′-PNA – DNA-3′ oligomers may involve HBTU activation in the presence of DIPEA in DMF – acetonitrile.[150] Solutions of PNA monomers and activating reagents should be used at concentrations of no less than 0.1 M (preferably, 0.2 M); preactivation is also desirable. A commercially available aminohexanol phosphoroamidite derivative can be used as a linker between the PNA and DNA fragments; this is attached to the 5′-end of a DNA fragment by a standard oligonucleotide synthesis protocol. Then the synthesis of a 5′-terminal PNA fragment of a chimeric molecule is followed.[150]

The MMT/acyl strategy is used in the synthesis of chimeric molecules with the composition 5′-PNA – DNA – PNA-3′.[109] In this case, both PNA fragments are synthesised in an automated regime using HBTU activation in the presence of DIPEA in DMF – acetonitrile mixture; this may require an eightfold excess

of the reagents with respect to the carrier loading. Preactivation of PNA monomers makes it possible to increase the condensation efficiency and the average yields in the attachment of monomeric fragments up to 96%.[109]

Obviously, the problem of efficiency of each individual approach to the synthesis of PNA molecules has no unambiguous solution. The choice of the most adequate strategy for the PNA synthesis depends on the goal and facilities as well as on the number of PNA oligomers to be synthesised, the scale of synthesis, composition and purity of PNA oligomers.

VIII. Conclusion

The design of the most efficient method for the synthesis of PNA oligomers requires that a rational compromise between the efficiency and economy of the synthetic process be found.

On the one hand, one has to reach the maximum yields of condensation products and the choice of synthetic strategy must take into account both the nature of the activating reagent and other factors discussed in this review.

On the other hand, the synthesis of PNA should be rational. This implies that the synthetic procedure should not only be efficient, but also fast and as cheap as possible. Examples of both the approaches to PNA synthesis have been presented in this review.

In low-budget laboratories, where the primary goal is economic PNA synthesis, it is the 'slow' synthesis that is most commonly used. Although this procedure is rather laborious, it gives excellent yields in the condensation step.

'Fast' processes are utilised in the majority of large biotechnological companies which manufacture PNA oligomers for commercial purposes. Here, large excesses of PNA monomers and the most potent activating reagents are employed in order to ensure high yields of the condensation products. However, quantitative yields cannot be attained due to a reduction of the condensation time; therefore, pure PNA oligomers can be obtained by virtue of complex isolation procedures.

The most rational synthetic strategies combine the best features of both approaches, *viz*., the 'fast' and the 'slow' syntheses of PNA.

This work has been written within the framework of the State Programme for Support of Leading Scientific Schools of the Russian Federation (Grant No. 00-15-97944).

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