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Synthesis of DNA–Peptide Conjugates by Solid-Phase Fragment Condensation

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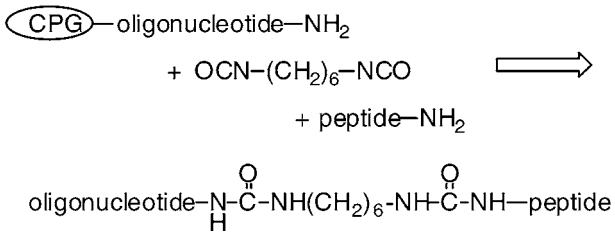
ABSTRACT



DNA–peptide conjugates were synthesized by condensing partially protected peptide fragments and oligonucleotides on a CPG support using diisocyanatoalkane as a linker. After cleavage and deprotection with aqueous ammonia, pure products were obtained by single RPHPLC purification in satisfactory yields and identified by a MALDI-TOF MS spectrometer. This method allows one to prepare DNA–peptide conjugates with any components and sequences of DNA and peptides.

An artificial control of genetic expression is a powerful tool for biological studies and medical therapies. Nucleic acid drugs such as antisense oligonucleotides, antigene oligonucleotides, ribozymes, decoys, and siRNAs, have attracted much attention and have been intensively studied for the past two decades.[1−3] Most of the efforts have been focused on the improvement of binding affinity and specificity of oligonucleotides to target sequences of mRNA or DNA and also on the improvement of resistance against digestion by cellular nucleases. It has been proven that chemical modifications make it possible to overcome such problems to a certain extent. For example, introduction of the phosphorothioate backbone in oligonucleotides enhances their nuclease resistance and retains RNase H activity. Modification of 2′-O hydroxyl groups of ribose moieties increases binding affinity to RNA and dsDNA. Nevertheless, difficulties in using oligonucleotides as therapeutic agents also involve transport of oligonucleotides through cellular membrane, delivery and localization in the targeted cellular structure, and targeting the specific sequence of mRNA or DNA with sufficient affinity and specificity. From such points of view, DNA−peptide conjugates have been attracting intensive attention as alternative and advanced materials for the technology of genetic medicines and novel functional nucleic acids.[4−7]

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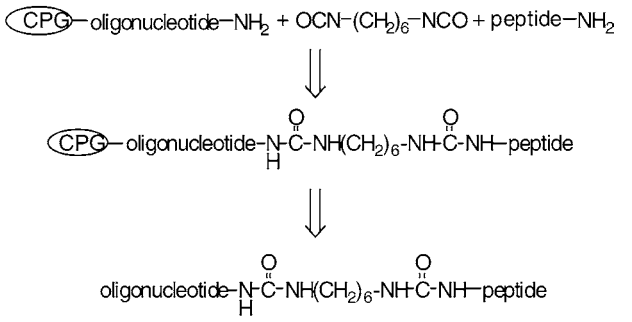
Synthetic methods studied so far can be classified into two categories, that is, solution-phase and solid-phase syntheses. The former mostly involves coupling procedures of DNA and peptide fragments by using small linker molecules having two different functionalities.[8] This method requires two coupling reactions and two isolation procedures and often suffers from a low overall yield of the desired product caused by insufficient reaction efficiencies at low concentration of substrates. M. J. Gait and his colleague successfully reported syntheses of a variety of DNA−peptide conjugates by "native ligation".[9] By their method, oligonucleotides modified with *S*-*tert*-butylsulfenyl L-cystein moiety at 5′-end can be reacted site-specifically with the thioester of a peptide fragment to give a covalent conjugate.

On the other hand, tandem syntheses of DNA−peptide conjugates on a solid phase have also been intensively studied. J. Haralambidis and co-workers achieved sequential synthesis of conjugates of oligonucleotides and viral fusion peptides.[10] In their report, peptides were prepared first on modified CPG support and then oligonucleotides were assembled by an automated DNA/RNA synthesizer. This semiautomated method still has some problems to be solved. One of the major problems is how to prepare fmoc derivatives of all amino acids having protective groups on side chains labile under treatment with ammonia. Recently, A. Azhayev et al. reported an improved method toward a general preparation of DNA−peptide conjugates.[11] They successfully prepared DNA−peptide conjugates containing amino acids bearing hydroxyl, carboxyl, imidazolyl, amino, and indolyl functions by replacing acid-labile protective groups on side chains of peptides with alkaline labile ones before assembly of oligonucleotides. M. J. Gait et al. also reported another improved method of stepwise solid-phase synthesis of DNA/RNA−peptide conjugates.[12]

Solid-phase synthesis of DNA−peptide conjugates in a sequential manner in which peptides are usually prepared first and oligonucleotides are prepared next cannot avoid the problem that the coupling efficiencies in peptide synthesis are not always high enough compared with those in DNA synthesis. Insufficient coupling yields result in lower overall yields of desired products. To overcome such problems as (1) low overall yields of products and (2) limitation of amino acid components in peptides, several attempts of fragment coupling on solid support have been performed. In the previous studies, amino-functionalized oligonucleotides were coupled with carboxyl-activated peptides by amide bond formation.[13] These methods also have limitations in components of peptides because they employ standard coupling conditions of solid-phase peptide synthesis for amide formation. In the present study, we attempted to develop a universal method for preparing DNA−peptide conjugates by "solid-phase fragment condensation" (SPFC).

The strategy of SPFC is that a DNA fragment having a free amino group prepared on a CPG support is reacted with α,ω-diisocyanatoalkane and then with a peptide fragment bearing a single reactive amino group. The resulting DNA−peptide conjugate covalently linked to a solid support is cleaved from CPG and deprotected by treatment with ammonia (Scheme 1).

Scheme 1. Solid-Phase Fragment Condensation



Reactions of SPFC are summarized in Scheme 2. Syntheses of oligonucleotides were performed in 1 μmol scale by an automated DNA/RNA synthesizer on a CPG support using standard cyanoethylphosphoramidite chemistry. Oligonucleotides were modified at the 5′-end by a cyanoethylphosphoramidite derivative of *N*-monomethoxytrityl-2-aminoethoxyethanol (5′-Amino Modifier 5, Glenn Research). After removal of the MMT group with 3% trichloroacetic acid in CH_2Cl_2, 1,6-diisocyanatohexane (50 equiv) in CH_3CN was injected into the reaction column via a syringe. After shaking the reaction column at 20 °C for 12 h, the CPG support was washed with anhydrous CH_3CN (1 mL × 5) to remove the excess diisocyanate. Cross-linked dimeric products, which can be formed by the reaction of 1,6-diisocyanatohexane and two neighboring oligonucleotides, were either negligible or not observed at this step for oligonucleotides larger than 5-mer. Peptide fragments were independently prepared by automated peptide synthesizer using standard fmoc chemistry on Wang resin and cleaved by

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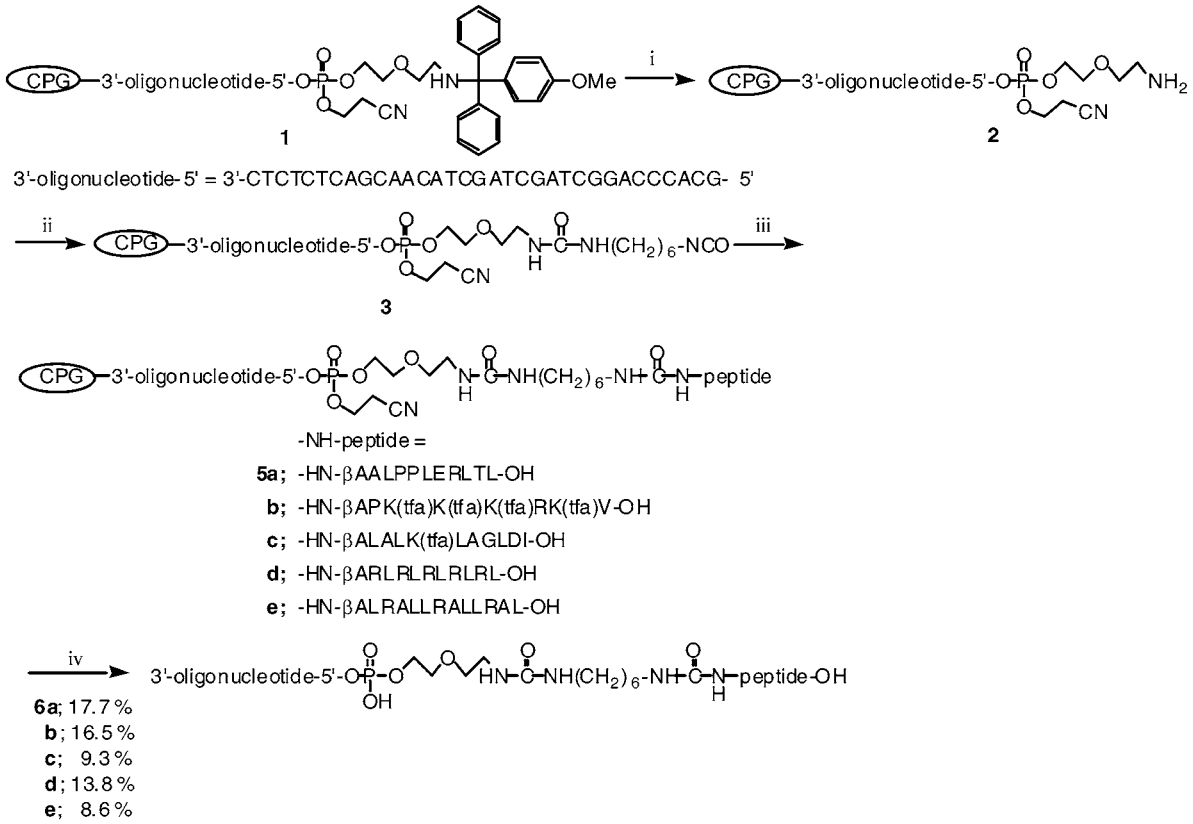
Scheme 2. Synthesis of DNA−Peptide Conjugates by Solid-Phase Fragment Condensation[a]



[a] Reagents and conditions: (i) 3% TCA/CH_3CN; (ii) 1.6-diisocyanatohexane (50 equiv), DIEA (10 equiv), anhydrous CH_3CN, rt, 12 h; (iii) peptide-NH_2 (**4a**−**e**) (10 equiv), DIEA (10 equiv), anhydrous CH_3CN, rt, 24 h; (iv) concentrated NH_4OH, 55 °C, 4 h.

trifluoroacetic acid (95%) and thioanisol. Partially protected peptide fragments were obtained by HPLC purification in satisfactory yields and characterized by a MALDI-TOF mass spectrometer (Table 1). Hydroxyl, carboxyl, and guanidinyl groups were deprotected with TFA, whereas ϵ-amino groups of lysines were still protected with trifluoroacetyl group. β-Alanine was attached at the N-terminus of each peptide, and its amino group was deprotected by treatment with piperidine prior to cleavage reaction. A solution of thus obtained partially protected peptide (10 equiv) in DMF was introduced into the reaction column using a syringe. The reaction column was shaken at 20 °C for 24 h, washed with anhydrous CH_3CN (1 mL × 5), and treated with aqueous ammonia at 55 °C for 4 h for cleavage and deprotection. Solid support was filtered off, and the obtained crude mixture was purified by reversed-phase HPLC. HPLC profiles of **6a** before and after purification are shown in Figure 1 as examples.

Table 1. MALDI TOF Mass Data of Peptides **4a**−**e**[a]

conjugate	MS calcd	MS found *m/z*
4a	1321.63	1322.72
4b	1338.29	1338.88
4c	1304.41	1305.23
4d	1435.83	1435.50
4e	1449.86	1449.78

[a] Matrix: saturated α-cyano-4-hydroxycinnamic acid in 50% CH_3CN/H_2O containing 1% TFA.

Overall yields of the products were determined by measuring absorbances at 260 nm. Isolated yields were from 8.6 to 17.7%. Each DNA−peptide conjugate was characterized by a MALDI TOF mass spectrometer and gave a satisfactory result (Table 2). It is to be pointed out that functional groups of peptide side chains except for an ϵ-amino group of Lys were inert under coupling reaction conditions with an isocyanate function linked to a solid support. It is probable that protonation of guanidinyl group of Arg at neutral pH decreased its nucleophilicity and retarded the attack on the isocyanate group.

As shown in Scheme 2, since reactivity of the N-terminal α-amino group of each peptide is not sufficient, all the peptides used were attached to β-alanine (βA) at their N-terminus to increase the nucleophilicities. Peptide moieties in conjugates **6a**−**e** contain glutamic acid (E), arginine (R), threonine (T), and lysine (K), as well as other nonprotected hydrophobic amino acids. Except for lysine, all the side chain residues were deprotected prior to the coupling reaction. ϵ-Amino groups of lysine were protected with trifluoroacetyl groups that were removed at the final treatment with

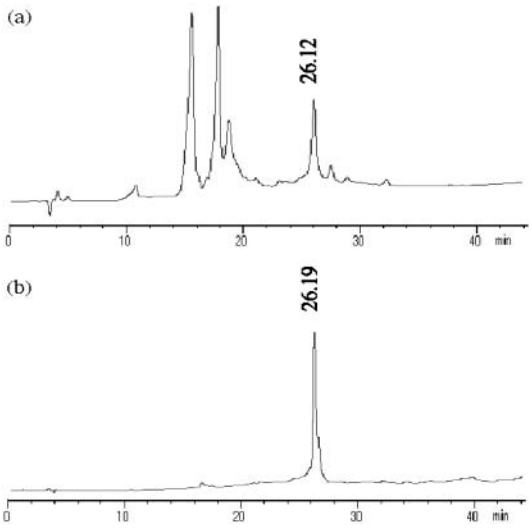


Figure 1. HPLC Profiles of **6a** before (a) and after (b) Purification. Conditions: ODS 4 × 125 mm; (A) 100 mM TEAA; (B) 30% 100 mM TEAA in CH_3CN, a linear gradient of B from 10 to 100% over 60 min.

ammonia. It can be advantageous to use amino groups of peptide fragments for SPFC because amino groups can be easily introduced at desired positions of peptides and selectively protected by both acid- and base-labile protective groups. This allows ones to selectively link oligonucleotides to peptides at desired positions. Peptide moieties in conjugates **6a**−**e** are derived from a nuclear export signal (NES) sequence of HIV-1 rev protein (**6a**),[14] an NES of PKIα (**6b**),[15] a nuclear localization signal (NLS) of SV40 T-antigen (**6c**),[16] and designed peptides (**6d**)[17] and (**6e**).[18] Peptides **6d** and **6e** were proven to form an antiparallel β-sheet and an amphipathic α-helical structure in the presence of DNA, respectively, and it was shown that both of them could bind to and stabilize dsDNA.

Studies on biological properties of these DNA−peptide conjugates, hybridization affinity, nuclease resistance, cellular uptake, and intracellular delivery are now in progress in our laboratory and will be published soon.

Table 2. MALDI TOF Mass Data of Conjugates **6a**−**e**[a]

conjugate	MS calcd	MS found *m*/*z*
6a	10307.17	10308.40
6b	10197.18	10199.38
6c	10065.09	10071.63
6d	10535.83	10556.79
6e	10550.16	10549.97

[a] Matrix: solution A:solution B = 1:1. Solution A: saturated 2′,6′-dihydroxyacetophenone in 50% CH_3OH/H_2O. Solution B: saturated diammonium hydrogen citrate in 50% CH_3OH/H_2O.

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Supporting Information Available: Synthetic details for peptides **4a**−**e** and conjugates **6a**−**e** and HPLC profiles of **6a**−**e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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