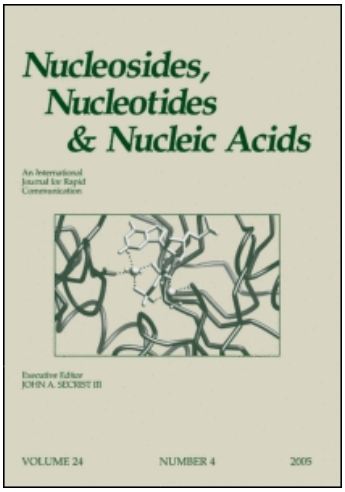

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Studies Towards the Synthesis of Peptide-Oligonucleotide Conjugates

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STUDIES TOWARDS THE SYNTHESIS OF PEPTIDE-OLIGONUCLEOTIDE CONJUGATES

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ABSTRACT: We describe a peptide fragment, solid-phase coupling strategy for synthesis of peptide-oligonucleotide conjugates. Model conjugates contained a hydrophobic tetrapeptide, a hydrophobic influenza virus fusion nonapeptide, or a basic octapeptide of the HIV-1 Tat protein coupled to either dT_{12} or a 16-mer anti-Tat oligodeoxyribonucleotide. Conjugation yields were improved by removal of internucleotide 2-cyanoethyl groups prior to peptide coupling and by use of a C12 spacer between peptide and oligonucleotide.

INTRODUCTION

The development of oligonucleotides as therapeutic agents has been limited by poor cellular uptake [1,2]. Phosphodiester and phosphorothioate oligonucleotides are known to enter cells *via* the endosomal pathway and passage into the cytosol has frequently been observed to be restricted in many cell lines in culture. Delivery mediated by cationic liposomes can boost this passage considerably[3]. Recently, a number of peptidic carriers of oligonucleotides into cells have been proposed, for use either with or without covalent conjugation[4-10]. Of particular interest are certain peptides that are able to translocate through cell membranes by a temperature-independent, non-endosomal route. These include a basic peptide from HIV-1 Tat [11,12], the third helix of the *Antennapedia* homeodomain (Antp-HD)[13], and a 27-residue composite peptide derived from the fusion sequence of HIV gp41 and a hydrophilic nuclear localization signal (NLS) from SV40 T-antigen[14]. Oligonucleotide delivery of disulphide-linked conjugates of Antp-HD into neurons[15,16] and of a composite fusion/NLS peptide into a range of other cells[17] has been demonstrated.

Covalent conjugation is often carried out in aqueous solution following separate synthesis of peptide and oligonucleotide moieties, each carrying a reactive functionality [4,6,18-20]. Alternatively, total stepwise solid-phase conjugate synthesis on a single support has been proposed also[21-25]. These routes suffer from solubility or incompatibility

problems that restricts the choice of conjugates that can be prepared. Following the precedence of Grandas *et al* [26], we have explored a fragment coupling strategy where oligonucleotide and peptide moieties are assembled separately by solid-phase synthesis. The peptide fragment is removed from its support, coupled to the support-bound oligonucleotide and the conjugate is cleaved from the support and deprotected.

RESULTS

A peptide fragment coupling strategy has been used previously in this laboratory for the assembly of small proteins[27,28]. Peptide fragments are synthesized by standard stepwise Fmoc solid-phase synthesis[29]. We investigated coupling of the carboxyl termini of pre-assembled peptide fragments to the 5'-end of support-bound oligonucleotides. A stable amide linkage is achieved *via* 5'-amino functionalization of the oligonucleotide. To avoid the possibility of C-terminal epimerization, we chose the amino acid derivative Fmoc-Gly and three peptides that contain a C-terminal Gly, the hydrophobic tetrapeptide $N\alpha$-Fmoc-Leu-Gly-Ile-Gly-OH, a basic octapeptide $N\alpha$-Fmoc-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-OH, corresponding to part of the basic domain of the HIV-1 *trans*-activator protein Tat, and a hydrophobic nonapeptide $N\alpha$-Fmoc-Phe-Gly-Ala-Ile-Ala-Gln-Phe-Leu-Gly, an influenza virus fusion peptide. As oligonucleotide models we chose dT_{12} and a 16-mer oligodeoxynucleotide sequence d(CTAGGATCTACTGGCT) complementary to part of the HIV-1 Tat gene[30].

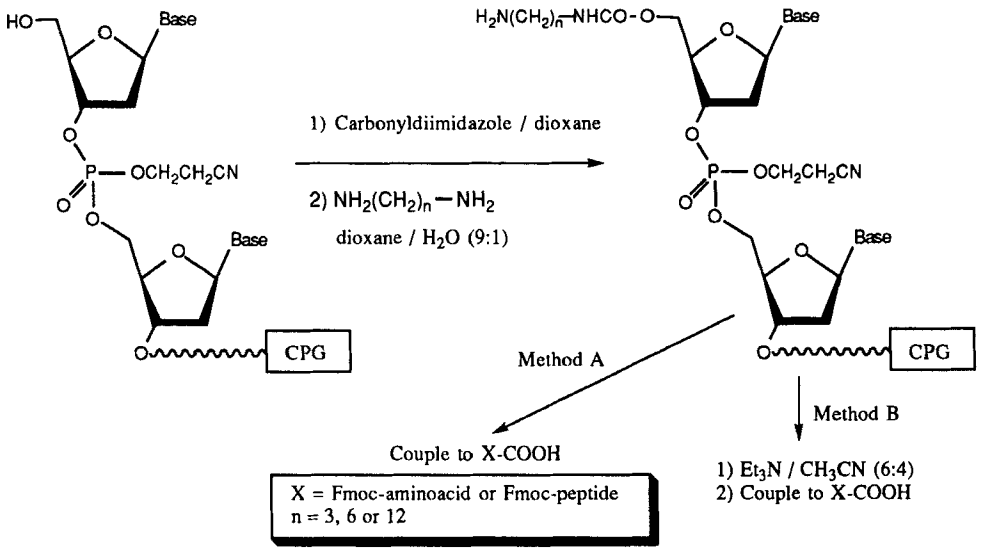

FIG. 1. Scheme for synthesis of peptide-oligonucleotide conjugates.

In assembly of peptide fragments, side-chain protecting groups were used for Gln (trityl), Glu (t-butyl) and Arg (2,2,5,7,8-pentamethylchroman-6-sulfonyl, Pmc). Since the acidic conditions for removal of Pmc caused depurination of oligodeoxyribonucleotides containing purine residues (data not shown), side-chain protecting groups were removed prior to conjugation. Oligonucleotides were assembled on a controlled pore glass (CPG) support by standard phosphoramidite synthesis. Then to generate a 5'-amino group, the terminal 5'-dimethoxytrityl group was removed by acid treatment and the resultant 5'-hydroxyl group reacted with 1,1'-carbonyldiimidazole followed by a diaminoalkane (Figure 1)[31]. We tested a number of conjugation conditions to CPG-bound dT_{12} based on standard peptide fragment coupling conditions[32]. With 5 equivalents of Fmoc-Gly or Fmoc-peptide over the support-bound oligodeoxyribonucleotide containing a 6-carbon atom amino linker, we found that the activator benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate (PyBOP) in the presence of 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF) gave the best results (Method A). After mild ammonia treatment to remove Fmoc and 2-cyanoethyl protecting groups and to cleave the conjugate from the support, analysis by reversed-phase HPLC and by MALDI-TOF mass spectrometry showed good conjugation yields for both Gly and peptide conjugates (Table 1). By contrast in the case of the mixed-base Tat d16-mer, the conjugates were formed only in very low yields (<5%). No improvements were obtained when the type of solid support was altered to polyethylene glycol-polystyrene (TentaGel™ or PEG-PS™), when the temperature of the reaction was increased to 40°C, or when the concentration of the peptide was doubled (data not shown). An increase in the number of equivalents of amino acid or peptide to 10 over support-bound oligonucleotide resulted in a slight improvement in yields (10%, Table 1).

TABLE 1. Effect of removal of 2–cyanoethyl groups on % yields of conjugates

Peptides / Oligos	**dT_{12}**		**Tat d16-mer**	
+ or - 2-cyanoethyl	+	-	+	-
Fmoc-Gly	90	80	10	50
Fmoc-LGIG	80	70	10	30
Fmoc-QR$_3$P$_2$QG	75	60	10	40

We tested the effect of increasing the polarity of the oligonucleotide by removing the 2-cyanoethyl protecting groups from internucleoside phosphates by treatment of the support-bound fully protected oligonucleotide with triethylamine-acetonitrile solution (4:6, v/v)[33]. Following conjugation and deprotection (Method B), conjugation yields were considerably improved (30-50%, Table 1), but yields for conjugation to the dT_{12} sequence were slightly

reduced.The effects of different lengths of spacer (3, 6 and 12 carbon atoms) were also tested. 5'-Aminopropyl, aminohexyl and aminododecyl support-bound oligonucleotides were synthesized for both dT_{12} and Tat d16-mer mixed base sequences, phosphate protecting groups removed, and conjugated to Fmoc-Gly and the three peptide fragments (Table 2). For dT_{12} conjugation yields were relatively unaffected. By contrast, conjugation of the mixed base d16-mer sequence to two of the peptides was significantly improved when the length of the alkyl spacer was increased but not for the hydrophobic nonapeptide.

TABLE 2. Effect of alkyl spacer length on % yields of conjugates.

Peptides / Oligos	**dT_{12}**			**Tat d16-mer**		
n	**3**	**6**	**12**	**3**	**6**	**12**
Fmoc-Gly	80	80	85	< 5	50	70
Fmoc-LGIG	50	70	60	20	30	85
Fmoc-QR3P2QG	45	60	65	< 5	40	40
Fmoc-FGAIAEFLG	nd	35	40	nd	30	10

DISCUSSION

To our knowledge this is the first demonstration that short hydrophobic and basic N_{α}-Fmoc, side-chain unprotected peptide fragments obtained by conventional solid-phase peptide synthesis can be conjugated to CPG-bound oligodeoxyribonucleotides. We have also been able to conjugate such peptides to an oligoribonucleotide[34]. However, a 14-mer basic peptide of Tat[35] failed to couple to the Tat 16-mer oligonucleotide despite several peptide activation conditions tried, whereas the same peptide coupled in high yield to a support bound peptide (data not shown). Thus the conjugation of larger peptides may require more active conjugation conditions.

Recently, dipeptide conjugates of oligodeoxyribonucleotides have been prepared *via* stepwise Fmoc-amino acid couplings to a 5'-amino-5'-deoxythymidine terminated oligodeoxyribonucleotide[36]. In this work, the first amino acid coupled in 50-90% yield but the second in >90% yield. This is in line with our results showing improvements to yield as spacer length is increased. Since removal of 2-cyanoethyl groups was also helpful in improving yields, it may be that a hydrophobic interaction of the support-bound oligonucleotide with incoming peptide adversely affects yields. However, our results give enouragement that a solid-phase fragment coupling approach may be suitable for synthesis of a wide range of peptide-oligonucleotide conjugates and analogues.

METHODS

Solid-phase peptide synthesis was carried out on 0.1 mmol scale by continuous-flow Fmoc methods on an N_{α}-Fmoc-Gly-Wang polystyrene resin (Novabiochem)[29,34]. Fmoc

peptides were analysed by HPLC on a RP-C8 column (Vydac) using gradients over acetonitrile in 0.1 % aqueous TFA. Fmoc-LGIG was 96% pure, Fmoc-QR3P2QG and Fmoc-FGAIAEFLG each 90%. Molecular masses were determined by MALDI-TOF mass spectrometry and showed the expected values. Oligonucleotides were synthesized on 1 μmol scale using standard phosphoramidite chemistry synthesis cycles[37] and 500 Å controlled pore glass (CPG) supports and deoxynucleoside amidite monomers (T, bzC, bzA and ibG, Cruachem). Derivatization of the 5'-end of the oligonucleotide[31,34] was carried out by manual flushing of the synthesis cartridge with: 1) dry dioxane, 2) 0.3 M carbonyl diimidazole (Aldrich) in dioxane for 45 min. at room temperature, 3) dioxane, 4) 0.2 M 1,3-diaminopropane, 1,6-diaminohexane or 1,12-diaminododecane (Aldrich) in dioxane/water (9:1, v/v) for 45 min. at room temperature.

Peptide-oligonucleotide conjugation: *Method A*. 100 μl activation reactions were prepared containing Fmoc Gly or Fmoc peptide (5 or 10 equivalents compared to oligonucleotide) dissolved in DMF to which diisopropylethylamine (DIEA, 10 equivalents) had been added, followed by a DMF solution of HOBt (0.196 M) and then of PyBOP (0.192 M) (each 1 equivalent compared to peptide). After 15 min. at room temperature, the mixture was 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. *Method B*. The support-bound oligonucleotide was treated with triethylamine/acetonitrile (TE/AC) (4:6, v/v, 1 ml) for 10 min, washed with acetonitrile (2 x 500 μl), treated again with TE/AC for 30 min., washed again with acetonitrile (2 x 500 μl) and conjugated as before. Supernatant was removed and the support rinsed with DMF (2 x 200 μl), diethyl ether (2 x 200 μl), dried in air, treated with concentrated aqueous ammonia (1 ml) for 4 h. (dT_{12}) or 24 h. (d16-mer) at room temperature and evaporated to dryness. Analytical and preparative HPLC of conjugates was carried out on an RP-C18 column (μBondapak™, Waters) using gradients of acetonitrile in 0.1M triethylammonium acetate solution (pH 6.5). Conjugate molecular masses were determined by MALDI-TOF MS using 2,6-DHAP (30 mg ml^{-1}) and diammonium citrate (40 mg ml^{-1}) as matrix (sample:matrix, 1:5, v/v).

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