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The Synthesis of Peptide-Oligonucleotide Conjugates by a Fragment Coupling Approach[1]

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Abstract: Solid-phase synthesis of several peptide-oligonucleotide conjugates has been achieved using a peptide fragment coupling strategy on a controlled pore glass support. The conjugates contain either a hydrophobic tetrapeptide LGIG or an 8-residue basic peptide of the HIV-1 Tat protein coupled to one of two oligodeoxyribonucleotides, an oligoribonucleotide or a mixed ribo/2'-*O*-methyl oligonucleotide. Improved yields were obtained when internucleotide β-cyanoethyl groups were removed from the support-bound oligonucleotide prior to peptide fragment coupling, and by use of a long alkyl spacer in the linkage between peptide and oligonucleotide.

INTRODUCTION

In the last decade major advances have been made in the development of oligonucleotides as potential therapeutic agents [2]. To be active as antisense agents, oligonucleotides have to enter cells in sufficient concentration, find their RNA target, and then hybridize to it. However, a frequent limitation of their use is poor cellular uptake [3,4]. A common strategy of oligonucleotide delivery is based on the use of cationic liposomes [5]. An alternative method involves conjugation with cationic polypeptides such as poly-L-lysine [6]. More recently, a number of peptidic carriers of oligonucleotides and DNA into cells have been suggested, for use either with or without covalent conjugation. These include sections of fusogenic proteins [7-9], the KDEL peptide motif involved in intracellular compartment shuttling [10,11], an anionic membrane-destabilizing peptide [12], a cationic condensation peptide [13], and a cationic amphipathic peptide [14]. Two other peptides are interesting in that, when coupled to an oligonucleotide through a reversible disulfide linker, each is able to enhance uptake by a temperature-independent, membrane translocation process [15,16].

Hitherto, covalent conjugation has been carried out mostly through linkage of a peptide to an oligonucleotide at its 3'- or 5'-end. The conjugation is carried out in aqueous solution, where the two species are fully deprotected and purified and each contains a specific functionality that is introduced synthetically. Various linkages have been formed such as disulphide [7,17], thioether [10,18] or maleimide [19,20], as well as amide bonds [21,22]. Such solution couplings can give poor yields and are prone to interference by the secondary structures of peptide and oligonucleotide parts. Further, some methods require the incorporation of a single unique cysteine residue on the peptide part.

Conjugate synthesis has been carried out by solid-phase chemistry, for example by initial assembly of the peptidic chain, using either *t*-butyloxycarbonyl (Boc) [23,24] or 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis [25,26], addition of a bifunctional linker to generate a hydroxyl function, and finally synthesis of the oligonucleotide part *via* standard phosphoramidite chemistry. Alternatively, synthesis of oligoalanyl-oligothymidine conjugates has been reported where solid-phase oligonucleotide synthesis precedes peptide synthesis [27]. In another approach, a bifunctional linker carrying differently protected hydroxyl and amino groups is first attached to a solid support, peptide and oligonucleotide synthesis carried out sequentially [28,29]. Cleavage from the support leaves a conjugate where the peptide is attached to the 3'-end of the

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oligonucleotide. All these methods have the drawback of incompatibilities between peptidic and oligonucleotidic protecting groups during the sequential assemblies, which leads to a restricted choice of sequences that can be prepared.

We wished to explore an alternative strategy for the preparation of peptide-oligonucleotide conjugates that involves a solid-phase coupling of an N_α-protected peptide fragment to the 5'-end of a preassembled oligonucleotide linked to a solid support. A potential advantage of such a route over a sequential assembly method is that the oligonucleotide does not need to be stable under the conditions of peptide assembly and *vice-versa*. In principle, this might allow the coupling of a wider variety of synthetic peptides to DNA, RNA as well as to a range of analogues. Further, the peptide fragment can be purified, stored and used for multiple conjugate syntheses. Following solid-phase coupling, the desired conjugate may be obtained by mild ammonia treatment to deprotect the oligonucleotide and to cleave the conjugate from the support. A fragment coupling route was first suggested by Grandas *et al.* [30] for conjugation of a protected tripeptide as a C-terminal amido phosphoramidite derivative to a protected 2'-deoxyhexanucleotide attached to a polystyrene support. We now present our first results of solid-phase couplings of short basic and hydrophobic peptide fragments to oligodeoxynucleotides, an oligoribonucleotide and a ribonucleoside-2'-*O*-methylnucleoside chimeric oligonucleotide towards the aim of a generally useful method for synthesis of peptide-oligonucleotide conjugates.

RESULTS AND DISCUSSION

The peptide fragment coupling strategy has proved to be very successful in this laboratory for the assembly of large peptides and small proteins [31,32]. Peptide fragments are synthesized on large scale (0.1 mmol) by standard Fmoc solid-phase chemistry, released from the support as N_α and side-chain protected fragments, and chromatographically purified. Our strategy for conjugate synthesis involves coupling of pre-assembled peptide fragments through their carboxy termini to support-bound protected oligonucleotides through the formation of a stable amide linkage. The formation of an amide linkage could be achieved by coupling to an oligonucleotide which has been functionalized with a primary amino group, for example at its 5'-end.

For initial studies, we chose for conjugation a series of oligonucleotide and peptide models (Table 1). Although coupling conditions for low C-terminal epimerization of peptide fragments have been published [33], we chose for this first study two peptides that contain a C-terminal glycine, where epimerization cannot take place, as well as the amino acid derivative Fmoc glycine. One peptide, N_α-Fmoc-Leu-Gly-Ile-Gly-OH (Fmoc-LGIG), is hydrophobic whereas the second, N_α-Fmoc-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-OH (Fmoc-QR$_3$P$_2$QG) is extremely basic and corresponds to a fragment of the basic domain of the HIV-1 *trans*-activator protein Tat that is a potential carrier for oligonucleotides into cells [16,17]. During assembly of the Tat peptide, the side-chains of glutamine and arginine were protected by the standard trityl and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) groups respectively. However, since the acidic conditions for removal of Pmc were found to be too harsh for oligodeoxynucleotides containing purine residues to remain intact (data not shown), side-chain protecting groups were removed prior to peptide coupling to the oligonucleotides. In a trial coupling of a 14-mer peptide fragment containing seven unprotected arginine side-chains to the N_α-amino group of the LGIG sequence attached to a solid support, we found that high yields of fragment

coupling were still obtained and there was no evidence for the generation of ornithine in the coupled product (data not shown).

Table 1. Synthetic Peptide-Oligonucleotide Conjugates.
NH_2-PEPTIDE-CONH-$(CH_2)_n$-NHCO-O-5'OLIGONUCLEOTIDE3'

CONJUGATE	OLIGONUCLEOTIDE	Fmoc-PEPTIDE
$\mathbf{1a_{n=3,6,12}}$	dT_{12}	Gly
$\mathbf{1b_n}$		LGIG
$\mathbf{1c_n}$		QR_3P_2QG
$\mathbf{2a_{n=3,6,12}}$	d(CTAGGATCTACTGGCT)	Gly
$\mathbf{2b_n}$	Tat d16-mer	LGIG
$\mathbf{2c_n}$		QR_3P_2QG
$\mathbf{3a_{n=6,12}}$	rU_9	Gly
$\mathbf{3b_n}$		LGIG
$\mathbf{3c_n}$		QR_3P_2QG
$\mathbf{4a_{n=6,12}}$	r(C*GA*A*AC*U*C*C*)	Gly
$\mathbf{4b_n}$	r/Me 9-mer	LGIG
$\mathbf{4c_n}$		QR_3P_2QG

rNu* = 2'-O-methylnucleoside

Four oligonucleotide models were chosen. Two of these, dT_{12} and rU_9, require no protecting groups on the nucleobases. A mixed base 16-mer oligodeoxynucleotide sequence (Tat d16-mer) was also chosen, which corresponds to an antisense oligonucleotide directed against the HIV-1_{BRU} Tat gene [34], as well as a 9-mer mixed base ribonucleoside-2'-*O*-methylnucleoside chimeric oligonucleotide (r/Me 9-mer) as an example of an oligoribonucleotide analogue. Each oligonucleotide was assembled by standard 1 μmol phosphoramidite synthesis on a controlled pore glass (CPG) support. To generate a 5'-amino group on the oligonucleotide attached to the support, we found best results were obtained by acidic removal of the terminal 5'-dimethoxytrityl group followed by activation of the resultant 5'-hydroxyl on the support-bound oligonucleotide using 1,1'-carbonyldiimidazole and subsequent reaction with a diaminoalkane [35]. This route allows the effect of length of the alkane spacer on yield of coupling of the peptide to be investigated by choice of different diaminoalkanes (Table 1).

To obtain the desired peptide-oligonucleotide conjugates, a number of different peptide activation and fragment coupling conditions to CPG-bound dT_{12} oligonucleotide were investigated based on experience from standard peptide fragment coupling conditions [33]. Of these the activator benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) in the presence of 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIEA) gave the best initial results. For solubility reasons the solvent *N,N*-dimethylformamide (DMF) was preferred over dichloromethane and five equivalents of Fmoc glycine or the Fmoc peptide were used in the conjugation reaction over the support-bound oligodeoxyribonucleotide containing a 6-carbon atom amino linker (Protocol A, Experimental Section). After overnight reaction at room temperature, the supernatant was removed by filtration and the support was treated under mild ammoniacal conditions to remove phosphate protecting groups and to cleave the conjugate from the solid support. In control experiments it was found that aqueous or methanolic ammonia treatment at room temperature for 24 h did not affect these and a range of other peptides (data not shown). The cleaved products were analysed by reversed-phase HPLC and by MALDI-TOF mass spectrometry (see Experimental Section). For coupling reactions to the support-bound amino-functionalized oligonucleotide dT_{12}, good yields were

obtained for both glycine and peptide conjugates (Figure 1, Table 2). By contrast in the case of the mixed-base Tat d16-mer, the conjugates were formed only in very low yields.

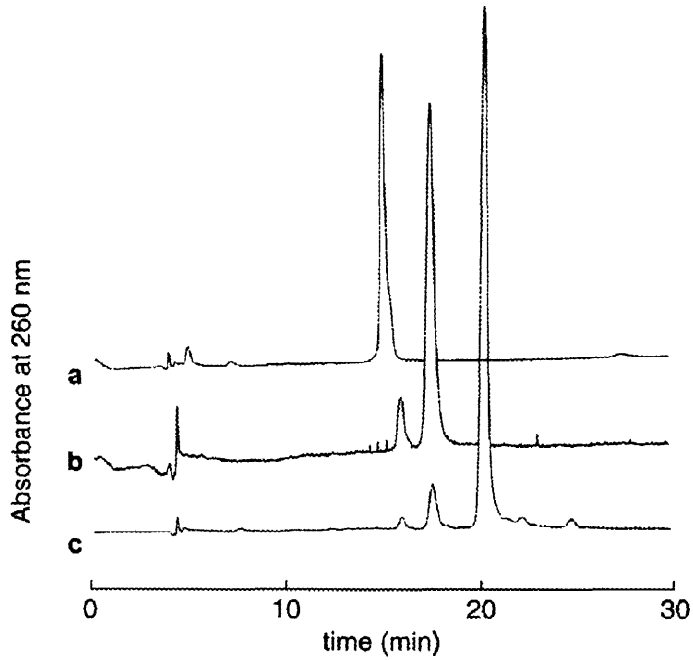

Fig. 1. HPLC profiles (gradient A) of a) 5'-OH dT_{12}, b) 5'-NH_2 (n=6) dT_{12}, c) Conjugate $1b_6$.

We next investigated whether the poor conjugation yields in the case of the mixed base Tat d16-mer was due to the nature of the support. Whereas some groups have reported good amino acid coupling yields in peptide assembly on CPG [9,24,26], others have reported some difficulties [23,29]. We therefore carried out oligonucleotide assemblies of 5'-amino linked d16-mer on two types of polyethylene glycol-polystyrene support (TentaGel™ and PEG-PS™) followed by coupling of Fmoc-Gly or Fmoc-LGIG. These two supports allow an increased nucleoside loading, good solvation properties in a large range of organic solvents, and have been suggested previously for conjugate synthesis [23,29,36]. However, in neither case were yields improved using Protocol A (data not shown). We thus concluded that the failure to couple peptides to the d16-mer sequence was independent of the support used.

Table 2. Percentage Yields in the Conjugation Reactions using Protocols A or B.

Peptides / Oligos	dT_{12} n=6	Tat d16-mer n=6		rU9 n=6	r/Me 9-mer n=6
	Protocol A	Protocol A	B	Protocol B	Protocol B
Fmoc-Gly	90[a]	< 5[b]	10	40	40
Fmoc-LGIG	80	< 5	10	90	50
Fmoc-QR3P2QG	75	< 5	10	10	20

a) Yields are based on HPLC analysis. b) The yield in a conjugation reaction was considered <5% when a signal corresponding to the desired conjugate was observed by mass spectral analysis but no peak was observed by HPLC analysis.

We therefore looked at the conditions for forming the amide bond between peptide fragment and d16-mer oligonucleotide. An increase in the temperature of the reaction (40°C) or doubling of the concentration of peptide had no effect (data not shown). An increase in the number of equivalents of amino acid or peptide to

10 over the support-bound oligonucleotide (Protocol B in the Experimental Section) resulted in only a marginal improvement in yields to 10% for the d16-mer sequence (Table 2). By contrast, for the reaction of the amino-substituted oligoribonucleotide rU9 with either Fmoc-Gly or Fmoc-LGIG moderate to good yields were obtained using Protocol B: 40% for Fmoc-Gly, 90% for Fmoc-LGIG, but only 10% yield for the basic peptide Fmoc-QR3P2QG (Table 2). Moderate yields (20-50%) were also obtained in conjugation of the ribonucleoside-2'-*O*-methylnucleoside chimeric oligonucleotide (r/Me 9-mer). Thus only in the case of the mixed sequence d16-mer oligodeoxyribonucleotide were uniformly poor conjugation yields obtained.

We wondered whether the lack of reactivity observed in conjugation of peptide fragments to the mixed-base oligodeoxyribonucleotide 16-mer might be due to a higher hydrophobicity of this kind of molecule which carries benzoyl and isobutyryl protecting groups on the C, A and G residues in addition to phosphate protecting groups. Proper solvation of the oligonucleotide might in some way be prevented, resulting in a decrease in the accessibility of the terminal amino group. To reduce the hydrophobicity, we decided therefore to remove the β-cyanoethyl protecting groups from internucleoside phosphates on the support-bound oligonucleotide chain. It was recently reported that specific removal of β-cyanoethyl protecting groups could be achieved by treatment of a support-bound fully protected oligonucleotide with triethylamine-acetonitrile solution (4:6, v/v) for 90 min., without damaging the linkage between the solid support and the oligonucleotide, or giving rise to loss of base protecting groups [37]. In our case we found that use of this procedure leads to the formation of a by-product resulting from addition of acrylonitrile, liberated in the deprotection reaction, to the terminal 5'-amino group of the linker (data not shown). To reduce this, we modified the procedure by treating the support for just 10 minutes, washing the support with acetonitrile and then treating again for 30 min. with fresh triethylamine-acetonitrile solution. We now measured the coupling yields of Fmoc-Gly and the two peptide fragments to the phosphate-deprotected, support-bound oligonucleotides (Protocol C, Table 3). Deprotection of the phosphate groups led to a significant increase in the yields of the peptide conjugation reactions to the mixed base d16-mer sequence (30-50%). By contrast, there were modest reductions in conjugation yield for the dT_{12} sequence. For the all RNA oligonucleotide rU9 and for the r/Me 9-mer oligonucleotide there was an increase in yields for coupling of the basic Fmoc-QR3P2QG peptide.

Table 3. Percentage Yields in the Conjugation Reactions using Protocol C.

Peptides / Oligos	dT_{12} n=6	Tat d16-mer n=6	rU9 n=6	r/Me 9-mer n=6
Fmoc-Gly	80	50	50	40
Fmoc-LGIG	70	30	90	50
Fmoc-QR3P2QG	60	40	25	30

We also examined the effect of altering the length of the spacer between the 5'- terminal amino group and the oligonucleotide on the support in case this spacing might also affect accessibility of the amino group. Thus three different lengths of linker (3, 6 and 12 carbon atoms) were tested by functionalization using different diaminoalkanes in the 5'-functionalization reaction. The 5'-aminopropyl, aminohexyl and aminododecyl support-bound oligonucleotides were therefore synthesized for both dT_{12} and d16-mer mixed base sequences, phosphate protecting groups removed, and the coupling yields in the reactions of Fmoc-Gly and the two peptide fragments measured following Protocol C (Table 4). Whereas in the case of dT_{12} the

reaction yields were relatively unaffected, in the case of the mixed base d16-mer sequence, the length of the linker had a dramatic effect on coupling yields. For Fmoc-LGIG yields increased from 20% to 85% when the length of the carbon chain was increased from 3 to 12 atoms. For the basic Tat peptide there was a big improvement in yields when the linker length was increased from 3 to 6 methylene units but no further increase in the case of the dodecyl chain. The results with rU9 and r/Me 9-mer were more variable. In most cases there were increases in yield as the linker length was increased, but there was a reduction in yield for coupling of Fmoc-LGIG to rU9 from 90% when n=6 to 50% when n=12, and the 30% yield for coupling of Fmoc-QR3P2QG was not altered when the linker length was increased (Table 4).

Table 4. Influence of the Length of the Alkyl Spacer on Percentage Yields in the Conjugation Reactions (Protocol C).

Peptides / Oligos	dT12			Tat d16-mer			rU9		r/Me 9-mer	
	n=3	n=6	n=12	n=3	n=6	n=12	n=6	n=12	n=6	n=12
Fmoc-Gly	80	80	85	< 5	50	70	50	75	40	90
Fmoc-LGIG	50	70	60	20	30	85	90	50	50	70
Fmoc-QR3P2QG	45	60	65	< 5	40	40	25	45	30	30

The results reported here show that by use of a fragment coupling strategy we were able to obtain a number of conjugates of the amino acid glycine, the hydrophobic peptide LGIG and the basic peptide QR3P2QG linked to either the oligodeoxyribonucleotide dT12, a mixed base Tat d16-mer sequence, the oligoribonucleotide rU9, or a ribonucleoside-2'-*O*-methylnucleoside chimeric 9-mer oligonucleotide. These conjugates were obtained in yields which were a significant improvement on alternative procedures and the studies open the way for a general solid-phase synthetic route to a larger range of peptide-oligonucleotide hybrids. Work is in progress in order to extend the fragment coupling approach to longer peptides and to explore alternative methods of peptide activation that might give rise to further improvements in yields.

EXPERIMENTAL SECTION

Peptide Synthesis and Characterization

Solid-phase peptide synthesis was carried out on 0.1 mmol scale using an LKB "Biolynx" 4170 automated Synthesizer and continuous-flow Fmoc-polyamide methods [38] on an $N\alpha$-Fmoc-Gly-Wang polystyrene resin (Novabiochem). $N\alpha$-Fmoc-amino acid pentafluorophenyl activated esters (Novabiochem) and HOBt were used throughout at five-fold molar excess over support-bound amino groups, except for $N\alpha$-Fmoc-NG-2,2,5,7,8-pentamethylchroman-6-sulfonyl-L-arginine (Fmoc-Arg(Pmc)) which was coupled using PyBOP/ HOBt / DIEA in DMF. Cleavage from the solid support and deprotection of the side chain protecting groups (Pmc on arginine and trityl on glutamine) was carried out in trifluoroacetic acid (TFA)-ethanedithiol (EDT)-triethylsilane (TES)-water (90:5:3:2, v/v/v/v, 10 ml) for 2 h. at room temperature. The filtrate was flushed with nitrogen and the concentrated fraction was precipitated by addition of ice-cold diethyl ether (40 ml) and washed with diethyl ether (4 x 40 ml). Finally, the solid was dissolved in 0.1% aqueous TFA (10 ml) and lyophilized.

Fmoc peptides were analysed by HPLC on a RP-C8 column (Vydac, cat 208TP54) using a 10 to 90 %B gradient over 25 min, where A= 0.1 % aqueous TFA and B= 90 % CH3CN-10 % A, 1.5 ml min^{-1} flow rate, and dual UV peak detection at 215 and 230 nm. The purity of Fmoc-LGIG was determined as 96% and that of Fmoc-QR3P2QG as 90% as judged by the area of the main peaks at 215 nm. Peptide molecular masses were determined by MALDI-TOF mass spectrometry on a Voyager DE spectrometer (PerSeptive Biosystems) in positive ion mode using sinapinic acid as matrix (sample:matrix, 1:5, v/v), Fmoc-LGIG: m/e calculated 580.2; found 581.8, Fmoc-QR3P2QG: m/e calculated 1215.1; found 1216.7. The quality of the Fmoc 8-mer peptide was also determined by hydrolysis in 6N HCl for 18 h. at 110 °C and amino-acid analysis was carried out on a Beckman Amino-Acid Analyser (System 7300): found (expected): Gln/Glu 2.18 (2); Gly 1.08 (1); Arg 2.75 (3); Pro 1.88 (2). The peptides were dissolved in a mixture of water and trifluoroethanol (3:1, v/v) for Fmoc-LGIG or water for Fmoc-QR3P2QG, lyophilized and stored at 4°C.

Oligonucleotide Synthesis and Characterization

Assembly of oligonucleotides was carried out on an Applied Biosystems 380B or 394 Synthesizer on a 1 μmol scale. Oligodeoxyribonucleotides were assembled using standard phosphoramidite chemistry synthesis cycles [39]. Deoxynucleoside amidite monomers (T, bzC, bzA and ibG) were obtained from Cruachem. 2'-*O*-silyl ribonucleoside amidites (U, iPrPacG and PacA) and 2'-*O*-methyl nucleoside amidites (U, bzA and bzC) were obtained from Glen Research (*via* Cambio) and oligoribonucleotides assembled by standard protocols using tetrazole as activator [40]. 2'-*O*-methyl ribonucleoside amidites were also coupled for 15 min. using tetrazole as activator. 500 Å long chain alkylamine controlled pore glass (CPG) supports loaded with dT (Cruachem) or rU or 2'-*O*-Me-bzC (Glen Research) were used in assemblies. TentaGel T (loading of dT : 220 μmole g^{-1}) was obtained from Rapp Polymere. Amino functionalized PEG-PS support (PerSeptive Biosystems) was loaded by reaction with 2'-deoxythymidine 3'-*O*-succinate by a standard protocol [41] to give a loading of 50 μmol g^{-1}. Cycles for TentaGel and PEG-PS were identical to those used for CPG except that the iodine oxidant was replaced by 1.5 M *t*-butyl hydroperoxide in dichloromethane [42]. In addition an extra wash with DMF was given immediately after each trichloroacetic acid deprotection step and after each oxidation step which helped to remove contaminants fully from these supports before coupling.

The derivatization of the 5'-end of the oligonucleotide chain was carried out using a previous method [35] by manual flushing of the synthesis cartridge containing the solid support using a syringe. Briefly, the cartridge was flushed with dry dioxane and then reacted sequentially with a 0.3 M solution of carbonyl diimidazole (Aldrich) in dioxane for 45 min. at room temperature, flushed again with dioxane, and then reacted with a 0.2 M solution of either 1,3-diaminopropane, 1,6-diaminohexane or 1,12-diaminododecane (Aldrich) in dioxane/water (9:1, v/v) for 45 min. at room temperature.

One batch of each synthesis was deprotected by standard protocols and analysed by reversed phase HPLC (Table 5) on a μBondapak™ C18 column (Waters), 125 Å, 10 μm using a gradient of acetonitrile in 0.1M triethylammonium acetate solution (pH 6.5). Gradient A= 10 to 35 % CH3CN over 25 min., gradient B= 0 to 40 % CH3CN over 30 min. and gradient C= 10 to 45 % CH3CN over 25 min., 1.0 ml min^{-1} flow rate, UV detection at 260 nm. Each gradient was followed by continuation to 50 % CH3CN for 5 min. The crude products were also characterized by MALDI-TOF mass spectrometry (Table 5) in negative ion mode (except positive ion mode for d16-mer) using 2,6-dihydroxyacetophenone (DHAP, 30 mg ml^{-1}) and diammonium citrate (40 mg ml^{-1}) as matrix (sample: matrix, 1:5, v/v).

Table 5. HPLC and Mass Spectral Characterization of Oligonucleotide Main Products.

	Oligos	dT_{12}*	Tat d16-mer**	rU_9*	r/Me 9-mer*
elution time (min)		15.1	17.6	6.9	14.5
m/e found	5'-HO	3585.7	4867.9	2695.9	2893.8
expected		*3587.6*	*4870.8*	*2693.3*	*2895.5*
elution time (min)		15.7	18.0		
m/e found	5'-NH_2 n=3	3685.5	4962.9		
expected		*3688.6*	*4971.8*		
elution time (min)		16.2	18.1	12.1	15.4
m/e found	5'-NH_2 n=6	3730.7	5010.9	2834.6	3035.4
expected		*3730.7*	*5013.9*	*2836.4*	*3038.6*
elution time (min)		25.1	23.5	19.2***	20.0***
m/e found	5'-NH_2 n=12	3813.5	5089.4	2920.4	3123.7
expected		*3814.7*	*5097.9*	*2920.5*	*3122.7*

* HPLC running on gradient A, ** HPLC running on gradient B, *** HPLC running on gradient C

Coupling of Peptide Fragments to Oligonucleotides

Protocol A. 100 µl volumes of activation reaction were prepared using the desired amount of Fmoc glycine or Fmoc peptide (5 equivalents compared to oligonucleotide) dissolved in DMF, with diisopropylethylamine (DIEA; 10 equivalents) being added, followed by a DMF solution of HOBt (0.196 M) and then of PyBOP (0.192 M) (each 1 equivalent compared to peptide). The reaction was allowed to proceed for 15 min. at room temperature, poured into the support-bound oligonucleotide (1 µmol) contained in a small glass vial (final peptide concentration 50 mM) and shaken overnight at room temperature.

Protocol B. The coupling reaction was carried out as described in Protocol A but with 10 equivalents of peptide compared to oligonucleotide. The final concentration of the peptide was maintained at 50 mM.

Protocol C. Cyanoethyl protecting groups were first removed from the support-bound oligonucleotide [37] by treatment with triethylamine/acetonitrile (TE/AC) (4:6, v/v, 1 ml) for 10 min, followed by washing with acetonitrile (2 x 500 µl), and treated again with TE/AC for 30 min. and then washed again with acetonitrile (2 x 500 µl) . The coupling reaction was carried out as described in Protocol B.

Cleavage of Conjugates from the Solid-Support and Deprotection

The supernatant was removed and the support was rinsed with DMF (2 x 200 µl) and then with diethyl ether (2 x 200 µl) and dried in air. For the peptide-DNA conjugates $\mathbf{1a_n,b_n,c_n\text{-}2a_n,b,_nc_n}$, the support was treated with concentrated aqueous ammonia (1 ml) for 4 h. (dT_{12}) or 24 h. (d16-mer) at room temperature and then evaporated to dryness. For the peptide-RNA conjugates $\mathbf{3a_n,b_n,c_n\text{-}4a,_nb_n,c_n}$, the support was treated with anhydrous methanolic ammonia (1.5 ml) for 24 h. and the supernatant was concentrated under reduced pressure and a solution of triethylamine trihydrofluoride (98%)-DMF (4:1, v/v, 1 ml) was added. The reaction was allowed to proceed for 1.5 h at 55°C. The product was precipitated by addition of butan-1-ol at -20°C, overnight [40].

Analysis and Characterisation of Peptide-Oligonucleotide Conjugates

Analytical and preparative HPLC of conjugates were carried out on an HPLC-RP-C18 column (µBondapak™) as described above for oligonucleotides. Conjugate molecular masses were determined by

MALDI-TOF MS (PerSeptive Biosystems) in negative mode (positive ion for d16-mer) using 2,6-DHAP (30 mg ml^{-1}) and diammonium citrate (40 mg ml^{-1}) as matrix (sample:matrix, 1:5, v/v).

Table 6. HPLC and Mass Spectral Characterization of Peptide-Oligonucleotide Conjugates

			n=3	n=6	n=12			n=6	n=12
elution time (min)	dT_{12}		16.0*	17.1*	23.5*	rU9		13.3*	20.5***
m/e found		**$1a_n$**	3741.9	3785.3	3873.2		**$3a_n$**	2890.2	2977.6
expected			*3745.6*	*3787.7*	*3871.8*			*2893.4*	*2977.5*
elution time (min)		**$1b_n$**	20.8	20.9	29.9		**$3b_n$**	18.9	24.5
m/e found			4024.6	4066.9	4153.9			3175.6	3260.3
expected			*4028.9*	*4070.9*	*4154.9*			*3176.6*	*3260.7*
elution time (min)		**$1c_n$**	16.4	24.3	27.6		**$3c_n$**	15.1	22.3
m/e found			4661.3	4704.2	4782.4			3803.7	3891.8
expected			*4664.2*	*4706.2*	*4790.2*			*3811.9*	*3896.0*
elution time (min)	Tat d16-mer		18.4**	18.6**	25.0**	r/Me 9-mer		16.3*	21.3***
m/e found		**$2a_n$**	5036.5	5073.5	5151.5		**$4a_n$**	3092.2	3179.1
expected			*5028.4*	*5070.9*	*5154.9*			*3095.6*	3179.7
elution time (min)		**$2b_n$**	20.2	21.7	29.3		**$4b_n$**	20.2	24.5
m/e found			5307.8	5352.7	5439.7			3377.5	3463.5
expected			*5311.7*	*5354.1*	*5437.6*			*3378.8*	*3462.9*
elution time (min)		**$2c_n$**	nd †	19.2	25.4		**$4c_n$**	17.5	22.6
m/e found			5944.1	5989.2	6072.1			4009.1	4098.7
expected			*5947.4*	*5989.5*	*6073.7*			*4014.1*	*4098.2*

* HPLC running on gradient A, ** HPLC running on gradient B, ***HPLC running on gradient C, † not determined

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REFERENCES AND NOTES

1. This paper is dedicated to the memory of Professor Wang Yu and his pioneering work on peptides and oligonucleotides.
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