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Article

High-Resolution HPLC for Separating Peptide−Oligonucleotide Conjugates

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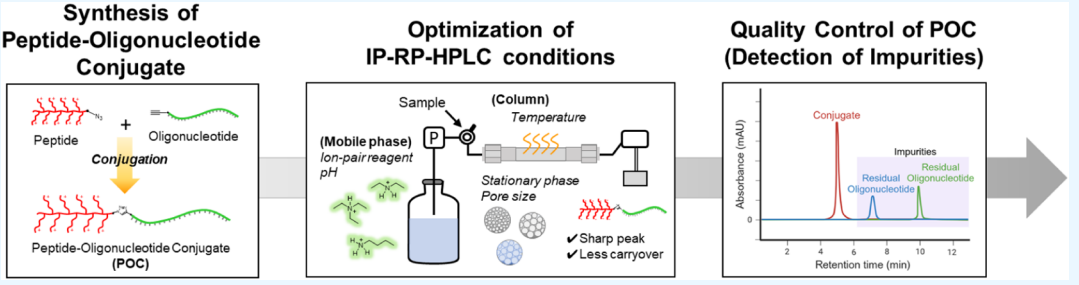

ABSTRACT: Peptide−oligonucleotide conjugates (POCs) are chimeric molecules that combine the specificity of oligonucleotides with the functionality of peptides, improving the delivery and therapeutic potential of nucleic acid-based drugs. However, the analysis of POCs, particularly those containing arginine-rich sequences, poses major challenges because of aggregation caused by electrostatic interactions. In this study, we developed an optimized high-performance liquid chromatography (HPLC) method for analyzing POCs. Using a conjugate of DNA and nona-arginine as a model compound, we systematically investigated the effects of various analytical parameters, including column type, column temperature, mobile-phase composition, and pH. A column packed with C18 resin with wide pores combined with butylammonium acetate as the ion-pairing reagent and an optimal column temperature of 80 °C provided superior peak resolution and sensitivity. The optimized conditions gave clear separation of POCs from unlinked oligonucleotides and enabled the detection of nucleic acid fragments lacking an alkyne moiety as a linkage part, which is critical for quality control. Our HPLC method is robust and reproducible and substantially reduces the complexity, time, and cost associated with the POC analysis. The method may improve the efficiency of quality control in the production of POCs, thereby supporting their development as promising therapeutic agents for clinical applications.

INTRODUCTION

Oligonucleotide-based therapeutics are rapidly emerging in the biopharmaceutical industry.[1−4] Several oligonucleotide-based therapeutics, including antisense oligonucleotides, morpholinos, and small interfering RNA, have been approved by the US Food and Drug Administration, and others are being tested in clinical trials.[5,6] In particular, oligonucleotide modalities are easy to design because their sequences can be decided based on existing target sequences.[7] Another advantage of oligonucleotide-based therapeutics is their versatility in modulating target molecules, such as RNA and DNA.[8] These therapeutics have shown great potential to treat various orphan diseases.[9]

A major challenge in developing oligonucleotide-based therapeutics is ensuring effective delivery to their targets at the cellular and systemic levels.[10] Because both oligonucleotides and cellular membranes are negatively charged, it is difficult for oligonucleotides to cross the membrane. Peptide−oligonucleotide conjugates (POCs) are a common solution for improving the membrane permeability of oligonucleotide-based drugs. Conjugating oligonucleotides and membrane-permeable peptides, such as oligoarginine, improves intracellular delivery, enhancing the therapeutic potential of oligonucleotides.[11−13] In addition, at the systemic level, POCs improve the delivery of oligonucleotides to impervious tissues, such as muscle and central nervous system tissues. Cell-penetrating peptide-conjugated oligonucleotides have been delivered to the central nervous system through the blood−brain barrier[14,15] and to muscle via systemic administration.[16,17]

POCs have been synthesized by various methods.[18,19] Most synthetic methods rely on postsynthetic coupling, in which oligonucleotides and peptides are prepared separately and then

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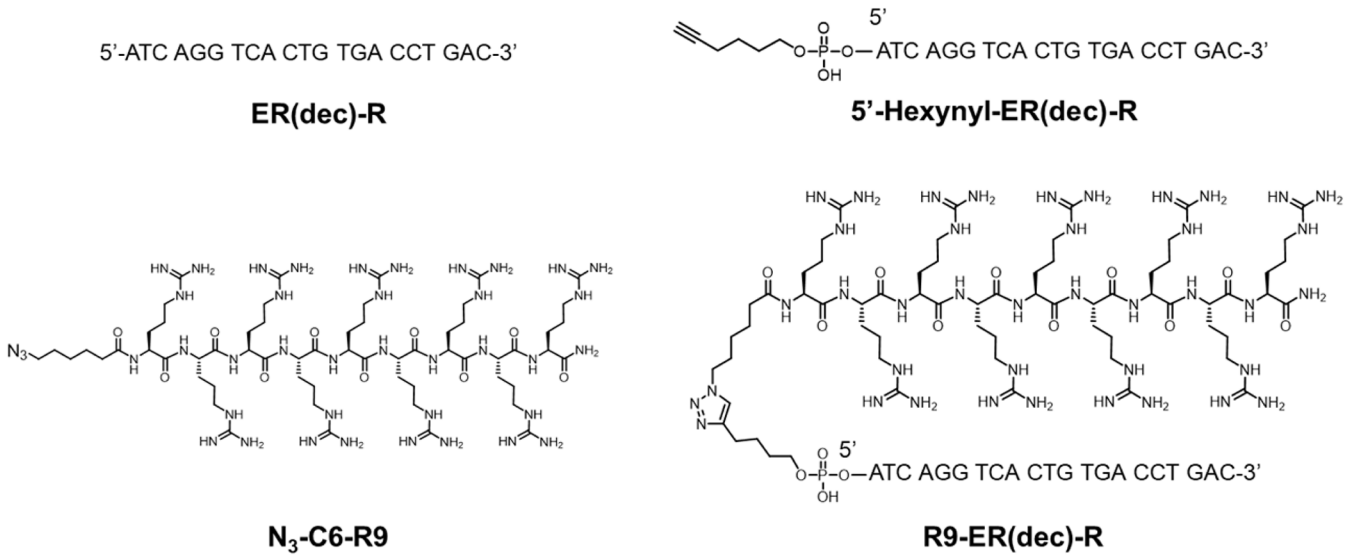

Figure 1. Compounds used for the LC analysis in this study.

conjugated. The model POC used in the present study is prepared by azide−alkyne cycloaddition, known as click chemistry, which employs this strategy. After postsynthetic coupling, unreacted ingredients (i.e., oligonucleotides and peptides modified for linkage reactions) can remain as impurities in the reaction mixture, and unmodified oligonucleotides and peptides are impurities associated with the ingredients. These impurities complicate the development of standardized quality control methods for the fabrication of POC drugs. Therefore, analytical methods for measuring residual oligonucleotides and peptides in their modified and unmodified forms are required for the proper quality control of POC drugs.

Here, we present the development of analytical methods for the quality control of POC drugs synthesized by conjugation using a click reaction. Ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) is widely used for oligonucleotide analysis. We optimized the method to achieve a sensitive analysis of POCs by exploring the stationary-phase modification groups, ion-pair reagents, mobile-phase organic solvent, mobile-phase pH, and column temperature, resulting in a robust and precise analytical method for characterizing POCs.

RESULTS AND DISCUSSION

DNA used in our previous studies (**ER(dec)-R**)[20] was used as the model nucleic acid (Figure 1). The length of this DNA sequence is 21-mer. We considered this sequence to be a suitable target for our model; nucleic acids with similar strand lengths have been used in marketed nucleic acid therapeutics.[21] First, to prepare a conjugate of DNA and nona-arginine (R9), a click reaction was performed between DNA modified with an alkyne group at the 5′-terminus (**5′-Hexynyl-ER(dec)-R**) and R9 modified with an azide group at the *N*-terminus (**N_3-C6-R9**) by copper-catalyzed azide−alkyne cycloaddition. To prevent the formation of aggregates in the reaction system, the linkage reaction between the nucleic acid and peptide was performed in a buffer solution containing 8 M urea (0.1 M K_2HPO_4, 0.3 M KBr, and 8 M urea).[22] The POC, **R9-ER(dec)-R**, in this reaction solution was analyzed by using the LC system.

In the reversed-phase HPLC analysis of **R9-ER(dec)-R**, analytical columns were screened first. Among analytical columns with various carbon contents and pore diameters, the C4 column showed substantial peak tailing (Figure 2c),

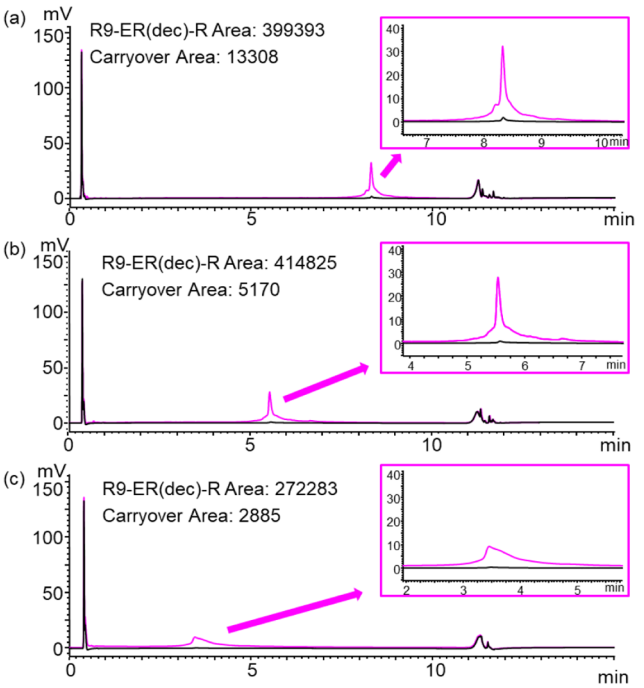

Figure 2. Chromatograms of the crude conjugated product (pink lines) and blank (black lines) obtained with different columns. (a) Accura Triart C18 (pore size: 12 nm), (b) Accura Triart Bio C18 (pore size: 30 nm), and (c) Accura Triart Bio C4 (pore size: 30 nm).

whereas the C18 column exhibited a good peak shape (Figure 2a,b). Additionally, the wide-pore C18 resin (Triart Bio C18) exhibited less carryover on the HPLC chromatograms (carryover area: 5170) than the Triart C18 resin (carryover area: 13,308). The pore size of the packing material differed between the Triart C18 (pore size: 12 nm) and Triart Bio C18 resins (pore size: 30 nm), which was expected to affect peak

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shape[23] and the amount of carryover of **R9-ER(dec)-R**. Based on these results, Triart Bio C18 resin was selected as the optimal solid support for analyzing **R9-ER(dec)-R**.

The mobile phase used in the LC analysis was also investigated. In nucleic acid analysis, ion-pair reagents for phosphate groups, such as triethylammonium acetate (TEAA), are commonly used to improve signal sensitivity and peak separation. We compared the following ion-pair reagent systems in the mobile phase: triethylamine (TEA)-1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/methanol (MeOH); TEAA/acetonitrile (ACN); diethylammonium acetate (DEAA)/ACN; and butylammonium acetate (BAA)/ACN.

The TEA-HFIP system generally provides better retention and separation based on oligonucleotide length, and better sensitivity in mass spectrometry compared with the TEAA system.[24] TEA-HFIP/MeOH and DEAA/ACN systems were examined (Figure 3a,b). Although the reproducibility of the retention time on the LC chromatograms was good, the peak area values were not reproducible. In contrast, the TEAA/ACN and BAA/ACN systems gave good peak shapes with the desired signals, and the retention time and peak area values were reproducible (Figure 3c,d). In BAA buffer, good results were obtained in the analysis of **R9-ER(dec)-R**, probably because of the hydrophobicity of the alkyl groups in the ion-pairing butylammonium salts and the formation of an ion pair with the phosphate moiety. The gradient of the mobile phase was optimized under BAA/ACN conditions in subsequent experiments owing to the sharper POC peak shape compared with the other mobile phases tested. The butylamine concentration in the mobile phase was also investigated. The peak shape deteriorated greatly at a butylamine concentration of 50 mM, possibly because no ion-pair formation occurred between the phosphate moiety of **R9-ER(dec)-R** and butylamine at such a low butylamine concentration. Based on these results, the optimum concentration of butylamine was determined as 100 mM, which produced a good peak shape.

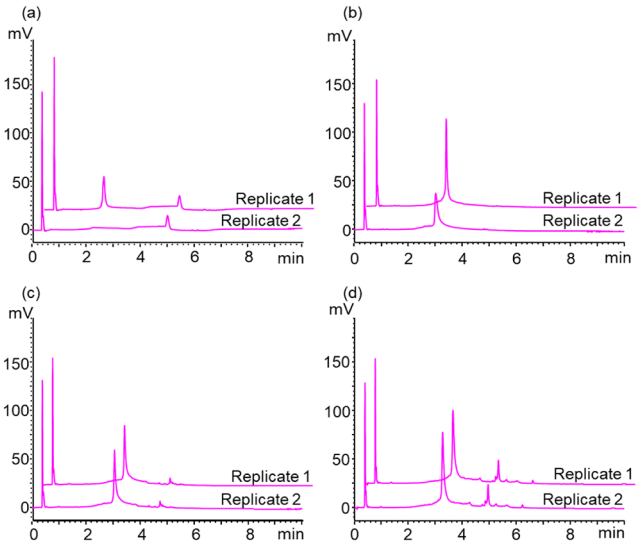

Figure 3. Chromatograms of the crude conjugated product obtained with different mobile-phase solutions: (a) 15 mM TEA, 400 mM HFIP/MeOH, (b) 100 mM DEAA (pH 7.0)/ACN, (c) 100 mM TEAA (pH 7.0)/ACN, and (d) 100 mM BAA (pH 7.0)/ACN.

With the BAA/ACN system, the target signal eluted with a sharp peak shape, even when a gentle gradient was applied (Figure 4). After testing several mobile-phase gradient settings, condition (a) (7−17% B from 0 to 10 min, 100% B from 10 to 15 min) was selected as the optimal mobile-phase gradient because the minor and main peaks tended to separate.

The effect of the BAA buffer pH on the LC analysis of POC is shown in Figure 5. In a pH range of 6.0−8.0, the conjugates had no major effect on retention time or peak shape during LC analysis. Comparing the chromatograms showed greater separation of the minor peak and the conjugate at pH 7.0 and 8.0 than at pH 6.0. Carryover of the conjugate was observed at each pH, with the highest carryover at pH 8.0. Based on these results, pH 7.0 was determined to be the optimal buffer pH because minor peaks were separated and the carryover was relatively low.

The effect of the column temperature on LC analysis of **R9-ER(dec)-R** was examined (Figure 6). Generally, increasing the column temperature is expected to improve peak shape, and this technique is particularly effective for high-molecular-weight compounds.[25,26] The retention time of **R9-ER(dec)-R** tended to shorten as the column temperature increased, and the peak corresponding to **R9-ER(dec)-R** was difficult to detect at 40 °C (Figure 6a). The peak shape remained broad at 60 °C (Figure 6b), but became sharp at 70 °C (Figure 6c) and 80 °C (Figure 6d). A column temperature of 80 °C was determined as optimal because the peak shape was sharp and the minor peaks tended to separate.

Finally, the elution of POC, **R9-ER(dec)-R**, and unlinked oligonucleotide, **5′-Hexynyl-ER(dec)-R**, on the chromatogram was confirmed under optimized analytical conditions. In some previously reported HPLC analyses of POCs, the elution times of POCs and unlinked oligonucleotides did not change substantially.[22,27] However, under the optimized analytical conditions in this study, unreacted oligonucleotide (**5′-Hexynyl-ER(dec)-R**) was separated from POC (**R9-ER(dec)-R**) (Figure 7). Furthermore, nucleic acid fragments without the 5′-hexynyl group (**ER(dec)-R**), which is the linking position on the oligonucleotide side, could be distinguished and analyzed. Because unmodified nucleic acid fragments are impurities in POC production, it is desirable to measure them by separation in a single analysis. Therefore, the analytical conditions established in this study may be suitable for the quality control evaluation of the POCs.

In conclusion, this study established a robust, reproducible HPLC analytical method for characterizing POCs using a conjugate of DNA and an arginine-rich peptide as the model compound. By optimizing key parameters, including the type of analytical column, column temperature, mobile-phase composition, and pH, we demonstrated that the BAA/ACN system provides superior peak resolution and sensitivity for analyzing POCs. A C18 resin with wide pores and an optimal temperature of 80 °C were critical in achieving sharp peak shapes and minimizing carryover, which are essential for accurate and reproducible analysis. For preparative chromatography on a large scale, maintaining a high column and mobile-phase temperature using a thermostatic bath is necessary. Nevertheless, the LC conditions employed in this study are expected to be applicable up to a certain scale. Regarding the scope of application of the analytical method developed in this study, for nucleic acid molecules with charged moieties that can interact with ion-pair reagents—such as RNA and other modified nucleic acids (e.g., 2′-*O*-alkyl, phosphorothioate)—

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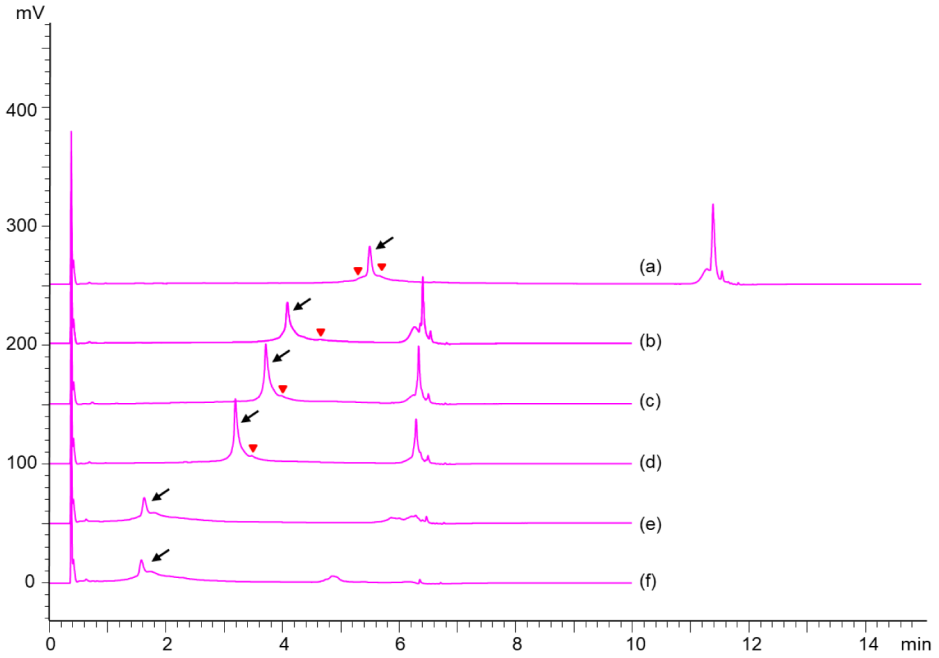

Figure 4. Chromatograms of the crude conjugated product obtained with different gradient elution conditions where the percentage of mobile-phase solution B (ACN) was gradually increased: (a) 7−17% B (0−10 min), 100% B (10−15 min); (b) 7−17% B (0−5 min), 100% B (5−10 min); (c) 5−25% B (0−5 min), 100% B (5−10 min); (d) 7−27% B (0−5 min), 100% B (5−10 min); (e) 10−30% B (0−5 min), 100% B (5−10 min); and (f) 10−40% B (0−5 min), 100% B (5−10 min). Black arrows indicate **R9-ER(dec)-R** peaks. Red triangles indicate shoulder peaks partially separated from the **R9-ER(dec)-R** peaks.

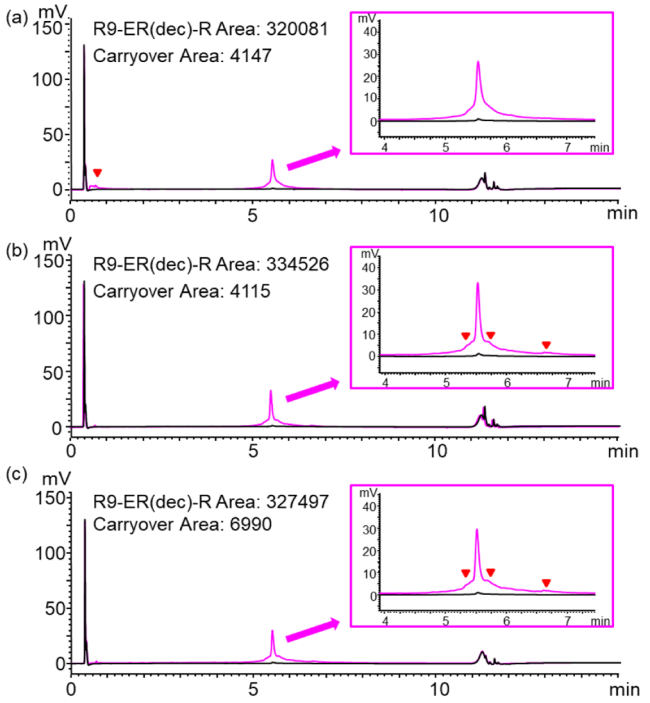

Figure 5. Chromatograms of the crude conjugated product (pink lines) and blank (black lines) obtained with BAA buffer at pH (a) 6.0, (b) 7.0, and (c) 8.0. Red triangles indicate shoulder peaks partially separated from the **R9-ER(dec)-R** peaks.

IP-RP-HPLC is expected to be applicable, although variations in elution time and chromatographic behavior are anticipated. Conversely, peptide nucleic acids (PNA) and morpholino nucleic acids do not possess charged moieties, making ion-pair chromatography ineffective. Therefore, alternative chromatographic conditions tailored to the linked peptide molecules are required for their analysis. Additionally, with regard to the nucleic acid chain length, 21-mer DNA was used as a model in this study. For the analysis of longer nucleic acid chains, it is essential to optimize the packing material and mobile-phase conditions based on the specific analyte,[29] and the same considerations would apply to the corresponding POCs.

Our findings highlight the importance of selecting appropriate ion-pair reagents and buffer systems in improving the separation and detection of POCs. The BAA buffer was particularly important because of its hydrophobic alkyl groups and effective ion-pair formation. Additionally, the optimized conditions facilitated the clear separation of unlinked oligonucleotides and POCs, which is crucial for the quality control of POC production using an LC system. This method offers a major improvement over previously reported HPLC techniques by eliminating the need for complex systems that combine multiple modes, such as two-dimensional HPLC methods,[28] and by reducing analysis time and cost. Overall, our HPLC method is a valuable tool for the analysis and quality control of POCs, paving the way for more efficient development and use of these promising therapeutic agents in clinical settings.

MATERIALS AND METHODS

Synthesis of Oligonucleotide. The model DNA sequence used in this study (5′-ATC AGG TCA CTG TGA CCT GAC-3′, **ER(dec)-R**)[20] was prepared by automated DNA/RNA synthesizer (*M-2-MX-E*, Nihon Techno Service). Solid-phase synthesis of the oligonucleotide was performed by standard phosphoramidite chemistry on a 1.0 μmol scale loaded on 500 Å controlled-pore glass (CPG) supports (High Load Glen UnySupport, Glen Research). Alkyne modification to the 5′ terminus of the DNA was performed using 5′-hexynyl

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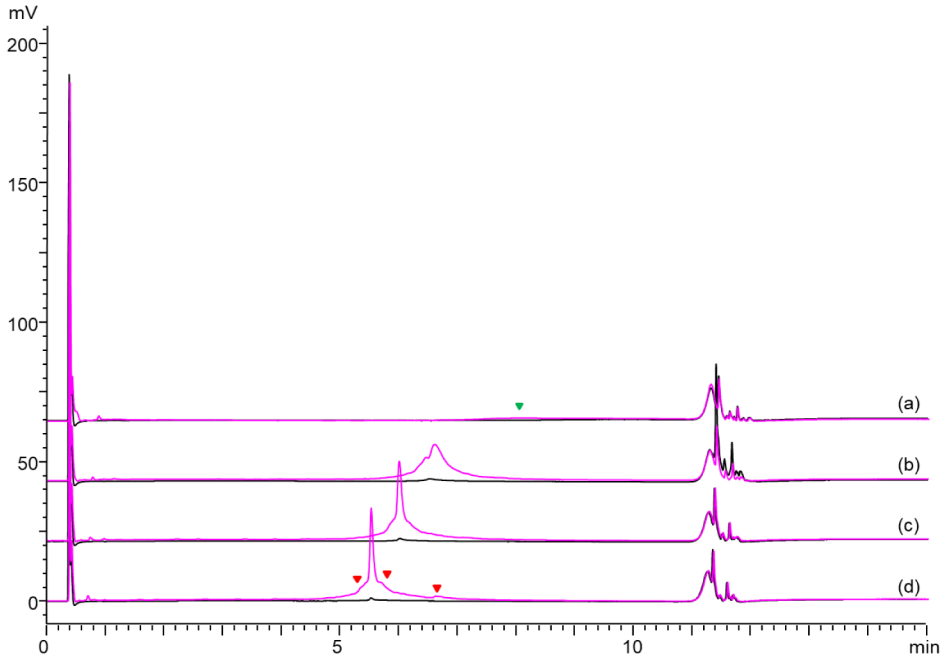

Figure 6. Chromatograms of the crude conjugated product (pink lines) and blank (black lines) obtained at column temperatures of (a) 40, (b) 60, (c) 70, and (d) 80 °C. Red triangles indicate shoulder peaks partially separated from the **R9-ER(dec)-R** peaks. At 40 °C, no elution of **R9-ER(dec)-R** was observed, although the UV absorption signal showed a slight increase from 7 to 9 min (green triangle).

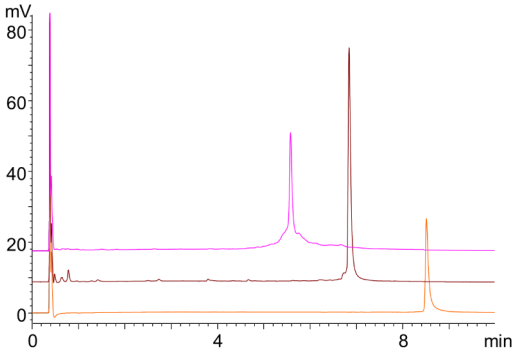

Figure 7. Chromatograms of the crude conjugated product (**R9-ER(dec)-R**, pink line), unmodified oligonucleotide (**ER(dec)-R**, brown line), and modified oligonucleotide (**5′-Hexynyl-ER(dec)-R**, orange line).

phosphoramidite (Glen Research). The oligonucleotides on CPG were treated with 28% aqueous ammonia (1 mL) at 55 °C for 16 h to release them from the solid support and deprotect the bases. After being cooled to room temperature, the resulting mixture was filtered and purified by reverse-phase HPLC (0.1 M TEAA (pH 7.0)/ACN system). The collected fraction containing the target material was lyophilized, and its purity was assessed using HPLC (JASCO) equipped with a CAPCELL PAK MG-II column (OSAKA Soda) (C18, 250 mm × 4.6 mm I.D., 5 μm, solvent A: 0.1 M TEAA (pH 7.0), solvent B: ACN, flow rate: 1.0 mL/min, gradient: 10−40% solvent B over 20 min). The obtained oligonucleotide was dissolved in nuclease-free water, and the concentration of the stock solution was estimated by UV absorbance at 260 nm using a NanoDrop ONE (Thermo Fisher Scientific). The structure of the obtained oligonucleotide (**5′-Hexynyl-ER-(dec)-R**) was confirmed by MALDI-TOF MS analysis (matrix: 3-hydroxypicolinic acid (Sigma-Aldrich)).

Synthesis of Peptide. Nona-arginine (R9) was prepared using microwave-assisted synthesis by Fmoc solid-phase peptide synthesis with Liberty Blue (CEM). A representative coupling and deprotection cycle is described as follows. Rink Amide ProTide (LL; 0.18 mmol/g) (CEM) was soaked for 30 min in CH_2Cl_2. After the resin had been washed with DMF, the Fmoc protecting group was removed by treatment with 25% piperidine in DMF for 15 min at room temperature. Amino acids were coupled for 1 h using 4 equiv of Fmoc-Arg(Pbf)−OH, 4 equiv of (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholinocarbenium hexafluorophosphate (COMU) as the activating agent, and 8 equiv of *N*,*N*-diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (NMP). Azide modification to the *N*-terminus of the peptide was performed using 6-azidohexanoic acid. The peptide on the resin was suspended in a cleavage cocktail (95% TFA, 2.5% water, 2.5% triisopropylsilane) at 42 °C for 30 min on Razor (CEM) to cleave it from the resin. TFA was evaporated to a small volume under a stream of N_2 and dripped into a cold ether to precipitate the peptide. The peptides were dissolved in dimethyl sulfoxide (DMSO) and purified using reverse-phase HPLC (Waters) using a Discovery BIO Wide Pore C18 column (Supelco) (250 mm × 21.2 mm I.D., 5 μm; solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/ACN, flow rate: 10 mL/min, gradient: 10−90% solvent B over 40 min). After purification, the peptide solutions were lyophilized, and peptide purity was assessed using UPLC (Waters) equipped with an ACQUITY UPLC BEH C18 column (Waters) (50 mm × 2.1 mm I.D., 1.7 μm; solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/ACN, flow rate: 0.5 mL/min, gradient: 10−90% solvent B over 4 min). The structure of the obtained peptide (**N_3-C6-R9**) was confirmed by IT-TOF MS (Shimadzu) equipped with an electrospray ionization source and a CAPCEL PAK MG-II column (C18, 35 mm × 2.0 mm I.D., 3 μm) (Osaka Soda) at 40 °C.

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Preparation of POC. Conjugation between 5′-hexynyl-DNA and *N*-terminus azide peptide was performed by copper-catalyzed azide−alkyne cycloaddition (CuAAC). To prevent aggregation between nucleic acids and cationic peptides, reactions were performed in urea buffer (0.1 M K_2HPO_4 (pH 7.4), 0.3 M KBr, 8 M urea).[22] Briefly, to a mixture of **5′-Hexynyl-ER(dec)-R** (1.0 mM, 10 μL) and **N_3-C6-R9** (1.0 mM, 10 μL) in urea buffer was added a mixture of 50 mM $CuSO_4$−$5H_2O$ and 100 mM sodium L-ascorbate in urea buffer (5 μL). The mixture was incubated at 55 °C for 2 h. The progress of the reaction and the formation of the nucleic acid−peptide conjugate (**R9-ER(dec)-R**) (molecular weight: 8128) were confirmed by 20% denaturing polyacrylamide gel electrophoresis (dPAGE) containing 7 M urea (Figures S5 and S6). After dPAGE analysis (300 V, 25 min), the bands were stained by SYBR Green I (Takara Bio) and visualized by a ChemiDoc MP (Bio-Rad). This reaction mixture was directly used as a sample for LC analysis.

Liquid Chromatography (LC) Analysis. LC analysis of POC was performed by a Nexera X2 (Shimadzu) for the LC system. Accura Triart C18 (50 × 2.1 mm I.D., 1.9 μm), Accura Triart Bio C18 (50 × 2.1 mm I.D., 1.9 μm), or Accura Triart Bio C4 (50 × 2.1 mm I.D., 1.9 μm) (YMC CO., LTD.) was used as LC column. The conditions for LC analysis of POC and its related compounds are as follows: mobile phase: 15 mM TEA-400 mM HFIP/MeOH, 100 mM TEAA (pH 7.0)/ACN, 100 mM DEAA (pH 7.0)/ACN, or 100 mM BAA (pH 6.0−8.0)/ACN, flow rate: 0.4 mL/min, monitoring wavelength: 260 nm, column temperature: 40−80 °C.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c01308.

Structure of compounds used in this study, LC trace and MS spectrum of synthesized oligonucleotide and peptide, dPAGE analysis of POC in the reaction mixture, Peak identification of compounds on LC chromatogram by dPAGE analysis (PDF)

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Author Contributions

M.N., D.W., M.A., and R.H. performed the experiments and analyzed the data. G.T., Y.D., Y.H., N.H., S.N., and T.N. designed the research. G.T., Y.D., Y.H., and T.N. wrote the paper. All authors discussed the results and commented on the manuscript.

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Notes

The authors declare the following competing financial interest(s): M.A., R.H., Y.H., N.H., S.N., and T.N. are employed by YMC CO., LTD., a manufacturer of the HPLC columns used in this study.

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■ ABBREVIATIONS

POCs, peptide−oligonucleotide conjugates; HPLC, high-performance liquid chromatography; R9, nona-arginine; TEA, triethylamine; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; TEAA, triethylammonium acetate; DEAA, diethylammonium acetate; BAA, butylammonium acetate; ACN, acetonitrile; MeOH, methanol

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