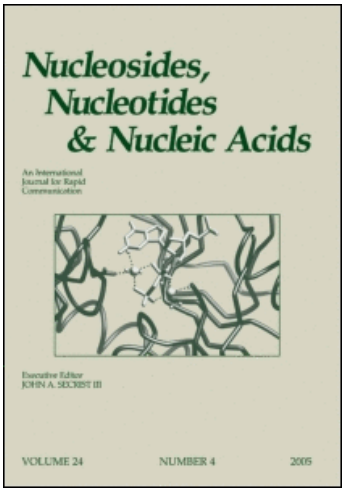

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The Solution Synthesis of Antisense Oligonucleotide-Peptide Conjugates Directly Linked via Phosphoramide Bond by Using a Fragment Coupling Approach

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ABSTRACT

To improve antisense oligonucleotide penetration inside cells, conjugates of oligonucleotides and cell-penetrating peptides, covalently linked through a phosphoramide bond, were prepared by a fragment coupling approach in the liquid phase. Two methods were used for this synthesis, i.e., phosphorylation of a peptide amino group by an oligonucleotide terminal phosphate 1-hydroxybenzotriazole ester in aqueous media or condensation of phosphate and amino groups in presence of triphenylphosphine, 2,2′-dithiopyridine and 4-dimethylaminopyridine in organic media. Several oligonucleotides, including a 18-mer antisense oligodeoxyribonucleotide complementary to an internal coding region of the reporter gene of the green fluorescent protein (GFP) were prepared. Peptides derived from the third helix of the homeodomain of

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Antennapedia, the influenza envelope hemagglutinin subunit as well as melittin and polymyxin B were used for the conjugates' synthesis. The peptides with various amino acid composition were chosen to confirm that these coupling methods are of a general use.

Key Words: Oligonucleotide-peptide conjugates; Antisense oligonucleotides; Conjugate synthesis.

INTRODUCTION

Covalent conjugates of oligonucleotides with different molecules, in particularly with peptides, have attracted an increasing attention during the last few years as potential therapeutic agents.[1–4] Synthesis and applications of a number of oligonucleotides covalently linked with peptides introducing such properties as heightened DNA or RNA binding, specific RNA cleavage, improved transport through cellular membrane have been described.[5,6] The overwhelming majority of peptide-oligonucleotide conjugate applications belong to the antisense biotechnology where the peptide sequence provides the conjugate penetration inside cells.[7,8]

Two main strategies have been proposed for preparing peptide-oligonucleotide hybrid molecules, i.e., the solid phase synthesis of the whole conjugate and the assembling of peptide and oligonucleotide fragments in the liquid phase.

The most direct way is a total on-line synthesis of peptide-oligonucleotide conjugates on a solid support.[9–16] In this approach, a conjugate synthesis is usually begun by preparation of a peptide part and followed by oligonucleotide synthesis,[9–12] or a peptide and an oligonucleotide may be synthesized in series on a bifunctional branch linker attached to the polymer support.[13–16] In some studies, a peptide synthesis follows the oligonucleotide synthesis in on-line approach.[17] The other solid phase approach consists in the previously synthesized peptide joining to an oligonucleotide attached to a solid support.[18–20]

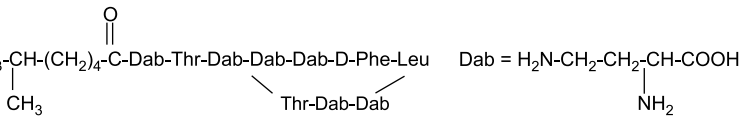
However, the incompatibilities of the peptide and oligonucleotide chemistries lead to a restricted number of sequences that can be prepared by the solid phase strategy. The liquid phase fragment conjugation strategy could avoid these limitations, because oligonucleotides and peptides are synthesized separately, and then they are combined. Nevertheless, this approach has also some problems mainly associated with the solubility of the reaction components. Since oligonucleotides are well water soluble, it would be better to carry out conjugation reactions in aqueous solutions. On the other hand, peptides commonly used for conjugation reactions are mainly soluble in organic solvents.

In order to be joined, the oligonucleotide and/or peptide need to be modified by some reactive moieties, such as amino,[21–23] maleimide,[24,25] iodoacetyl,[26,27] or thiol[28–30] groups. Nevertheless, there is a possibility to avoid this additional modification and to combine directly a terminal oligonucleotide phosphate and a peptide amino group via a phosphoramide bond, if the phosphate is previously activated. There are at least two methods to produce the oligonucleotide phosphate activation that allows it to react efficiently with amino groups in different media. The first one is the activation by 1-hydroxybenzotriazole,[31,32] when the resulting active ester can be used for conjugation with a peptide in water or water-organic solutions. The second method is the

I H-Gly-Leu-Phe-Gly-OH

II H-Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-OH

III H-Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Asn-Gly-Trp-Asp-OH

IV 

V H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2

VI H-Arg-Gln-Ile-Lys(Fmoc)-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys(Fmoc)-Trp-Lys(Fmoc)-Lys(Fmoc)-NH_2

VII H-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2

VIII 5' CTCCTTCTG 3'

IX 5' GAGCTGCACGCTGCCGTC 3' GFP antisense

X 5' CTGCCGTCGCACGTCGAG 3' GFP control

Figure 1. Structures of peptides and oligonucleotides used for the conjugate synthesis.

oligonucleotide phosphate activation in organic media using triphenylphosphine, 2,2′-dithiopyridine and 4-dimethylaminopyridine.[33]

The present work is aimed on the elaboration of the direct oligonucleotide and peptide coupling via the phosphoramide bond in aqueous, aqueous-organic and organic media. A model 9-mer oligonucleotide and two 18-mer oligonucleotides, an antisense complementary to an internal coding region of the reporter gene of the green fluorescent protein (GFP) and a control oligonucleotide with the inverted sequence, were used to prepare conjugates (Fig. 1, compound VIII, IX and X respectively). Cell penetrating peptides, such as: *Antennapedia* third helix peptide 43–58 (penetratin-1), hemagglutinin envelope fusion peptide, melittin and polymyxin B, were chosen as peptide fragments of the conjugates (Fig. 1, compound I–VII).

EXPERIMENTAL PROCEDURES

Materials

Controlled pore glass (CPG) support derivatized with protected 2′-deoxynucleosides and nucleosides 2-cyanoethyl-N,N′-diisopropylphosphoramidites for the oligonucleotide synthesis were purchased from Applied Biosystems. [γ-^{32}P]ATP (1000 Ci/μmol) was supplied by Isotop (Russia). T4 polynucleotide kinase (10000 units/ml) was obtained from Sibenzyme (Russia). Boc-protected amino acids were obtained from Reanal. Proteinase K from Tritidanium album (>30 units/mg protein), DCC, EDC, and CTAB were from Sigma. Anhydrous solvents were prepared using standard techniques.

Oligonucleotides

Oligonucleotides 5′-CTCCTTCTG-3′ (VIII), 5′-GAGCTGCACGCTGCCGTCp-3′ (IX) and 5′-CTGCCGTCGCACGTCGAGp-3′ (X) were synthesized using Applied Biosystems 380B DNA synthesizer by the standard cyanoethyl phosphoramidite procedure and purified by reversed phase and ion-pair HPLC using Altex (USA) and Tracor (Netherlands) apparatus. The 5′-end of the oligonucleotides were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by the shipper.

Peptides

Polymyxin B sulfate (IV) was supplied by Sigma and melittin (V) was obtained from Fluka (Switzerland). Peptides (I–III) from HA-2 subunit of influenza hemagglutinin were synthesized by the solution fragment condensation method using HOBt/DCC as coupling reagents and Boc- and methyl or benzyl ester groups for amino and carboxyl group protection, respectively.[34] Fmoc-protected peptide 43–58 from homeodomain of *Antennapedia* (VI) was synthesized by manual solid phase technique utilizing Boc-strategy,[35] starting with MBHA-resin. Peptide VI was cleaved from the resin using HF/p-cresol mixture and purified by reverse phase HPLC on a Lichrosorb RP-18 column using a Knauer HPLC apparatus (Germany). Peptide 43–58 from homeodomain of *Antennapedia* (VII) was obtained after deprotection of the peptide VI by its treatment with 20% piperidine solution in DMF. The mass spectra were recorded on a VG ZAB SE FAB mass spectrometer: M (exp) = 3184.5; M (theor) = 3183.9 and M (exp) = 2244.8; M (theor) 2244.3 for peptides VI and VII, respectively. Based on amino acids and HPLC analysis, the purity of the peptides I–III, VI and VII was higher than 95%.

Oligonucleotide-Peptide Conjugate Synthesis

Method 1

An oligonucleotides (1–50 nmol) were solubilized in 10 μl of water and 60 μl of 1 M HOBt solution in 60% aqueous DMF, pH 4.5, were added. The mixture was thoroughly mixed and supplemented with 5 mg (25 μmol) of EDC. After incubation for

Table 1. Syntheses of the oligonucleotide CTCCTTCTG (VIII) conjugates with ornithine and short hydrophobic peptides (I) and (II), incubation time 16 h, 18°C.*

Amino compound	Method	Amino compound concentration, M	Medium			Coupling efficiency** %
			Buffer***	DMF % (v)	pH	
ornithine	1	10^{-2}	N-MeIm	0	9	60–65
					11	95
			N-MeIm	50	9	85
					11	95
			N-MeIm	85	9	90
					11	95
		10^{-3}	N-MeIm	0	11	65–70
				85	11	80–85
		10^{-4}	N-MeIm	85	11	5
H-GLFG-OH (I)	1	5×10^{-3}	N-MeIm	70	9	45–50
			N-MeIm	95	11	25–30
			TEA	95	11	25–30
	2	5×10^{-3}	–	100	–	90–95
H-GLFGAIAG-OH (II)	1	5.7×10^{-3}	N-MeIm	95	9	20–25
	2	5.7×10^{-3}	–	100	–	80–85

*Oligonucleotide component concentration was 2×10^{-5} M.
**Calculated on the base of gel electrophoresis analysis of the ^{32}P-labeled oligonucleotide. The coupling efficiency was expressed in % of the total oligonucleotide radioactivity.
***pH value was achieved by adding TEA to the reaction mixture or by using 0.4 M N-methylimidazole buffer, containing 0.1 M NaCl.

2.5–3 h at 8°C, HOBt esters of oligonucleotides were twice precipitated with 10-fold excess of 2% $LiClO_4$ solution in acetone and washed by acetone.

Then the oligonucleotide HOBt esters (1–50 nmol) were incubated with 50–80 μl of a solution of a peptide (or ornithine) (see Table 1 for the peptide concentration and medium composition) for 10–16 h at 18°C. After the incubation, oligonucleotide-peptide conjugates were precipitated with 0.5 ml of 2% $LiClO_4$ solution in acetone, washed by acetone and purified by electrophoresis in 20% PAGE, 7 M urea, TBE.

Method 2

An oligonucleotide (1–50 nmol) was precipitated from its aqueous solution by CTAB (1.5 equiv. of CTAB per one oligonucleotide phosphate group), the sediment was dissolved in 50 μl of DMF and the solution was supplemented by 5 mg (41 μmol) of DMAP in 15 μl of DMF, 6.6 mg (30 μmol) of Py_2S_2 in 15 μl of DMF and 7.9 mg (30 μmol) of Ph_3P in 15 μl of DMF.

A peptide (0.5–5 μmol) and an equimolar amount of DIEA were dissolved in 20–30 μl of DMF or DMF:DMSO (1:1) mixture and added to the oligonucleotide activated as upper described. After incubation for 10–16 h at 18°C, oligonucleotide-peptide conjugates were precipitated from the reaction mixture and purified as described above.

Analysis of Oligonucleotide-Peptide Conjugates

Acid Hydrolysis

The oligonucleotide-peptide conjugates were treated by 15% acetic acid for 40 min at 50°C. After hydrolysis, oligonucleotide products were precipitated with 10-fold excess of 2% $LiClO_4$ solution in acetone, washed by acetone and analyzed by electrophoresis in denaturing 20% PAGE, 7 M urea, TBE.

Enzymatic Digestion

The oligonucleotide-peptide conjugates were incubated with proteinase K (1 mg/ml) in 50 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$ for 2 h at 37°C. After hydrolysis, oligonucleotide products were precipitated and analyzed as upper described.

ESI-MS

The electrospray mass spectra of samples were performed on the quadrupole mass spectrometer (QUATTRO-LCZ, Micromass, Manchester, U.K.) equipped with the Z-spray ion source. The analyzer was calibrated with ultrapure-grade caesium iodide (Prolabo, Paris, France). Negative ion electrospray spectra of oligonucleotides were obtained by scanning from m/z 500 to 1500. The sprayer voltage and the cone voltage were set to 2800 V and 35 V respectively. The samples, dissolved at 10 μM concentration in water:acetonitrile (1:1) with 0.2% triethylamine, were introduced at 5 μl/min with an infusion pump (Harvard, Ealing, les Ulis, France). The experimental molecular mass was then calculated with the mass spectrometer Masslynx software. The theoretical molecular masses were calculated with the Biolynx software (Micromass, Manchester, U.K.).

RESULTS

Our strategy of conjugate synthesis involved coupling of oligonucleotides with peptides of various amino acid composition possessing different solubility in aqueous and organic media; therefore, we used two methods in order to generate a phosphoramide bond between two conjugate fragments. Both methods included the activation of an oligonucleotide terminal phosphate followed by its reaction with a peptide amino group (Fig. 2). The first method consisted in the formation of the oligonucleotide HOBt phosphodiester that can further interact with amino groups in aqueous media.[31,32] In the second method the oligonucleotide phosphate was activated by DMAP/Ph_3P/Py_2S_2 and reacted with amines in organic media.[33] It should be noted that these methods have never been used to prepare oligonucleotide conjugates with cell penetrating peptides. To optimize the coupling reaction conditions, some preliminary studies were performed with a short model oligonucleotide (VIII) (Fig. 1), which was conjugated with diaminocarboxylic acid $NH_2CH[(CH_2)_3NH_2]COOH$, ornithine, mimicking peptides rich in positively charged aminoacids (*Antennapedia*

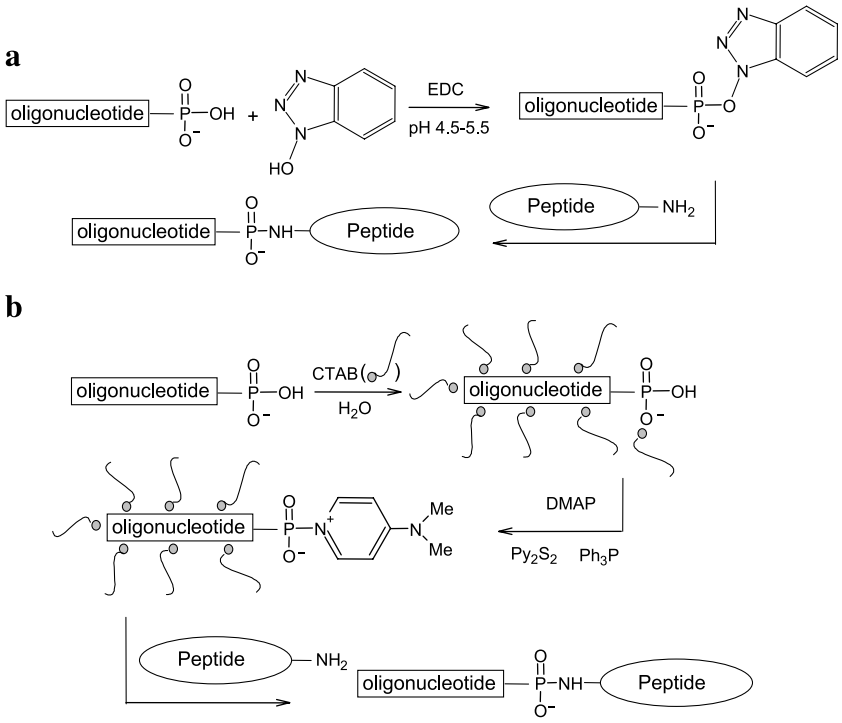


Figure 2. Scheme of the oligonucleotide and peptide conjugation using methods of the oligonucleotide terminal phosphate activation by HOBt (a) and Ph_3P/Py_2S_2/DMAP (b).

peptide, melittin and polymyxin B), as well as short hydrophobic peptides corresponding to a fragment of the HA2 subunit of hemagglutinin (I).

The method involving oligonucleotide HOBt activation, as earlier described, gives good results for oligonucleotide and peptide conjugation in aqueous solutions.[36–39] To investigate the possibility of this method application in aqueous-organic media, we examined the influence of organic solvent-water ratio, peptide (amino acid) concentration and pH on the coupling efficiency (Table 1). To reach a good efficiency of the reaction between HOBt activated oligonucleotides and amines, the latter need to be deprotonated.[32] Ornithine has two amino groups with different pKa values (pKa = 8.65 for α-NH_2 and pKa = 10.7 for δ-NH_2). When the reaction was carried out in a buffer at pH 11, both amino groups were deprotonated and could therefore interact with the activated phosphate. At pH 8–9, the δ-NH_2 group was for the most part protonated, and probably it was a reason for the coupling efficiency decrease (40–45% in water solution). Increasing the DMF content in the reaction mixture, we observed a coupling efficiency augmentation. In presence of 85% DMF the oligonucleotide-ornithine conjugate was synthesized with 90% efficiency even at pH 9 where the α-NH_2 was mainly active. This effect might be a result of suppression of the concurrent process of the oligonucleotide HOBt phosphodiester hydrolysis in aqueous-organic

media. It was also found that a diminution of ornithine concentration lower then 10^{-3} M caused a dramatic decrease of the reaction efficiency.

Coupling of shorts peptides (I, II) on the oligonucleotide (VIII) was performed by both methods (Table 1). Because of hydrophobic properties of these peptides, we used a high concentration of DMF in the reaction mixtures when preparing conjugates by HOBt method. To maintain a constant pH value in the media, two tertiary amines: TEA and N-MeIm, were used. At pH 9 N-MeIm provided a higher coupling efficiency (Table 1), probably, because it could act as nucleophilic catalyst. In summary, the efficiency of the oligonucleotide (VIII) and hydrophobic peptides coupling by the HOBt ester method was only moderate. By contrast, the method of the oligonucleotide phosphate activation with Ph_3P/Py_2S_2/DMAP resulted in high yields of the conjugates (Table 1). The reaction in DMF was carried out in presence of a tertiary amine DIEA in order to deprotonate peptide amino groups.

Based on the results obtained from the preliminary study with the model oligonucleotide (VIII), several rules were drawn to synthesize conjugates of 18-mer oligonucleotides and membrane active peptides. In order to aim the reaction at the α-amino group of peptides containing side-chain amino groups and soluble in aqueous-organic media (melittin, polymyxin, *Antennapedia peptide*), it seemed better to use the HOBt activation of oligonucleotide phosphates followed by peptide coupling in a buffer

Table 2. Syntheses of oligonucleotide conjugates with different peptides, incubation time 16 h, 18°C.

Peptide	Method	Concentration, M	Medium	Coupling efficiency for different oligonucleotides*,** % (VIII)	(IX)	(X)
H-GLFG-OH (I)	2	5×10^{-3}	DMF	90–95	90–92	88–90
H-GLFGAIAG-OH (II)	2	5.7×10^{-3}	DMF	80–85	76–78	75–77
Hemagglutinin peptide (III)	2	2×10^{-3}	DMSO-DMF, 1:1	–	21–23	18–20
Polymyxin B (IV)	1	3×10^{-3}	Buffer***	–	84–86	85–87
	2	3×10^{-3}	DMF	–	87–89	85–87
Melittin (V)	1	7.2×10^{-4}	DMF-buffer***, 1:1	–	81–83	80–82
	2	7.2×10^{-4}	DMF	–	87–89	–
Fmoc-protected *Antennapedia* peptide (VI)	2	6.4×10^{-4}	DMF	–	15–16	–
Antennapedia peptide (VII)	2	6.4×10^{-4}	DMF	–	58–60	–

*Calculated on the base of gel electrophoresis analysis of the ^{32}P-labeled oligonucleotide. The coupling efficiency was expressed in % of the total oligonucleotide radioactivity.

**Oligonucleotide concentration was 2×10^{-5} M.

***Buffer: 0.4 M aqueous N-methylimidazole, 0.1 M NaCl, pH 9.0.

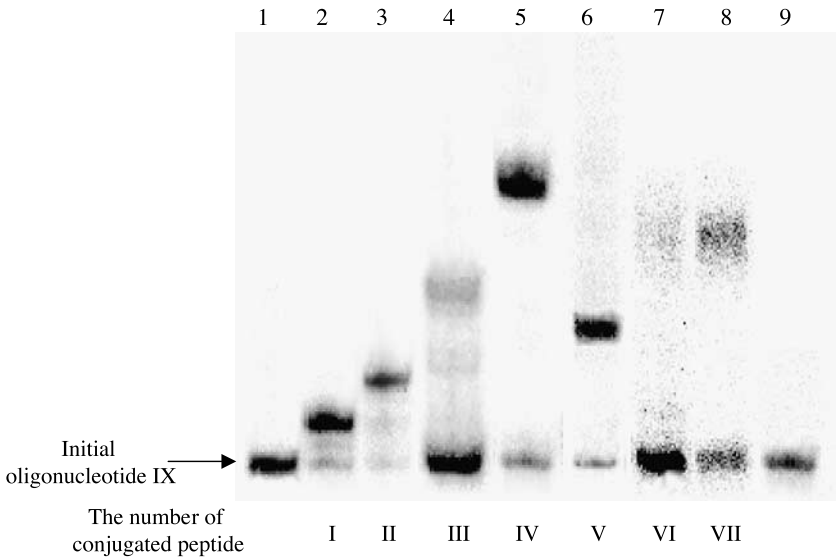


Figure 3. Autoradiograph of a 20% polyacrylamide gel analysis of the oligonucleotide (IX) conjugation with peptides I–VII: lane 2—with H-GLFG-OH (I); lane 3—with H-GLFGAIAG-OH (II); lane 4—with hemagglutinin peptide (III); lane 5—with polymyxin B (IV); lane 6—with melittin (V); lane 7—with Fmoc-protected *Antennapedia* peptide (VI); lane 8—with *Antennapedia* peptide (VII). Lanes 1, 9—initial oligonucleotide (IX).

or buffer/DMF mixture with a pH value near 9 (method 1). For hydrophobic peptides the phosphate activation with Ph_3P/Py_2S_2/DMAP followed by peptide coupling in anhydrous organic solvents (method 2) was preferable.

Syntheses of conjugates of antisense (IX) and inverse (X) oligonucleotides with membrane active peptides (I–VII) were performed under different reaction conditions depending on the peptide solubility (Table 2). Results obtained for the oligonucleotide (IX) conjugates synthesis by using the method 2 are shown in the Fig. 3. To evaluate the applicability of both methods for the conjugation of lengthy peptides and oligonucleotides, 5′-[^{32}P]-phosphorylated oligonucleotides (IX) and (X) were used. Since T4 polynucleotide kinase had 3′-phosphatase activity, 3′-phosphate was eliminated from these oligonucleotides during their phosphorylation, and consequently the peptide coupling occurred onto 5′-[^{32}P]-phosphate. Conjugates prepared for the MS analysis (see below) were synthesized using 3′-phosphorylated oligonucleotides (Fig. 1). The peptide-oligonucleotide ratio in all conjugates was 1:1.

Conjugates of 18-mer oligonucleotides (IX and X) with short peptides (I and II) from HA-2 sequence were prepared using method 2, and the reaction efficiency was about the same as for the model 9-mer oligonucleotide (VIII). The coupling of the 15-mer peptide (III) corresponding to HA-2 subunit of influenza hemagglutinin was performed using the same method, however, the conjugate yield was only moderate (Table 2).

Polymyxin B (IV) is well soluble both in water and in DMF. Besides, it lacks a terminal α-amino group, and only γ-amino groups of α,γ-bis-aminobutanoic acid (Dab) can be joined to the oligonucleotide phosphate. Conjugates of this peptide with

the 18-mer oligonucleotides (IX, X) were synthesized with high yields by using both methods (Table 2).

Melittin (V) was also combined with oligonucleotides by HOBt method in aqueous-organic media and by Ph_3P/Py_2S_2/DMAP method in anhydrous DMF in presence of a tertiary amine DIEA in order to deprotonate melittin α-NH_2-group. Due to the high solubility of the peptide, the coupling efficiency did not depend on the phosphate activation method (Table 2). However, it should be noted that DIEA being a strong amine with pKa $\cong$ 12 could deprotonate not only α-NH_2 but also ε-NH_2 of lysine residues, and as a result, they might participate in the coupling. In contrast, the first method is more favorable to produce phosphate conjugation with peptide α-NH_2-groups.

To verify if the conjugation was really effectuated through the melittin α-amino group, the conjugate formed with the oligonucleotide (X), was synthesized by method 1, isolated, characterized by ESI-MS. The experimental molecular mass (M = 8384.7) agreed with the calculated mass (8385.1). The conjugate was then treated with proteinase K to hydrolyze its peptide part. The enzymatic degradation products were analyzed by the ESI-MS method. Mass spectrometry analysis showed two products of the conjugate digestion, corresponding to the oligonucleotide (X) linked with melittin N-terminal tripeptide (GlyIleGly) (70%) and Gly (20%): 5782.7 [M (theor) = 5782.8] and 5612.6 [M (theor) = 5612.6], respectively. A conjugate of the oligonucleotide (X) with melittin N-terminal dipeptide (GlyIle) (5%) [M (exp) = 5726.2; M (theor) = 5726.0] was also detected.

Coupling of the oligonucleotide (IX) with *Antennapedia* peptide (VI), in which side-chain Lys residues were protected, was performed in organic media because of the high hydrophobicy of the protected peptide. However the reaction efficiency was low

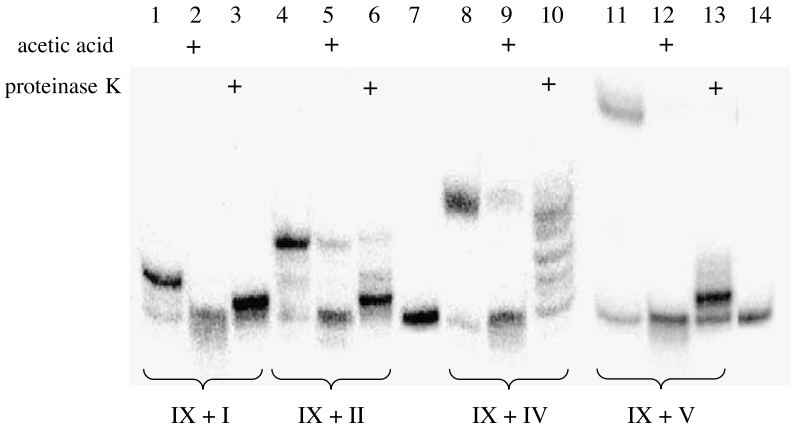


Figure 4. Autoradiograph of a 20% polyacrylamide gel analysis of acid and proteinase K hydrolysis of the oligonucleotide IX conjugates with peptides: H-GLFG-OH (I) (lanes 1–3), H-GLFGAIAG-OH (II) (lanes 4–6), polymyxin B (IV) (lanes 8–10) and melittin (V) (lanes 11–13). Reaction mixtures obtained as a result of conjugation—lanes 1, 4, 8, 11; products of the treatment of the reaction mixtures with 15% acetic acid lanes 2, 5, 9, 12; products of proteinase K cleavage—lanes 3, 6, 10, 13. Lanes 7, 14—initial oligonucleotide (IX).

(15%) (Table 2). For comparison, the completely deprotected peptide (VII) was coupled to the oligonucleotide (IX) under the same reaction conditions. It resulted in a considerable conjugation efficiency augmentation. However, a coupling through ε-amino group of lysine residues could not be excluded in this case.

The formation of peptide-oligonucleotide conjugates was confirmed by acid treatment and proteinase K digestion. The conditions of the acid treatment utilized in the present work were earlier developed for the selective phosphoramide bond cleavage in oligonucleotide derivatives with amino compounds.[32] As expected, the treatment of the conjugates with 15% acetic acid resulted in products with the same electrophoretic mobility as non-modified initial oligonucleotides (Fig. 4). Hydrolysis of peptide fragments of the conjugates by proteinase K gave products with slightly lower mobility than the initial oligonucleotides. It could be due to the incapacity of proteinase K to cleave the phosphoramide bond and conformational inaccessibility of some amino acids adjacent to the oligonucleotide phosphate for the enzymatic digestion. As a result, oligonucleotide derivatives containing one or several amino acid residues might be formed. This suggestion was confirmed by the MS analysis of the products of the enzymatic hydrolysis of the melittin containing conjugate (see above).

DISCUSSION

Peptides usually used for the oligonucleotide transport into cells possess various amino acid compositions and differ by their membrane interaction mechanism. They can be broadly classified as either hydrophobic, amphipathic or cationic. In the present work two methods were proposed to synthesize oligonucleotide conjugates with peptides of various sequences (Fig. 1).

A fusogenic peptide derived from the influenza virus hemagglutinin envelope protein (III) is known as a pH-sensitive peptide and has been used for plasmid and oligonucleotide intracellular delivery.[40–42] This peptide changes its conformation at acidic pH and destabilizes the endosomal membranes thus resulting in an increased cytoplasmic gene delivery. Its sequence includes a hydrophobic region at the N-terminus. Two short peptides (I and II) derived from this sequence were chosen as model hydrophobic peptides to optimize conjugation conditions.

Polymyxin B (IV) is an antibiotic with bacterial action. It has a cationic peptide sequence, which interacts with the negatively charged lipid bilayer and induces pore formation in cell membranes.[43–45]

Melittin (V) from honeybee venom is a basic amphipathic peptide, which alters membrane permeability.[46–48]

The 16-mer peptide (VII), corresponding to amino acids 43–58 of the homeodomain (HD) of the *Drosophila* transcription factor encoded by the *Antennapedia* gene, is found to have translocation properties and to be able to deliver different compounds, such as peptides, oligonucleotides and peptide nucleic acids, across the plasma membrane. It is thereafter called penetratine-1 (or 'third helix'). This peptide is non-cell-type specific and highly efficient to effect direct delivery of compounds into the cytoplasm and nucleus.[49–52] It might be due to its amphipathic structure arisen from the alteration of hydrophobic and cationic residues in its sequence. As ε-amino groups of lysine residues are involved in interactions with the

membrane lipids, they must not be affected during the conjugate synthesis. For this purpose two approaches may be proposed, i.e., the application of protected groups, for example Fmoc groups, to prevent ε-amino group reaction with the activated phosphate (structure VI, Fig. 1) or the use of a method providing a selective oligonucleotide phosphate reaction with peptide α-amino group.

To construct peptide-oligonucleotide hybrid molecules, a strategy involving the coupling of an unprotected oligonucleotide bearing previously activated terminal phosphate upon a peptide amino group through a phosphoramide bond formation was applied. Two methods of the phosphate activation were used.

The first one includes the oligonucleotide phosphate activation by 1-hydroxybenzotriazole. The yield of the active product, HOBt ester, usually is more than 90%,[31,32] so it does not need any isolation and may be directly treated with a peptide in aqueous solutions (Fig. 2a). This method is found to be effective for nucleophilic substitution at the phosphorus atom[31,32,36] and has been successfully used for peptide-oligonucleotide conjugate synthesis in aqueous media.[37–39] It has been earlier shown that HOBt activated oligonucleotides interact only with free (deprotonated) amines.[32] Consequently, the reaction efficiency was pH dependent (usually pH $\leq$ pKa of amines used for the conjugation), and moreover, in the case of compounds with two amino groups with different pKa values, only one of them, which has a lower pKa, may be predominantly phosphorylated.[32] It means that theoretically one can couple oligonucleotides on α-NH_2 of peptides without touching upon ε-amino groups of Lys. It is especially important in the case of peptides penetrating into cells, because the Lys modification can change their structure and suppress their intracellular transport.

The second method consists in the oligonucleotide terminal phosphate activation with Py_2S_2, Ph_3P and DMAP in anhydrous DMF or DMF-DMSO mixture.[33] It should be noted that oligonucleotides must be preliminary adapted to organic media by CTAB treatment. An activated oligonucleotide can interact with amino compounds directly in the reaction mixture. This method has been used to link unprotected oligonucleotides to different DNA-binding, fluorescent, chemically active ligands[53] and short peptides.[54]

To synthesize peptide-oligonucleotide conjugates, we used both these methods employing reaction conditions found as a result of preliminary studies on the model compounds (see section ''Results'').

The antisense oligonucleotide (IX) and the inverse sequence oligonucleotide (X) (Fig. 1) were used for conjugate synthesis, and it was found that the product yields did not depend on the oligonucleotide sequence (Table 2). Moreover, when comparing the efficiency of short peptides (I) and (II) coupling on to the 18-mer oligonucleotides (IX), (X), on one hand, and the model 9-mer oligonucleotide (VIII), on the other hand, one can conclude that it did not depend significantly on the oligonucleotide length (Table 2).

The coupling of short peptides (I) and (II) as well as 15-mer peptide (III) on the oligonucleotide phosphates was performed according the method 2 because of the high hydrophobicy of these compounds. The reaction efficiency was slightly lower for the peptide (II) regarding the peptide I, and a considerable efficiency decrease was detected for the peptide (III) (Table 2). It might arise from the diminution of the steric accessibility of the amino group because of the peptide folding or self-association in the case of lengthier sequences. Besides, the efficiency decrease might be caused by the weak solubility of the peptide (III), which resulted in its 3-fold lower concentration when compared with peptides (I) and (II).

Both methods provided high conjugation efficiency for polymyxin B. Apparently, the presence of six γ-amino groups, high solubility in water and DMF as well as the absence of a complicated secondary structure resulted in the efficient conjugation both in organic and aqueous media (Table 2).

The most noteworthy result was obtained for melittin, because we demonstrated that a phosphoramide bond formation between oligonucleotides and lengthy amphipatic peptides might proceed with high efficiency in aqueous-organic media (Table 2, method 1). Since melittin contains three lysine residues, the coupling could occur not only through α-amino group, but also through any ε-amino group. To prevent this undesirable process, the pH value of the reaction mixture was maintained below the pKa of the Lys side-chain amino groups. The following ESI-MS analysis of products of the conjugate digestion by proteinase K confirmed that the conjugation was effectuated through the N-terminal amino group of melittin. Hence, the method of the oligonucleotide phosphate HOBt activation can be proposed for specific conjugation of oligonucleotides and α-amino groups of peptides containing side-chain amino groups.

Unfortunately we did not succeed in the coupling of the cationic peptide (VII) on oligonucleotides by using the HOBt activation method. Addition of this peptide, containing 7 positively charged residues (4 Lys and 3 Arg) per 16 amino acids, to polyanionic oligonucleotides in aqueous media resulted in their aggregation and precipitation. For this reason, the method 2 alone was applied for conjugation of the oligonucleotide (IX) and the *Antennapedia* peptide. In order to avoid phosphorylation of Lys amino groups, the Fmoc-protected peptide (VI) was used for the conjugation. Unfortunately, we failed to reach a high coupling efficiency (Table 2). This may be explained by possible steric problems caused by bulky Fmoc-groups. For comparison, after ε-amino groups deprotection by the peptide (VI) treatment with a 20% piperidine solution in DMF, the conjugation of the resulting peptide (VII) with the oligonucleotide (IX) occurred more efficiently (Table 2). However, the oligonucleotide coupling through lysine ε-amino groups could not be excluded in this case, because DIEA used in the reaction could deprotonate all amino groups in the peptide. It should be noted that in organic solvents we did not detect any aggregate formation between the peptide and oligonucleotide.

Thus, it was demonstrated that by applying the fragment condensation strategy, it is possible to synthesize conjugates of oligonucleotides with peptides of various natures in the liquid phase. Depending on the amino acid composition and solubility in aqueous and organic media, two conjugation methods may be employed, i.e., HOBt ester or Ph_3P/Py_2S_2/DMAP oligonucleotide phosphate activation. The use of the most suitable method allows the efficient synthesis of conjugates of antisense oligonucleotides with different cell-penetrating peptides.

ABBREVIATIONS

Boc	*tert*-butyloxycarbonyl
BPB	bromophenol blue
CTAB	hexadecyltrimethylammonium bromide
Dab	2,4-diaminobutyric acid
DCC	N,N′-dicyclohexylcarbodiimide

DIEA	N,N-diisopropylethylamine
DMF	N,N-dimethylformamide
DMAP	4-dimethylaminopyridine
DMSO	dimethylsulfoxide
Fmoc	9-fluorenylmethoxycarbonyl
EDC	1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide
ESI-MS	electrospray ionization mass spectrometry
HOBT	1-hydroxybenzotriazole
MBHA	4-methyl-benzhydryl amine
N-MeIm	N-methylimidazole
PAGE	polyacrylamide gel electrophoresis
Ph_3P	triphenylphosphine
Py_2S_2	2,2′-dipyridyldisulfide
TBE	TRIS borate–EDTA buffer
TEA	triethylamine
XC	xylenecyanol FF

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