Versatile Phosphoramidation Reactions for Nucleic Acid Conjugations with Peptides, Proteins, Chromophores, and Biotin Derivatives

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Chemical conjugations of nucleic acids with macromolecules or small molecules are common approaches to study nucleic acids in chemistry and biology and to exploit nucleic acids for medical applications. The conjugation of nucleic acids such as oligonucleotides with peptides is especially useful to circumvent cell delivery and specificity problems of oligonucleotides as therapeutic agents. However, current approaches are limited and inefficient in their ability to afford peptide−oligonucleotide conjugates (POCs). Here, we report an effective and reproducible approach to prepare POCs and other nucleic acid conjugates based on a newly developed nucleic acid phosphoramidation method. The development of a new nucleic acid phosphoramidation reaction was achieved by our successful synthesis of a novel amine-containing biotin derivative used to systematically optimize the reactions. The improved phosphoramidation reactions dramatically increased yields of nucleic acid−biotin conjugates up to 80% after 3 h reaction. Any nucleic acids with a terminal phosphate group are suitable reactants in phosphoramidation reactions to conjugate with amine-containing molecules such as biotin and fluorescein derivatives, proteins, and, most importantly, peptides to enable the synthesis of POCs for therapeutic applications. Polymerase chain reactions (PCRs) to study incorporation of biotin or fluorescein-tagged DNA primers into the reaction products demonstrated that appropriate controls of nucleic acid phosphoramidation reactions incur minimum adverse effects on inherited base-pairing characteristics of nucleotides in nucleic acids. The phosphoramidation approach preserves the integrity of hybridization specificity in nucleic acids when preparing POCs. By retaining integrity of the nucleic acids, their effectiveness as therapeutic reagents for gene silencing, gene therapy, and RNA interference is ensured. The potential for POC use was demonstrated by two-step phosphoramidation reactions to successfully synthesize nucleic acid−tetraglycine conjugates. In addition, phosphoramidation reactions provided a facile approach to prepare nucleic acid−BSA conjugates with good yields. In summary, the new approach to phosphoramidation reactions offers a universal method to prepare POCs and other nucleic acid conjugates with high yields in aqueous solutions. The methods can be easily adapted to typical chemistry or biology laboratory setups which will expedite the applications of POCs for basic research and medicine.

INTRODUCTION

Covalent modifications of nucleic acids have been used to explore the diverse functions of nucleic acids and to characterize their intrinsic biochemical properties in biological systems. Continuous development of covalent conjugation methods for nucleic acids further facilitates applications of nucleic acids as research tools for chemical and biomedical studies, improves potency, and promotes specificity of nucleic acids as therapeutic reagents in medicine. For example, the combination of covalently modified nucleic acids and in vitro selection methods has contributed to the discovery of novel catalytic nucleic acids such as ribozymes (catalytic RNA) able to perform the Diels−Alder cycloaddition (*1*), the Michael addition (*2*), the aldol condensation (*3*), NAD^+-dependent alcohol dehydrogenation (*4*), and NADH-dependent aldehyde hydrogenation (*5*). Conjugation of fluorophores with nucleic acids has provided sensitive detection methods to study tertiary structure transitions relevant to RNA folding (*6*−*9*) and ribozyme catalysis (*7, 10, 11*), genomics (*12, 13*), and DNA microarray preparation (*14*). Finally, oligonucleotides conjugated with peptides to afford peptide−oligonucleotide conjugates (POCs) hold promise as an effective therapeutic reagent to treat viral infections and genetic diseases (*15*−*17*). Applications of POCs in basic and clinical research have produced nucleic acids with improved biological stability (*18*), cellular uptake efficiency (*18, 19*), and in vivo cell-specific targeting (*20*).

Preparation of POCs is essential for therapeutic applications of nucleic acids such as oligonucleotides. The direct use of oligonucleotides in medicine generally falls short of disease treatment expectations due to poor cell specificity and uptake of nucleic acids, and inaccessibility of nucleic acids to cell nuclei (*15*−*17*). The conjugation of oligonucleotides with peptides is thus the most common approach to circumvent cell delivery and specificity problems of oligonucleotides (*15*−*17*). However, methods currently available to prepare POCs are inefficient and inconvenient for typical research laboratories. The difficulties experienced when preparing desired effective POCs restrict the broad applications of POCs in achieving their much-needed medical applications.

POCs are generally prepared by post solid-phase synthesis to couple peptides with oligonucleotides (fragment coupling

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strategy) or stepwise solid-phase synthesis (online solid-phase synthesis) (*15*−*17*). POC preparation is generally more feasible using fragment coupling strategy to avoid chemical intolerance between peptides and oligonucleotides during standard solid-phase synthesis (*15, 16*). The generic fragment coupling strategy separately synthesizes peptides and oligonucleotides by standard solid-phase synthesis protocols, and then, both are covalently linked together post solid-phase synthesis. There are limited reactions available for POC formation because peptides primarily use unstable phosphodiester linkages when conjugating to 5′- or 3′-termini or ester linkages when linking to 2′-positions of riboses in oligonucleotides (*17*). Diverse and stable chemical linkages between peptides and oligonucleotides can be attained only if nonconventional nucleotides are used during solid-phase synthesis of oligonucleotides in order to introduce additional functionalities. The need to incorporate nonconventional nucleotides into oligonucleotides increases the cost of oligonucleotide solid-phase synthesis and deters current use of POCs. The development of a facile approach to use hydroxyl or phosphate groups in standard oligonucleotides to afford stable POCs with high purity and yields is highly desirable.

An ideal method to prepare POCs is nucleic acid phosphoramidation which offers the advantage of providing a stable phosphoramidate bond for conjugations between peptides and oligonucleotides. Stability of the phosphoramidate linkage has long been recognized and extensively used to acquire POCs by solid-phase synthesis (*17*). The methodology and applications of nucleic acid phosphoramidation in aqueous solutions were previously studied by Orgel's lab (*21*). They exploited 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) activation to transiently generate reactive phosphorimidazolide intermediates of nucleotides, followed by the coupling reaction with a nucleophile at rt for 24 h to result in oligonucleotide conjugates with maximum yields of 50% (*21*). The concept of phosphoramidation reactions for nucleic acid conjugations is analogous to the effective amidation reaction achieved by the intermediate step to form isolatable reactive *N*-hydroxysuccinimide (NHS) esters of carboxylic acids (*22*). The aqueous-phase phosphoramidation reaction, however, has received little attention and failed to gain popularity as a standard method for nucleic acid conjugation. Failure to gain support was likely due to cumbersome and time-consuming procedures and low yields. A later report of an aqueous-phase phosphoramidation method claimed to have quantitative yields of reactions between RNA and ethylenediamine after 4−8 h of incubation (*23*). However, further exploration of more extensive applications of phosphoramidation for nucleic acid conjugations was not conducted.

An effective and efficient aqueous-phase nucleic acid phosphoramidation method became important during our studies of RNA conjugations and ribozyme catalysis. We thus synthesized a new biotin derivative (**11**) to systematically optimize the nucleic acid phosphoramidation methods of Chu et al. (*21*) with the goal to improve effectiveness and efficiency. The synthesis of **11** was successfully accomplished with an excellent overall yield of 38.7%. The afforded **11** has a biotin tag and a primary amine which can be used to optimize phosphoramidation reactions with nucleic acids. The new aqueous-phase phosphoramidation method provided up to 80% yield for **11**−nucleic acid conjugates after 3 h of reaction. The method was able to complete conversions for 1,6-hexanediamine-nucleic acid conjugates after only 10−20 min of reaction. The phosphoramidation reaction between a model peptide tetraglycine (**12**) and nucleic acids was also successful in producing the desired POCs. The integrity of hybridization characteristics of nucleic acids after phosphoramidation reactions was demonstrated by polymerase chain reactions (PCRs) in which almost all the phosphoramidation-prepared DNA−**11** conjugate primers were integrated into PCR products. The phosphoramidation reactions were also applied to effective preparation of nucleic acid−BSA conjugates. The hallmark of the nucleic acid phosphoramidation reactions is that they offer a facile and universal approach to prepare POCs with no restrictions on compositions of either nucleic acids or peptides and without compromising base-pairing preference in nucleotides. The new aqueous-phase phosphoramidation reaction will provide an advantageous method for nucleic acid conjugate preparation and expedite medical applications of POCs as therapeutic reagents for gene silencing, gene therapy, and RNA interference (*15*−*17, 24*).

MATERIALS AND METHODS

All reagents were purchased from commercial sources (Sigma-Aldrich, Acros, Alfa Aesar, Mallinckrodt Baker, and Merck KGaA) except where noted and were further purified if necessary. Both disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG) for oligonucleotide−peptide conjugates were synthesized according to published methods (*25*). The procedures modified from those of Boger et al. (*26*) were used to synthesize H_2N-Gly-Gly-Gly-Gly-OH (tetraglycine; **12**). ^1H and ^{13}C NMR spectra were recorded using either a Varian 200 or 400 MHz spectrometer (Varian, Inc., Palo Alto, CA, USA) at Kaohsiung Medical University, Taiwan. NMR samples were prepared in CD_3OD, DMSO-d_6, or $CDCl_3$, and the chemical shifts of ^1H signals were given in parts per million downfield from TMS. ^{13}C signals were given in parts per million based on the internal standard of each deuteriated solvent. ESI high-resolution mass spectra were acquired from Department of Chemistry, National Sun Yat-Sen University, Taiwan, on a Bruker APEX II Fourier-transfer mass spectrometry (FT-MS; Bruker Daltonics Inc., Taiwan). Nucleic acid conjugates were analyzed by urea polyacrylamide gel electrophoresis (PAGE) or streptavidin (SAv) gel shift assay in urea PAGE, visualized, and quantified by an Amersham Typhoon phosphoimager (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Synthesis of the Biotin Derivative (11). *5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic Acid 2,5-Dioxo-pyrrolidin-1-yl Ester (***2***).* The method for synthesis of **2** was modified from that of Bayer and Wilchek (*27*). In brief, biotin (**1**; 0.31 g, 1.26 mmol) was completely dissolved in DMF (9 mL) with gentle warming. After cooling the **1** solution to room temperature without reprecipitation, it was added to *N*,*N*′-dicyclohexylcarbodiimide (DCC; 0.26 g, 1.28 mmol) and pyridine (0.10 mL, 1.26 mmol) while stirring for 5 min at room temperature, followed by the addition of *N*-hydroxysuccinimide (NHS; 0.19 g, 1.65 mmol) with continuous stirring for 24 h. The reaction product was filtered and concentrated under reduced pressure. The obtained solid was redissolved in 2-propanol (50 mL) with gentle heating, cooled down to room temperature, and reprecipitated at 4 °C overnight. The product was scraped out of the glassware, washed with cold 2-propanol, and air-dried to give **2** as white solid (0.36 g; 82.4%). ^1H NMR (400 MHz) (DMSO) δ: 6.46 (s, 1H, CONH), 6.39 (s, 1H, CONH), 4.31 (t, 1H, CHN), 4.16 (t, 1H, CHN), 3.12 (dd, 1H, CHS), 2.83 (s, 4H, CH_2 of NHS), 2.71 (d, 1H, C*H*HS), 2.69 (t, 2H, CH_2CO), 2.59 (d, 1H, C*H*HS), 1.43−1.68 (m, 6H). ^{13}C NMR (100.67 MHz) (DMSO) δ: 170.42, 162.85, 61.01, 59.28, 55.33, 30.08, 27.91, 27.68, 25.51. HRMS (ESI) calculated for $C_{14}H_{19}N_3O_5S$, $[M+Na]^+$ 364.0943 (calcd.), 364.0940 (found).

*6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoic Acid (***3***).* 5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (**2**; 0.35 g, 1.03 mmol) was completely dissolved in DMF (3 mL) with gentle warming (*28*). After cooling the **2** solution to room temperature without reprecipitation, it was mixed with a solution of ε-aminocaproic acid (0.18 g, 1.37 mmol) in sodium bicarbon-

ate buffer (0.1 M, pH 8.0; 4 mL) while stirring. After 4 h at room temperature, the reaction mixture was acidified by HCl solution (1 N) to assist in the formation of white precipitate. The white solid was washed with cold HCl solution, dried over ether, and air-dried to afford **3** (0.30 g; 80.5%). ^{1}H NMR (400 MHz) (DMSO) δ: 7.78 (s, 1H, CONH), 6.46 (s, 1H, CONH), 6.38 (s, 1H, CONH), 4.32 (t, 1H, CHN), 4.14 (t, 1H, CHN), 3.11 (dd, 1H, CHS), 3.02 (t, 2H, C*H*$_2$NH), 2.84 (d, 1H, C*H*HS), 2.59 (d, 1H, C*H*HS), 2.18 (t, 2H, C*H*$_2$COOH), 2.05 (t, 2H, CH$_2$CO), 1.23−1.68 (m, 12H). ^{13}C NMR (100.67 MHz) (DMSO) δ: 174.45, 171.79, 162.70, 61.03, 59.18, 55.43, 35.21, 33.60, 28.90, 28.02, 25.97, 25.33, 24.22. HRMS (ESI) calculated for $C_{16}H_{27}N_3O_4S$, $[M+Na]^+$ 380.1620 (calcd.), 380.1617 (found).

*6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoic Acid 2,5-Dioxo-pyrrolidin-1-yl Ester (***4***).* Synthesis of **4** with high purity was achieved following the method of Wilchek and Bayer with critical changes in workup procedures (*28*). 6-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexanoic acid (**3**; 0.31 g, 0.87 mmol) was completely dissolved in DMF (20 mL) with gentle warming. After cooling the **3** solution to room temperature without reprecipitation, DCC (0.34 g, 1.65 mmol) and pyridine (0.07 mL, 0.83 mmol) were added to the solution while stirring for 5 min, followed by the addition of NHS (0.16 g, 1.46 mmol) with continuous stirring for 18 h. The reaction product was filtered and concentrated under reduced pressure. The remaining solid was redissolved in 2-propanol (40 mL) with gentle heating, cooled down to room temperature, and reprecipitated at 4 °C overnight. The product was scraped out of the glassware, washed with cold 2-propanol, and air-dried to obtain **4** (0.22 g; 56.8%) as a white solid. ^{1}H NMR (400 MHz) (DMSO) δ: 7.77 (s, 1H, CONH), 6.45 (s, 1H, CONH), 6.38 (s, 1H, CONH), 4.31 (t, 1H, CHN), 4.15 (t, 1H, CHN), 3.11 (dd, 1H, CHS), 3.03 (t, 2H, C*H*$_2$NH), 2.83 (s, 4H, CH$_2$ of NHS), 2.79 (d, 1H, C*H*HS), 2.67 (t, 2H, C*H*$_2$COOH), 2.60 (d, 1H, C*H*HS), 2.06 (t, 2H, CH$_2$CO), 1.04−1.75 (m, 12H). ^{13}C NMR (100.67 MHz) (DMSO) δ: 171.90, 170.36, 169.03, 162.79, 61.13, 59.27, 55.53, 38.14, 35.32, 30.24, 28.74, 28.32, 28.14, 25.54, 25.41, 24.05. HRMS (ESI) calculated for $C_{20}H_{30}N_4O_6S$, $[M+Na]^+$ 477.1784 (calcd.), 477.1784 (found).

*(6-Amino-hexyl)-carbamic Acid tert-Butyl Ester (***5***).* 1,6-Hexanediamine (1.0 g, 8.61 mmol) was completely dissolved in dichloromethane (DCM; 7 mL) (*29*). The solution was chilled to 0 °C, followed by the addition of di-*tert*-butyl dicarbonate (0.63 g, 2.87 mmol) with stirring. The reaction was allowed to proceed at room temperature overnight. The acquired reaction product was diluted with chloroform (7.5 mL), extracted with 5% Na_2CO_3 twice, and its organic phase concentrated under reduced pressure. The obtained solid was dissolved in 1 N HCl and extracted with ether twice. The pH of the acquired aqueous phase was adjusted to 10 with NaOH and extracted with ethyl acetate (EA) five times. The final organic phase was dried over $MgSO_4$ and concentrated under reduced pressure to give oil-like **5** (0.25 g; 39.8%). ^{1}H NMR (400 MHz) ($CDCl_3$) δ: 4.54 (s, 1H, CONH), 3.11 (t, 2H, C*H*$_2$NH), 2.68 (t, 2H, C*H*$_2$NH$_2$), 1.40−1.50 (s, 9H, CH$_3$; m, 4H, CH$_2$), 1.25−1.37 (m, 4H, CH$_2$). ^{13}C NMR (100.67 MHz) (DMSO) δ: 77.00, 41.98, 33.55, 29.95, 28.33, 26.48. HRMS (ESI) calculated for $C_{11}H_{24}N_2O_2$, $[M+H]^+$ 217.1916 (calcd.), 217.1917 (found).

*(6-{6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoylamino}-hexyl)-carbamic Acid tert-Butyl Ester (***6***).* 6-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (**4**; 0.39 g, 0.86 mmol) was completely dissolved in DMF (15 mL) with gentle warming. After cooling the solution to room temperature without reprecipitation, it was mixed with triethylamine (Et_3N; 0.52 g, 5.15 mmol) followed by the addition of **5** (0.25 g, 1.14 mmol) in DMF (3 mL) dropwise while stirring. After overnight reaction at room temperature, the mixture was filtered, evaporated in vacuo, and loaded to a silica column for further purification. The products were separated by eluting with mobile phases of 5−20% MeOH in DCM to afford **6** (0.45 g; 93.8%) as a yellowish solid. ^{1}H NMR (400 MHz) (CD_3OD) δ: 4.53 (t, 1H, CHN), 4.34 (t, 1H, CHN), 3.25 (dd, 1H, CHS), 3.20 (t, 2H, C*H*$_2$NH), 3.08 (t, 2H, C*H*$_2$NH), 2.96 (d, 1H, C*H*HS), 2.73 (d, 1H, C*H*HS), 2.22 (t, 4H, CH$_2$CO), 1.38−1.80 (m, 29H). ^{13}C NMR (100.67 MHz) (DMSO) δ: 176.02, 175.97, 166.05, 158.50, 79.50, 63.39, 61.63, 57.01, 41.24, 41.04, 40.24, 36.99, 36.82, 30.90, 30.35, 30.13, 29.78, 29.49, 28.80, 27.56, 26.92, 26.74. HRMS (ESI) calculated for $C_{27}H_{49}N_5O_5S$, $[M+Na]^+$ 578.3352 (calcd.), 578.3349 (found).

*6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoic Acid (6-amino-hexyl)-amide, TFA Salt (***7***).* (6-{6-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexanoylamino}-hexyl)-carbamic acid *tert*-butyl ester (**6**; 0.23 g, 0.39 mmol) was dissolved in trifluoroacetic acid (TFA) and stirred at room temperature for 45 min. The product was concentrated under reduced pressure, washed by ether, and again concentrated under reduced pressure to obtain oil-like **7** (0.22 g; 100%) as TFA salts. ^{1}H NMR (400 MHz) (CD_3OD) δ: 4.58 (t, 1H, CHN), 4.35 (t, 1H, CHN), 3.25 (dd, 1H, CHS), 3.21 (t, 4H, C*H*$_2$NH), 2.96 (d, 1H, C*H*HS), 2.95 (t, 2H, CH$_2$NH^{3+}), 2.75 (d, 1H, C*H*HS), 1.22−1.80 (m, 20H). ^{13}C NMR (100.67 MHz) (DMSO) δ: 176.67, 166.14, 63.38, 61.63, 57.03, 41.05, 40.64, 40.19, 40.01, 36.98, 36.81, 30.17, 29.78, 29.50, 28.50, 27.53, 27.33, 26.96, 26.72, 26.27. HRMS (ESI) calculated for $C_{22}H_{41}N_5O_3S$, $[M+H]^+$ 456.3008 (calcd.), 456.3009 (found).

*Carbamoylmethyl-carbamic Acid tert-Butyl Ester (***8a***).* Glycine (0.45 g, 6.05 mmol) was dissolved in 6 mL of dioxane/water mixture (dioxane:water = 4:2) with stirring, followed by the addition of 1 N NaOH (2 mL). The acquired glycine solution was immersed in an ice−water bath and mixed with di-*tert*-butyl dicarbonate (1.38 g, 6.31 mmol) dropwise while stirring to initiate the reaction. The reaction was allowed to proceed at room temperature overnight. The reaction was diluted with water (2 mL) and EA (10 mL), extracted with 1 N HCl twice, and extracted with water twice. The final organic phase was dried over $MgSO_4$ and evaporated under reduced pressure to give oil-like **8a** (0.94 g; 90%). ^{1}H NMR (400 MHz) ($CDCl_3$) δ: 5.42 (s, 1H, CONH), 3.91 (d, 2H, CH$_2$CO), 1.44 (s, 9H, CCH$_3$). ^{13}C NMR (100.67 MHz) ($CDCl_3$) δ: 177.0, 156.0, 79.6, 35.8, 34.3, 28.2.

*tert-Butoxycarbonylamino-acetic Acid 2,5-Dioxo-pyrrolidin-1-yl Ester (***8b***).* Carbamoylmethyl-carbamic acid *tert*-butyl ester (**8a**; 0.61 g, 3.48 mmol) and NHS (0.61 g, 5.34 mmol) were dissolved in THF (7 mL), followed by dropwise addition of DCC (1.42 g, 6.9 mmol) in THF (7 mL) with stirring (*30*). After overnight reaction at room temperature, the mixture was quenched by the addition of three drops of glacial acetic acid, stirred for 1 h, and filtered to remove suspension. The acquired filtrate was concentrated under reduced pressure, resuspended in 2-propanol (25 mL) while stirring for 1 h, and filtered to separate the suspension from its filtrate. The remaining solid was washed by 2-propanol and dried to afford white-colored **8b** (0.62 g; 68.8%). ^{1}H NMR (400 MHz) ($CDCl_3$) δ: 5.00 (s, 1H, CONH), 4.27 (d, 2H, CH$_2$CO), 2.85 (s, 4H, CNOC*H*$_2$CH$_2$), 1.43 (s, 9H, CCH$_3$). ^{13}C NMR (100.67 MHz) ($CDCl_3$) δ: 168.6 (CONH), 77.0 (CH$_2$N), 40.2 (CH$_2$CO), 37.4 (CH$_2$CO), 25.5 (CH$_3$CO).

*[(6-{6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoylamino}-hexylcarbamoyl)-methyl]-carbamic Acid tert-Butyl Ester (***9***).* *tert*-Butoxycarbonylamino-acetic acid 2,5-dioxo-pyrrolidin-1-yl ester (**8b**; 0.18 g, 0.69 mmol) dissolved in DMF (5 mL) was added slowly to a DMF solution (3 mL)

containing 6-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexanoic acid (6-amino-hexyl)-amide (**7**; 0.16 g, 0.27 mmol) and Et_3N (0.1 mL, 0.72 mmol) while stirring at room temperature. The reaction was stopped after 3 h by evaporating DMF in vacuo, and the remaining residue was redissolved in a limited volume of a DCM/MeOH (19:1) solution and loaded to a preequilibrated silica column. The products were separated by eluting with mobile phases of 1−6.67% MeOH in DCM to afford **9** (0.10 g; 62.2%) as a yellow solid. ^{1}H NMR (400 MHz) (CD_3OD) δ: 4.30 dd, 1H, CHN), 4.15 (dd, 1H, CHN), 3.94 (d, 2H, CH_2CO), 3.32 (quin, 4H, C*H*$_2$NH), 2.99 (q, 2H, CH_2N), 2.92 (d, 1H, C*H*HS), 2.75 (d, 1H, CH*H*S), 2.20 (t, 4H, CH_2CO), 1.44−1.88 (m, 29H). ^{13}C NMR (100.67 MHz) (CD_3OD) δ: 175.9 (CO), 63.7 (CHN), 61.6 (CHN), 56.9 (CHS), 49.0 (CH_2N), 41.0 (CH_2CO), 40.2 (CH_2N), 36.9 (CH_2S), 30.1 (CH_2CO), 28.6 (CH_3CO), 26.7 (CH_2CH_2). HRMS (ESI) calculated for $C_{29}H_{52}N_6O_6S$, $[M+Na]^+$ 635.3567 (calcd.), 635.3564 (found).

*6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoic Acid [6-(2-Amino-acetylamino)-hexyl]-amide, TFA Salt (***10***).* [(6-{6-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexanoylamino}-hexylcarbamoyl)-methyl]-carbamic acid *tert*-butyl ester (**9**; 0.38 mg, 0.62 mmol) was dissolved in TFA (3 mL) and stirred for 45 min. The product was evaporated under reduced pressure, washed by chloroform, and again concentrated under reduced pressure to remove chloroform and to obtain brown oil-like **10** (0.32 g, 100%). ^{1}H NMR (400 MHz) (CD_3OD) δ: 4.50 (dd, 1H, CHN), 4.30 (dd, 1H, CHN), 3.64 (s, 2H, CH_2CO), 3.13−3.23 (m, 4H, C*H*$_2$NH), 2.99 (q, 2H, CH_2N), 2.93 (dd, 1H, C*H*HS), 2.72 (d, 1H, CH*H*S), 2.20 (t, 4H, CH_2CO), 1.29−1.88 (m, 20H). ^{13}C NMR (100.67 MHz) (CD_3OD) δ: 175.9 (CO), 63.7 (CHN), 61.6 (CHN), 56.9 (CHS), 49.0 (CH_2N), 41.0 (CH_2CO), 40.2 (CH_2N), 36.9 (CH_2S), 30.1 (CH_2CO), 26.7 (CH_2CH_2). HRMS (ESI) calculated for $C_{24}H_{44}N_6O_4S$, $[M+H]^+$ 513.3223 (calcd.), 513.3226 (found).

*6-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexanoic Acid [6-(2-Amino-acetylamino)-hexyl]-amide (***11***).* The TFA salt of 6-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexanoic acid [6-(2-amino-acetylamino)-hexyl]-amide, TFA salt (**10**; 0.52 g, 0.88 mmol) was dissolved in 5 mL of DCM/MeOH mixture (DCM:MeOH = 1:1) and mixed with 10 equiv of Amberlyst A-21 (1.1 mg, 8.8 mmol) while shaking for 30 min (*31*). After filtering to remove Amberlyst A-21, the filtrate of the reaction products was concentrated under reduced pressure to afford yellow oil-like **11** (0.42 mg, 93.8%). ^{1}H NMR (400 MHz) (CD_3OD) δ: 4.49 (dd, 1H, CHN), 4.30 (dd, 1H, CHN), 3.32 (s, 2H, CH_2CO), 3.14−3.29 (m, 4H, CH_2N), 2.99 (q, 2H, CH_2N), 2.86 (s, 1H, CHS), 2.72 (d, 1H, CHS), 2.20 (q, 4H, CH_2CO), 1.29−1.88 (m, 20H). ^{13}C NMR (100.67 MHz) (CD_3OD) δ: 175.9 (CO), 63.7 (CHN), 61.6 (CHN), 56.9 (CHS), 49.0 (CH_2N), 41.0 (CH_2CO), 40.2 (CH_2N), 36.9 (CH_2S), 30.1 (CH_2CO), 26.7 (CH_2CH_2). HRMS (ESI) calculated for $C_{24}H_{44}N_6O_4S$, $[M+H]^+$ 513.3223 (calcd.), 513.3226 (found).

Nucleic Acid Preparation. Single-stranded DNA (the 5′ primer, 5′-AACACGCATATGTAATACGACTCACTAT-AGGGATCGTCAGTGCATTGAG-3′; the 3′ primer, 5′-TAC-CCCTTGGGGATACCACC-3′) was purchased from Purigo Biotech, Inc., Taiwan, and purified by PAGE. Both the 5′ and 3′ primers were used in a standard PCR reaction to amplify the double-stranded TW17 DNA (119 bp; 5′-GGTAACACG-CATATGTAATACGACTCACTATAGGGATCGTCAGTGCATT-GAGAATGTCAGTGTCTTGCGCTGGGTTCGAGCGGTCCG-TGGTGCTGGCCCGGTGGTATCCCCAAGGGGTA-3′) from a plasmid derived from the pGEM-T vector (Promega, Madison, WI, USA; Wang et al., unpublished results). PCR products were extracted by phenol−chloroform solutions, precipitated in the presence of ethanol, and redissolved in 50 mM KCl. The GMP-primed TW17 RNA (87-mer; 5′-GGGAUCGUCAGUGCA-UUGAGAAGUGCAGUGUCUUGCGCUGGGUUCGAGCG-GUCCGUGGUGCUGGCCCGGUGGUAUCCCCAAGGGGUA-3′) was transcribed from the PCR-amplified double-stranded TW17 DNA carrying T7 promoter sequences under the T7 RNA polymerase runoff reaction in the presence of 6.25 mM each of ATP, CTP, GTP, and UTP, 10 mM GMP (guanosine monophosphate) and with or without 5 μCi [α-^{32}P]UTP (Izotop, Hungary). The GMP-primed TW17 RNA transcript was separated by 8% urea PAGE (220 V, 120 min) followed by ethanol precipitation. Acquired nucleic acids were quantified by their absorption at A_{260}.

Nucleic Acid Radiolabeling. The TW17 RNA was body-labeled with ^{32}P during T7 RNA polymerase runoff transcription stated previously. The double-stranded and single-stranded DNA molecules were ^{32}P-labeled at their 5′-ends with the procedures following the manufacture's instruction (Promega, Madison, WI, USA) and briefly described below. The double-stranded or single-stranded DNA (200 pmol) was dissolved in 20 μL of DEPC (diethyl dicarbonate) water, followed by the addition of 10× reaction buffer (3 μL), alkaline phosphatase (3 U), and DEPC water (4 μL). The reaction was stopped after 2 h at 37 °C, and the reaction product was purified by phenol/chloroform and chloroform extractions, followed by ethanol precipitation. The 5′-OH nucleic acids were redissolved in 7 μL of DEPC water, mixed with 10× reaction buffer (1 μL), [γ-^{32}P]ATP (Izotop, Hungary) and T4 polynucleotide kinase (10 U; Promega, Madison, WI, USA), and reacted 2 h at 37 °C. ^{32}P-labeled double-stranded DNA was purified by phenol/chloroform and chloroform extractions, followed by ethanol precipitation. Single-stranded DNA with ^{32}P-labeled 5′-end was purified by 20% urea PAGE.

Nucleic Acid Phosphoramidation Reactions. *One-Step Phosphoramidation Reactions.* The standard one-step phosphoramidation reaction for RNA was carried out by dissolving the GMP-primed TW17 RNA (92 pmol) and EDC (6.52 μmol) in 20 μL urea−imidazole buffer (8 M urea, 0.1 M imidazole, pH 6.0) and activating at rt for 90 min, followed by the addition of 7.5 μL urea−EPPS buffer (8 M urea, 100 mM EPPS, 2 mM EDTA, pH 8.0) and 5 μL of **11** (187.2 mM in DMF) to react at 41 °C for 3 h. The double-stranded TW17 DNA was also conjugated with **11** according to the same phosphoramidation reaction stated above. For single-stranded DNA (the 3′-primer DNA) the phosphoramidation reaction was modified by dissolving single-stranded DNA (92 pmol) and EDC (6.52 μmol) in 20 μL imidazole buffer (0.1 M imidazole, pH 6.0) and activating at rt for 90 min, followed by the addition of 7.5 μL EPPS buffer (100 mM EPPS, 2 mM EDTA, pH 8.0) and 5 μL of **11** (187.2 mM in DMF) with subsequent reactions at 41 °C for 3 h. No urea was used here to ensure higher reaction yields. All resulting nucleic acid−substrate conjugates were purified twice by ethanol precipitation, and their reaction yields were analyzed by SAv gel shift assay (8% urea PAGE for the TW17 DNA and RNA, and 20%/8% biphasic urea PAGE for single-stranded DNA), visualized, and quantified by an Amersham Typhoon phosphoimager.

Two-Step Phosphoramidation Reactions. The standard two-step phosphoramidation reaction for RNA was carried out by dissolving the GMP-primed TW17 RNA (92 pmol) and EDC (6.52 μmol) in 20 μL urea−imidazole buffer (8 M urea, 0.1 M imidazole, pH 6.0) and activating at rt for 90 min. The resulting 5′-phosphorimidazolide was purified by ethanol precipitation, redissolved in 27.5 μL of urea−EPPS buffer (8 M urea, 100 mM EPPS, 2 mM EDTA, pH 7.5), and added to 5 μL of **11** (187.2 mM in DMF) under reaction temperature at 41 °C for 3 h. For the single-stranded 3′-primer DNA, the two-step

phosphoramidation reaction was modified by dissolving single-stranded DNA (92 pmol) and EDC (6.52 μmol) in 20 μL imidazole buffer (0.1 M imidazole, pH 6.0) and activating at rt for 90 min. Again, the resulting 5′-phosphorimidazolides were also purified by ethanol precipitation, redissolved in 27.5 μL of EPPS buffer (100 mM EPPS, 2 mM EDTA, pH 7.5), and added to 5 μL of **11** (187.2 mM in DMF) to react at 41 °C for 3 h. The double-stranded TW17 DNA was also conjugated with **11** according to the same two-step phosphoramidation reaction for the single-stranded DNA. It is noted that urea was not used in two-step phosphoramidation reactions for the doubled-stranded and single-stranded DNA molecules to attain higher reaction yields. All acquired nucleic acid−**11** conjugates were purified twice by ethanol precipitation, and their reaction yields were analyzed by SAv gel shift assay in the same urea PAGE described for one-step phosphoramidation reaction analysis, visualized, and quantified by an Amersham Typhoon phosphoimager.

Nucleic Acid-1,6-Hexanediamine Conjugate Preparation. The phosphoramidation reaction between the TW17 RNA and 1,6-hexanediamine followed the standard one-step phosphoramidation method stated above but with **11** replaced by 1,6-hexanediamine. The phosphoramidation reaction between DNA and 1,6-hexanediamine was carried out by the standard one-step phosphoramidation method with the following modifications: (1) **11** replaced by 1,6-hexanediamine, (2) EDC activation time shortened to 15 min, (3) nucleophilic coupling reaction time shortened to 10 min for both the double-stranded TW17 DNA and the single-stranded 3′ primer DNA. The afforded nucleic acid−1,6-hexanediamine conjugates were purified twice by ethanol precipitation, and the reaction yields were analyzed by 8% (the TW17 DNA and RNA) or 20% (the 3′-primer DNA) urea PAGE, visualized, and quantified by an Amersham Typhoon phosphoimager.

Single-Stranded 3′-Primer DNA−Fluorescein Conjugate Preparation. The single-stranded 3′-primer DNA−fluorescein conjugate was prepared from its corresponding single-stranded 3′-primer DNA−1,6-hexanediamine conjugate described previously. In a typical reaction for the preparation of single-stranded DNA−fluorescein conjugates, the single-stranded 3′-primer DNA−1,6-hexanediamine conjugate was redissolved in 20 μL sodium carbonate buffer (0.1 M, pH 9.0) to give a final concentration of 2 μM (*32*). Under a darkened laboratory setup, fluorescein isothiocyanate (FITC, Acros) was weighted and dissolved in dry DMSO for a final concentration of 1 mg/mL (2.57 μM). In the same darkened laboratory, 20 μL of the FITC solution was slowly added to the singled-stranded 3′-primer DNA−1,6-hexanediamine conjugate solution while gentle mixing. The reaction was allowed to proceed for 8 h at 4 °C in the dark. The afforded single-stranded DNA−fluorescein conjugate was purified twice by ethanol precipitation.

PCR Reactions to Determine DNA Amplification Efficiency for the Biotin-Labeled 3′-Primer DNA or to Demonstrate Formation of the 3′-Primer DNA−Fluorescein Conjugate. Standard PCR procedures were applied to determine DNA amplification efficiency using the **11**-modified 3′-primer DNA under the condition that the normal 3′-primer (100 μM, 0.75 μL) was substituted with a mixture of the normal 3′-primer (100 μM, 0.15 μL) and ^{32}P-labeled and **11**-modified 3′-primer (1.9 μM, 10 μL). After 15 cycles of the PCR reaction, the product was purified by a standard phenol−chloroform extraction procedure, analyzed by biphasic urea PAGE (top gels of 6% acrylamide and bottom gels of 20% acrylamide), and visualized through an Amersham Typhoon phosphoimager. For the fluorescein-labeled 3′-primer DNA, its formation was studied by the same PCR reaction except that ^{32}P-labeled and **11**-modified 3′-primer was substituted with nonradiolabeled fluorescein-modified 3′-primer (1.9 μM, 10 μL). The PCR reaction product was also purified by a standard phenol−chloroform extraction procedure, analyzed by urea PAGE, and visualized under an Amersham Typhoon phosphoimager using an excitation wavelength of 488 nm and an emission filter set at 525 nm.

Preparation of Nucleic Acid−Tetraglycine (*12*) Conjugates by Phosphoramidation Reactions. The phosphoramidation reaction between nucleic acids and tetraglycine **12** followed the standard two-step phosphoramidation method stated above with **11** substituted with **12**. The afforded nucleic acid−tetraglycine conjugates were purified twice by ethanol precipitation, and the reaction yields were analyzed by high-resolution 8% urea PAGE for the TW17 RNA−**12** and the TW17 DNA−**12** conjugates or high-resolution 20% urea PAGE for the 3′-primer DNA−**12** conjugate. All electrophorized samples were visualized and quantified by an Amersham Typhoon phosphoimager.

Nucleic Acid−BSA (Bovine Serum Albumin) Conjugate Preparation. The standard procedures for preparation of nucleic acid−BSA conjugates were modified from those of Jablonski et al. (*33*). Oligonucleotide−1,6-hexanediamine conjugates were dissolved in 10 μL of EDTA−bicarbonate buffer (0.1 M sodium bicarbonate, 2 mM EDTA, pH 8.25) to give a concentration of 0.02 mM. Nucleic acid−1,6-hexanediamine conjugate solutions were then reacted with 20 μL of DSS or DSG solutions (182 mM in DMSO) for 5 min at rt in the dark. Reaction products were immediately purified twice by ethanol precipitation to avoid hydrolysis of the remaining succinimidyl esters. The afforded nucleic acid conjugates were again redissolved in 30 μL of BSA (10 mg/mL) in sodium phosphate buffer (0.1 M, pH 7.5) to give a molar ratio of BSA to nucleic acid conjugates of 2. The reaction was allowed to proceed for 2−16 h at rt, and the reaction product was purified twice by ethanol precipitation. Yields of nucleic acid−BSA conjugates were also determined by urea PAGE analysis and visualized through an Amersham Typhoon phosphoimager.

RESULTS

Synthesis of the Biotin Derivative (11) and Coupling to the TW17 RNA for Optimization of Phosphoramidation Reactions. The synthesis of **11** started from biotin (**1**) which was activated by carbodiimide to give its NHS ester (**2**), followed by coupling **2** with 4-aminocaproic acid to produce **3** which was again activated by carbodiimide to afford the corresponding NHS ester **4** (Scheme 1). We initially observed that the yield for **4** was always higher than 100%, an indication of ineffective product purification despite following the method of Wilchek and Bayer (*28*). We thus changed the workup procedures by reprecipitating the product in 2-propanol to acquire a fairly clean **4** (56.8%) as confirmed by ^{1}H and ^{13}C NMR and HRMS (ESI). Compound **4** was then reacted with previously synthesized **5** to afford **6** (93.8%), followed by TFA Boc-deprotection (**7**) and amidation with previously synthesized **8b** to give **9** (86.4%). The final product **11** (93.8%) was almost quantitatively obtained after TFA deprotection of **9** and TFA removal of **10**, as confirmed by ^{1}H and ^{13}C NMR and HRMS (ESI).

Successful synthesis of **11** paved the way for the optimization of aqueous-phase nucleic acid phosphoramidation reactions. As noted previously, aqueous-phase nucleic acid phosphoramidation reactions exploit EDC to activate 5′-terminal phosphate groups in nucleic acids and to transiently form reactive phosphorimidazolide intermediates of nucleic acids (Supporting Information Scheme S1). Phosphorimidazolide intermediates of nucleic acids can be purified or used directly to couple with amine-containing nucleophiles to result in desired nucleic acid conjugates. We believed that a key factor affecting final yields for phosphoramidation-prepared nucleic acid conjugates would rely on

Scheme 1. Synthesis of the Biotin Derivative 11 for Optimization of Nucleic Acid Phosphoramidation Reactions

providing a reasonable high concentration of phosphorimidazolide intermediates for nucleophilic attack by **11**. We thus refined phosphoramidation reactions to provide a steady supply of phosphorimidazolide intermediates to **11** during the course of the reactions. Refinements included systematic modifications of reaction conditions such as reactant and EDC concentrations, activation time, reaction time with nucleophile **11**, reaction temperature, pH, and surfactant effects. Both one-step and two-step phosphoramidation reactions were studied with the ultimate goal of improving reaction efficiency and effectiveness.

The optimized one-step phosphoramidation reaction provided an excellent yield of 79% for conjugation of the TW17 RNA with **11** after 3 h reactions (Figure 1A). Further improvement in the phosphoramidation reaction yield was not achieved, probably reflecting the lability of phosphorimidazolide intermediates and inaccessibility of phosphorimidazolide intermediates to **11**. A constant reaction temperature is essential for attaining high phosphoramidation reaction yields. The choice of 41 °C as a reaction temperature balances an enhanced reaction rate and prevention of phosphorimidazolide hydrolysis and RNA hydrolysis at higher temperatures. Maintenance of denaturing conditions by 6.77 M urea during the phosphoramidation reaction plays a pivotal role in achieving higher yields by preventing formation of RNA secondary structures. A very small portion of the GMP-primed TW17 RNA suffered from degradation during the reaction, an amount too insignificant to decrease overall reaction efficiency and yield.

Chu et al. also performed a two-step nucleic acid phosphoramidation method attained by purification of phosphorimidazolide intermediates of DNA which were subsequently reacted with nucleophiles to afford DNA phosphoramidation conjugates (*21*). We therefore also exploited the two-step phosphoramidation method for the TW17 RNA conjugation with **11** to study its effectiveness in preventing RNA degradation observed during the one-step phosphoramidation reaction. Yields of the two-step phosphoramidation reactions for the TW17 RNA−**11** conjugate preparation were consistent with expectations of lower

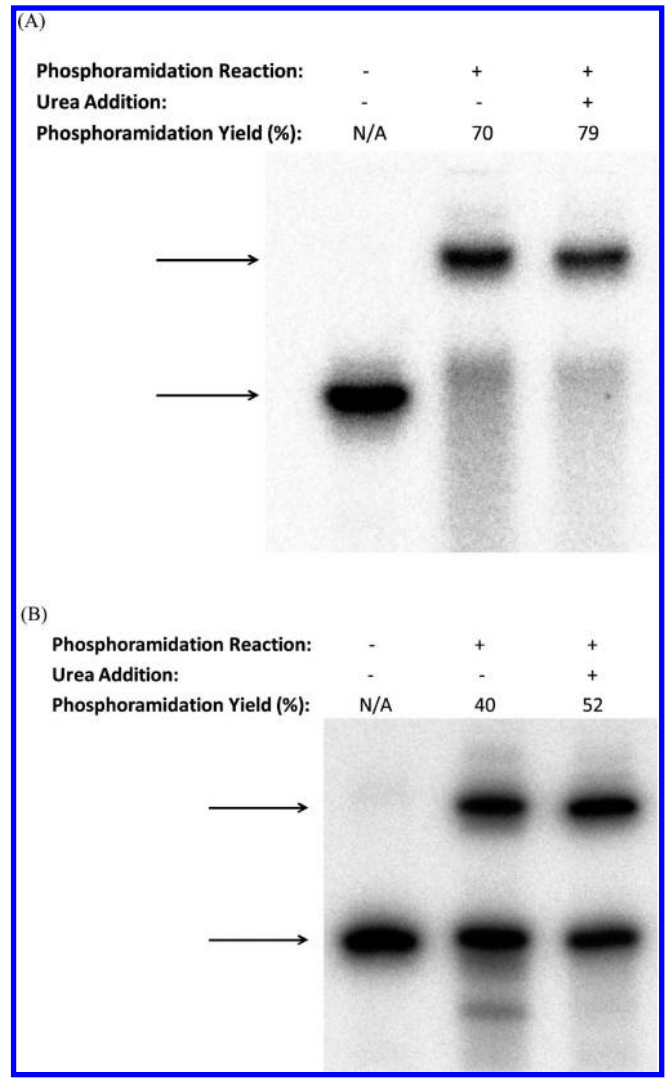


Figure 1. Phosphoramidation reactions of the ^{32}P-labeled GMP-primed TW17 RNA with **11**. (A) Results from the one-step phosphoramidation reaction were analyzed by streptavidin (SAv) gel shift assay in 8% urea PAGE and visualized by autoradiogram. (B) Results from the two-step phosphoramidation reaction were again analyzed by SAv gel shift assay in 8% urea PAGE and autoradiogram. The samples in each figure, starting from the left, are the TW17 RNA without the phosphoramidation reaction, the TW17 RNA conjugated with **11** by the phosphoramidation reaction without the addition of 6.77 M urea, and the TW17 RNA conjugated with **11** by the phosphoramidation reaction in the presence of 6.77 M urea, respectively. In both figures, the top arrow indicates the location of the TW17 RNA−**11** conjugate; the bottom arrow represents migration of the GMP-primed TW17 RNA.

yields than the one-step phosphoramidation reactions (52% in the presence of 6.77 M urea and 40% without 6.77 M urea; Figure 1B). Lower yields for two-step phosphoramidation reactions are attributed to phosphorimidazolide intermediate hydrolysis during its workup step. Both two-step phosphoramidation reactions, with or without 6.77 M urea, still provided reasonably good yields for the TW17 RNA−**11** conjugates, comparable to those prepared by the one-step phosphoramidation reactions. Two-step phosphoramidation reactions can be an advantageous approach to conjugate carbodiimide-sensitive nucleophiles with nucleic acids (vide infra). However, similar to the one-step reactions, RNA degradation was also observed. RNA degradation was thus not caused by prolonged carbodiimide activation but rather by the nucleophile attack step or by a higher reaction temperature at 41 °C.

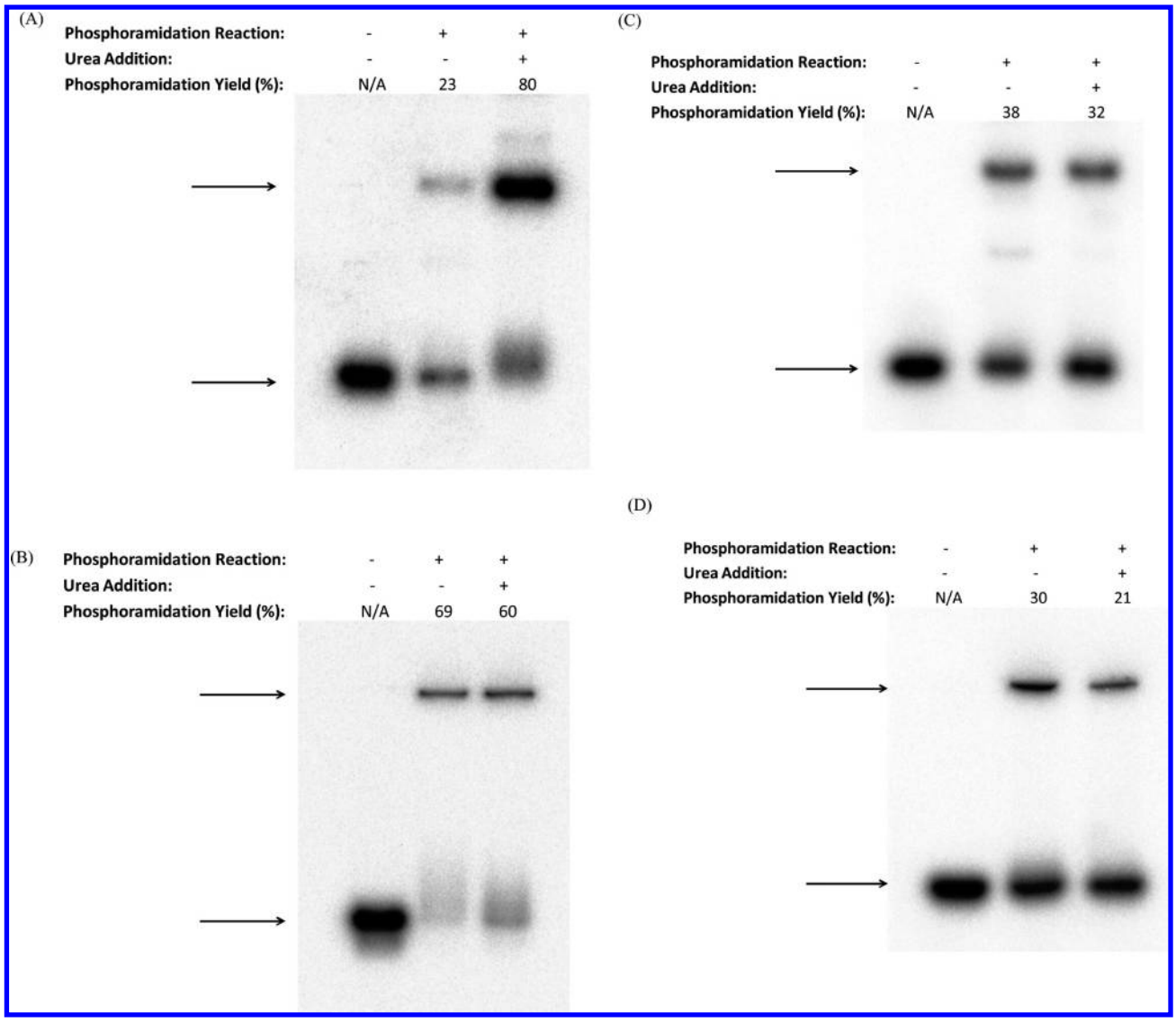


Figure 2. Phosphoramidation reactions of the ^{32}P-labeled DNA with **11**. Results from one-step phosphoramidation reactions were analyzed by SAv gel shift assays in (A) 8% urea PAGE for the double-stranded DNA (the TW17 DNA) conjugations or (B) 20% urea PAGE for the single-stranded DNA (the 3′-primer) conjugations, and visualized by autoradiogram. Results from two-step phosphoramidation reactions were again analyzed by SAv gel shift assays in (C) 8% urea PAGE for the double-stranded DNA (the TW17 DNA) conjugations or (D) 20% urea PAGE for the single-stranded DNA (the 3′-primer) conjugations, and also visualized by autoradiogram. The samples in each figure, starting from the left, are the DNA without the phosphoramidation reaction, the DNA conjugated with **11** by a phosphoramidation reaction without the addition of 6.77 M urea, and the DNA conjugated with **11** by a phosphoramidation reaction in the presence of 6.77 M urea, respectively. Also in each figure, the top arrow indicates the location of the DNA−**11** conjugate; the bottom arrow represents migration of the DNA without phosphoramidation reactions.

Preparation of Phosphoramidated DNA−11 Conjugates. One-step and two-step phosphoramidation reactions were carried out to prepare DNA−**11** conjugates and study universal applications of the phosphoramidation reaction protocol developed for the TW17 RNA−**11** conjugate. The one-step phosphoramidation reactions for the conjugations of either the single-stranded 3′-primer or the double-stranded TW17 DNA with **11** in the presence of 6.77 M urea offered a yield of 80% for the TW17 DNA−**11** conjugate and 60% for the 3′-primer DNA−**11** conjugate (Figure 2A,B). These yields were comparable to the 79% yield for the TW17 RNA−**11** conjugate. The addition of 6.77 M urea significantly improved the yields for the TW17 DNA−**11** conjugates from 23%, without urea, to 80% in the presence of urea. These yields parallel the positive effect of urea during the phosphoramidation reactions to prepare the TW17 RNA−**11** conjugates and are consistent with the proposed function of urea to disassemble secondary structures in nucleic acids and increase the availability of terminal phosphate groups for phosphoramidation reactions. The positive effect of urea on phosphoramidation reaction yields was, however, not observed upon preparation of the 3′-primer DNA−**11** conjugates. Slight deterioration of the phosphoramidation reaction yield for the 3′-primer DNA−**11** conjugates was observed in the presence of urea. This finding indicates very limited secondary structure formation in a relatively small 3′-primer DNA and adverse effects of urea on single-stranded DNA phosphoramidation reactions. In contrast to results of the TW17 RNA phosphoramidation reactions, DNA degradation was not observed during the one-step DNA phosphoramidation reactions.

The two-step phosphoramidation reactions for conjugation of either the 3′-primer or the TW17 DNA with **11** again provided yields lower than those for the one-step reactions (Figure 2C,D). Interestingly, both phosphoramidation reactions performed better in the absence of 6.77 M urea in which the yield for the TW17 DNA−**11** conjugate was 38% and that for the 3′-primer DNA−**11** conjugate was 30%. The inhibitory effect of urea on phosphoramidation reactions was clearly more prominent than its beneficial effect on denaturation of nucleic acids to disrupt

their secondary structures in the two-step DNA phosphoramidation reaction. DNA degradation during the two-step DNA phosphoramidation reaction was not observed. Overall, we demonstrated the feasibility of using two phosphoramidation reaction formats to prepare the DNA−**11** conjugates and the stability of DNA during phosphoramidation reactions.

Preparation of Nucleic Acid-1,6-Hexanediamine Conjugates. The success in using **11** to develop effective nucleic acid phosphoramidation methods encouraged us the further applications of the phosphoramidation reaction to the preparation of nucleic acid−1,6-hexanediamine conjugates. These conjugates can serve as key intermediates for covalently linking chromophores or oligopeptides to nucleic acids. One-step phosphoramidation reactions were chosen to attain higher phosphoramidation yields for nucleic acid−1,6-hexanediamine conjugates. We were encouraged by observing quantitative production of the TW17 RNA−1,6-hexanediamine conjugates according to the same phosphoramidation reaction for the TW17 RNA−**11** conjugate (Figure 3A). Quantitative formation of the TW17 DNA−1,6-hexanediamine or the 3′-primer DNA−1,6-hexanediamine conjugates was also attained even under the conditions of reduced times for EDC activation (15 min) and for 1,6-hexanediamine coupling (10 min for both DNA molecules) in the phosphoramidation reactions (Figure 3B,C). Unexpectedly, multiple conjugations of 1,6-hexanediamine to either the TW17 DNA or the 3′-primer DNA were very significant, but the conjugates were never observed to produce TW17 RNA−1,6-hexanediamine conjugates. Substitutions of 1,6-hexanediamine with ethylenediamine significantly decreased phosphoramidation reaction yields for all nucleic acid conjugates (Wang et al., unpublished results). These results imply that the pK_a, lipophilicity and diamine characteristics of 1,6-hexadiamine play an essential role in accomplishing higher yields and multiple conjugations in nucleic acid phosphoramidation reactions.

Preparation of the 3′-Primer DNA−Fluorescein Conjugate. The quantitative 1,6-hexanediamine−3′ primer DNA conjugates were applied to conjugations with FITC through covalent thiourea linkages between newly acquired primary amines in the 5′-termini of nucleic acids and FITC. Conventional high-resolution urea PAGE methods to determine yields for the 3′-primer DNA conjugate preparation could not be used due to multiple conjugations of the 3′-primer DNA with 1,6-hexanediamine (Figure 3C). Instead of obtaining quantitative measurements, we pursued qualitative data to demonstrate formation of the 3′-primer DNA−fluorescein conjugates by applying the fluorescein-tagged 3′-primer DNA to PCR amplification. Formation of the 3′-primer DNA−fluorescein conjugates was clearly shown in the results of PCR amplification. The fluorescence signals from either the PCR products or the fluorescein-tagged 3′-primer DNA were vividly observed (Supporting Information Figure S1). The signals corresponding to the PCR products were less than 30% of the total fluorescein signal, a sharp contrast to quantitative yields for the 3′-primer DNA−1,6-hexanediamine conjugate preparation. Nevertheless, the PCR results supported formation of the covalently linked 3′-primer DNA−fluorescein conjugates.

Characteristics of Hybridization Specificity in Nucleic Acids after Phosphoramidation Reactions. The effectiveness of POCs as therapeutic reagents strongly depends on intact hybridization specificity in nucleic acids after preparation. Thus, assurance of the integrity of hybridization specificity in nucleic acids after phosphoramidation reactions is essential to POC preparation reactions. Retention of hybridization specificity in the 3′-primer DNA after phosphoramidation and followup coupling reactions was ambiguous according to our qualitative studies of the 3′-primer DNA−fluorescein conjugate preparation (Supporting Information Figure S1). The low PCR product yield

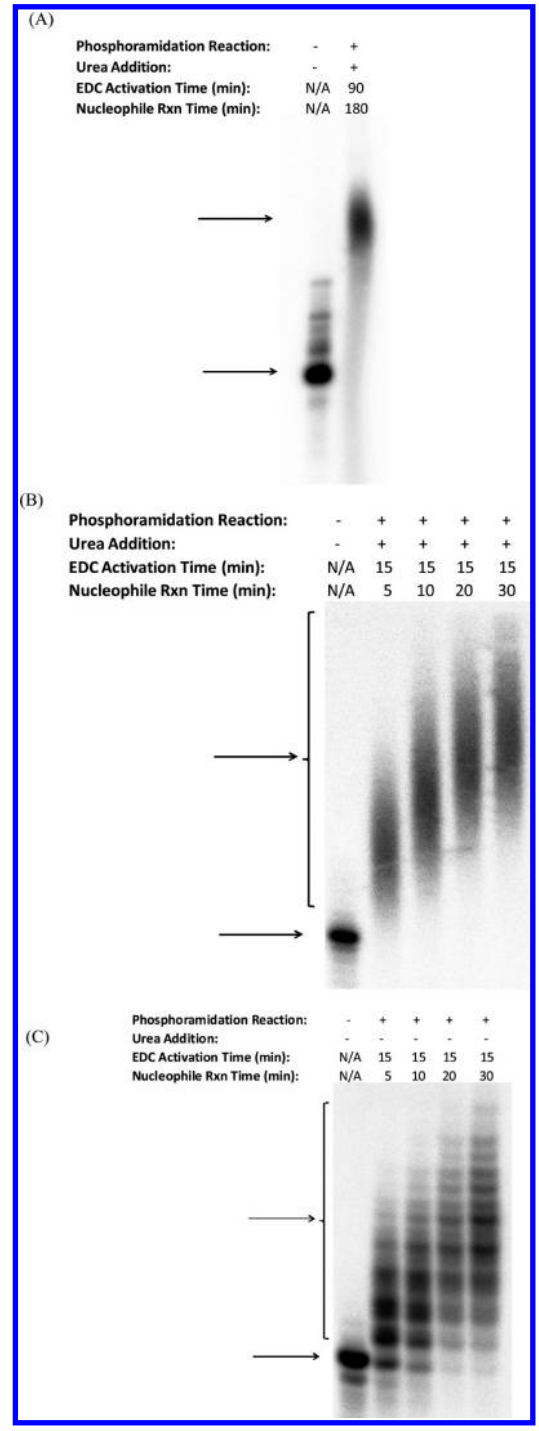


Figure 3. Phosphoramidation reactions of the ^{32}P-labeled DNA or RNA with 1,6-hexanediamine. Products from each one-step phosphoramidation reaction were analyzed by (A) 8% urea PAGE for the GMP-primed TW17 RNA conjugations, (B) 8% urea PAGE for the doubled-stranded TW17 DNA conjugations, or (C) 20% high-resolution urea PAGE for the single-stranded 3′-primer DNA conjugations, and visualized by autoradiogram. The samples in Figure 3A, starting from the left, are the RNA without phosphoramidation reactions, and formation of the RNA−1,6-hexanediamine conjugate by the phosphoramidation reaction with 60 min EDC activation and 180 min coupling reactions of with 1,6-hexanediamine in the presence of 6.77 M urea, respectively. The samples in Figure 3B,C, starting from the left, are DNA without phosphoramidation reactions, and formation of DNA−1,6-hexanediamine conjugates by the phosphoramidation reactions with 15 min EDC activation and 5, 10, 20, and 30 min coupling reactions of 1,6-hexanediamine in the presence of 6.77 M urea, respectively. Also in each figure, the top arrow indicates location(s) of nucleic acid−1,6-hexanediamine conjugate(s); the bottom arrow represents migration of the nucleic acid without phosphoramidation reactions.

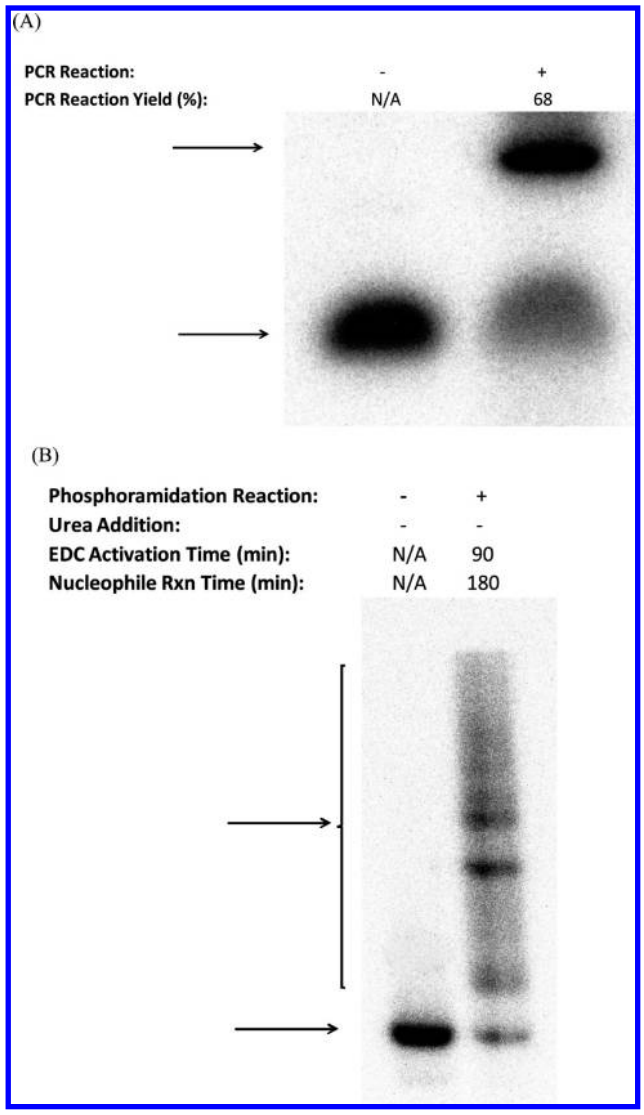


Figure 4. Demonstration of intact hybridization characteristics and minor multiple conjugations for the 3′-primer DNA after the phosphoramidation reaction with **11**. (A) The one-step phosphoramidation-prepared 3′-primer DNA−**11** conjugate (19 pmol) was mixed into the nonlabeled 3′-primer DNA (15 pmol) and used for PCR reactions. The samples of the 3′-primer DNA−**11** conjugates before (the left sample) and after (the right sample) a PCR reaction were analyzed by a biphasic urea PAGE with the top gel of 6% acrylamide and the bottom gel of 20% acrylamide to attain good resolutions. The top arrow indicates the location of the 119-mer DNA PCR products; the bottom arrow represents migration of the 3′-primer DNA−**11** conjugate. (B) The same samples in Figure 2B were analyzed by a 20% urea PAGE without the addition of SAv and visualized by autoradiogram.

could not be attributed to the poor coupling efficiency of FITC with the 3′-primer DNA−1,6-hexanediamine conjugates or to changes in hybridization specificity of the 3′-primer DNA−fluorescein conjugate. We thus applied the 3′-primer DNA−**11** conjugate, with a known product yield (Figure 2B), to PCR reactions to clearly demonstrate intact hybridization specificity. Results from the PCR reaction indicated that 68% of the radiolabeled 3′-primer DNA was incorporated into the PCR DNA product (Figure 4A). As the yield for the 3′-primer DNA−**11** conjugate preparation, in the absence of 6.77 M urea, was 69% (Figure 2B), we concluded that all of the phosphoramidation-prepared 3′-primer DNA−**11** conjugates had effectively hybridized with the provided DNA template to produce the desired DNA product. The impressive PCR amplification efficiency of the 3′-primer DNA−**11** conjugate is in sharp contrast to the poor PCR amplification efficiency of the 3′-primer DNA−fluorescein conjugate. Since the discrepancy in PCR product yields was not likely caused by differences between biotin and fluorescence moieties in nucleic acid conjugates, we questioned whether changes of PCR amplification efficiency could be attributed to the extent of multiple conjugations from phosphoramidation reactions. Consistent with predictions, the phosphoramidation reaction between the 3′-primer DNA and **11** entailed fewer extents of multiple conjugations, likely a key factor in sustaining hybridization specificity in nucleic acids (Figure 4B). Nucleic acid phosphoramidation reactions under appropriate controls provide an outstanding method to efficiently and effectively prepare nucleic acid conjugates, including POCs, without loss of hybridization specificity in nucleic acids moieties.

Preparation of Nucleic Acid−Tetraglycine (12) Conjugates. Understanding the basic chemistry for nucleic acid phosphoramidation provided essential information to develop appropriate and simple protocols for POC preparation. Our results initially implied that one-step phosphoramidation reactions were superior in preparing POCs because of their better reaction yields (Figures 1 and 2). Phosphoramidation reactions of DNA with 1,6-hexanediamine, however, revealed that the one-step approach was too reactive to produce multiple conjugations in DNA molecules and had adverse effects on hybridization specificity (Supporting Information Figure S1). In addition, generic one-step nucleic acid phosphoramidation reactions have restrictions on nucleophile carbodiimide-sensitive functionalities, which demands the use of carboxyl-protected peptides to participate in one-step nucleic acid phosphoramidation reactions for POC preparation. The need to modify peptides for one-step nucleic acid phosphoramidation reactions can impair the intrinsic binding affinity to their natural counterpart receptors on cell membranes (increase of K_d) to prohibit effective POC uptake by cells. Furthermore, peptide modifications can transform peptides into ligands of undesirable receptors on nontargeted cell membranes to incur side effects or lethal complications in clinical applications. It is a delicate balance between producing POCs with high yields and developing POCs for effective medical treatments with minimum adverse effects.

We anticipated that the combination of two-step nucleic acid phosphoramidation reactions and natural peptides free from any chemical modifications could be harnessed to afford POCs devoid of the drawbacks of using modified peptides. To test the concept, we synthesized tetraglycine (**12**) as a model peptide (*26*) and allowed it to react with nucleic acids by two-step phosphoramidation reactions. Consistent with the previous studies of nucleic acid conjugations with **11** by two-step phosphoramidation reactions, the acquired nucleic acid−**12** conjugates after 3 h of coupling reactions with nucleophile **12** had the following yields: the TW17 RNA−**12** conjugate of 44%, the TW17 DNA−**12** conjugate of 39%, and the 3′-primer DNA−**12** conjugate of 27% (Figure 5). The molecular mass difference between two DNA strands in the double-stranded TW17 DNA, which is over 500 Da, contributes to the observation of a dual DNA signal before and after the phosphoramidation reaction (Figure 5B; calculated molecular mass: the positive strand of the TW17 DNA, 36 963.8 g/mol; the negative strand of the TW17 DNA, 36 448.6 g/mol). The product yields are comparable to the two-step phosphoramidation reactions for nucleic acid−**11** conjugate preparation. Extending the reaction time for **12** to overnight (16 h) improved DNA−**12** conjugate yields, but not for the TW17 RNA−**12** conjugate. A lower yield for the TW17 RNA−**12** conjugate after the overnight coupling reaction could be attributed to instability of RNA during a prolonged coupling period. However, 3 h of reactions with peptide nucleophiles in two-step nucleic acid phosphoramidation

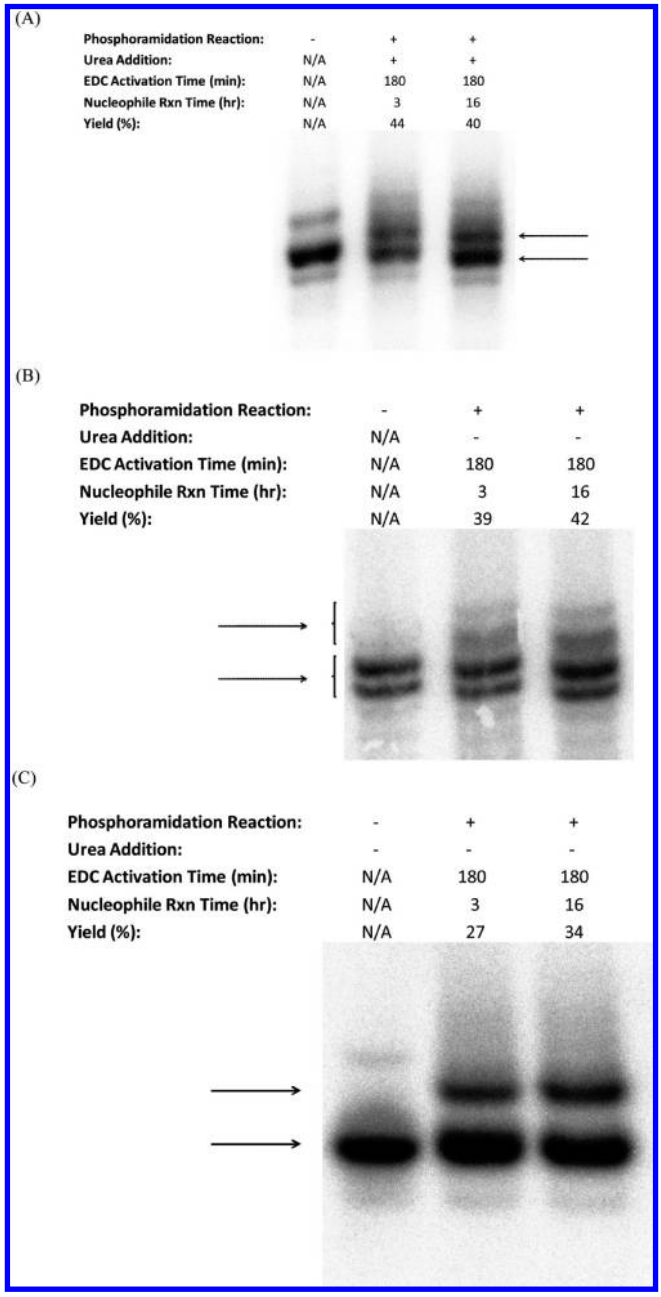


Figure 5. Phosphoramidation reactions of the ^{32}P-labeled DNA or RNA with tetraglycine (**12**) to prepare nucleic acid−tetraglycine conjugates. Products from each two-step phosphoramidation reaction were analyzed by (A) 8% high-resolution urea PAGE for the GMP-primed TW17 RNA conjugations, (B) 8% high-resolution urea PAGE for the doubled-stranded TW17 DNA conjugations, or (C) 20% high-resolution urea PAGE for the single-stranded 3′-primer DNA conjugations, and visualized by autoradiogram. The samples in each figure, starting from the left, are the nucleic acid without phosphoramidation reactions, the nucleic acid conjugated with **12** by phosphoramidation reactions without the addition of 6.77 M urea, and the nucleic acid conjugated with **12** by phosphoramidation reactions in the presence of 6.77 M urea, respectively. Also in each figure, the top arrow indicates the location of the nucleic acid−**12** conjugate; the bottom arrow represents migration of the original nucleic acid.

is enough to prepare suitable amounts of POCs. No multiple conjugations were observed in any nucleic acid−**12** conjugates, implying the integrity of hybridization specificity in nucleic acid moieties. These results clearly validate the two-step phosphoramidation reaction to prepare appropriate POCs for medical applications.

Phosphoramidation Reactions for Preparation of Nucleic Acid−BSA Conjugates. We further explored the use of phosphoramidation reactions to develop a generic approach for the preparation of nucleic acid−protein conjugates. Starting with the previously prepared nucleic acid−1,6-hexanediamine conjugates (Figure 3), they were first reacted with either DSS or DSG to effectively convert -NH_2 to the NHS ester of a carboxylic acid in 5′-termini of the nucleic acids (*33*). Activated acyl group characters of NHS esters allowed easy formation of new amide linkages between nucleic acids and proteins such as BSA to acquire nucleic acid−protein conjugates. Incorporation of 3 M NaCl in coupling reactions was proposed to be indispensible to diminish electric repulsions between nucleic acids and proteins to attain higher yields for nucleic acid−protein conjugates (*33*). Therefore, we performed the coupling reactions in the presence or absence of 3 M NaCl to ascertain any benefit.

The yields for the TW17 RNA−BSA conjugates were only 8% using DSS linkers and 22% using DSG linkers after 2 h reactions in the absence of 3 M NaCl (Figure 6A). Extension of the reaction time from 2 to 16 h, however, improved the yields for the TW17 RNA−BSA conjugates to 44% using DSS linkers and 34% using DSG linkers. Despite neutral pH reaction conditions (pH = 7.4), RNA suffered severe degradation for both DSS and DSG linker conjugates after prolonged coupling reactions. Preparation of the TW17 RNA−BSA conjugates after 2 h reactions in the presence of 3 M NaCl offered reasonable good yields by both DSS and DSG linkers and was comparable to the overnight (16 h) coupling reactions in the absence of 3 M NaCl (Figure 6B). Unexpectedly, extending the reaction time to 16 h was not necessary to improve reaction yields. The loss of the TW17 RNA−BSA conjugates during overnight reactions was observable; RNA degradation was again prevalent and more intensive during the longer reaction time. Overall, additions of 3 M NaCl when preparing the TW17 RNA−BSA conjugates, although not significantly increasing the conjugate yields, reduced the coupling time from 16 to 2 h, which is critical in preventing RNA degradation during POCs preparation.

Similar coupling yields were never obtained for the preparation of the TW17 DNA−BSA conjugates. Yields were less than 20% when using DSS or DSG linkers in the absence of 3 M NaCl (Supporting Information Figure S2A). No improvements in yields for the coupling reactions between BSA and the TW17 DNA derivatives were observed when the reaction times were increased from 2 to 16 h or for reactions carried out in the presence of 3 M NaCl (Supporting Information Figure S2B). Such results defy the beneficial effects of prolonged reaction times and the use of 3 M NaCl to enhance the formation of DNA−alkaline phosphatase conjugates (*33*). The studies of nucleic acid−BSA conjugate preparations underscore the fundamental difference between DNA and RNA toward phosphoramidation reactions which significantly effect yields of nucleic acid−BSA conjugates. Yields for all phosphoramidation reactions are provided in Table 1.

DISCUSSION

We have successfully synthesized a new amine-containing biotin derivative **11** suitable for conjugation with biomolecules such as proteins or nucleic acids for versatile applications. We harnessed the amine functionality and the biotin moiety in **11** to develop the phosphoramidation reactions for preparation of nucleic acid−**11** conjugates with excellent yields and efficiency, but without compromising hybridization specificity of nucleic acids. Both two-step and one-step phosphoramidation reactions were studied, and as expected, one-step phosphoramidation reactions always performed better than two-step formats, which can be attributed to phosphorimidazolide intermediate lability in aqueous solutions. A one-step phosphoramidation reaction

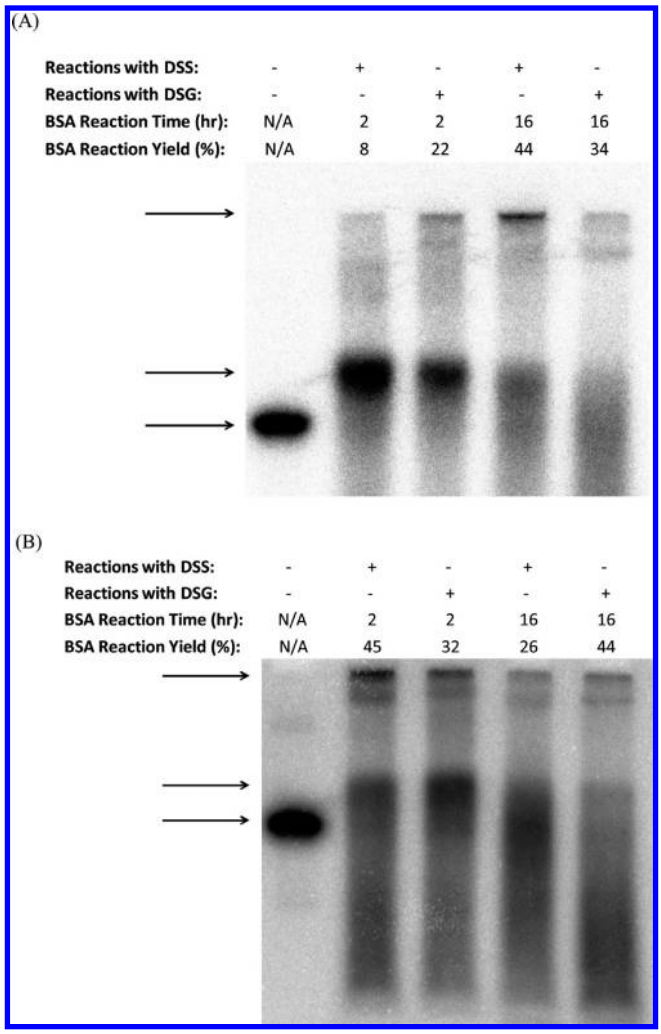


Figure 6. Preparation of the TW17 RNA−BSA conjugates. The ^{32}P-labeled GMP-primed TW17 RNA was conjugated with 1,6-hexanediamine using the one-step phosphoramidation reaction stated previously, followed by reactions with DSS or DSG and finally coupled with BSA (A) in the absence of 3 M NaCl or (B) in the presence of 3 M NaCl. The samples in both (A) and (B) were analyzed by 8% urea PAGE and visualized by autoradiogram. The samples in each figure, starting from the left, are the TW17 RNA without any modification reactions and formation of the TW17 RNA−BSA conjugates by using the DSS linker with 2 h coupling reactions, the DSG linker with 2 h coupling reactions, the DSS linker with 16 h coupling reactions, and the DSG linker with 16 h coupling reactions, respectively. The top arrow indicates the location of the TW17 RNA−BSA conjugate; the middle arrow stands for the migration of the TW17 RNA conjugates before coupling with BSA; the bottom arrow represents migration of the original GMP-primed TW17 RNA before phosphoramidation reactions.

is recommended when higher conjugation yields are a priority. Two-step phosphoramidation reactions, in spite of lower reaction yields, can be an advantageous approach. Two-step formats circumvent the problems of nucleophiles when containing a carbodiimide-sensitive functionality and the intricate phenomena of multiple conjugations, which deteriorate hybridization specificity in nucleic acids.

The outstanding properties of phosphoramidation reactions demonstrate their potential to afford diverse POCs not limited by sequences of peptides and nucleic acids or by properties of nucleic acids as DNA or RNA. We demonstrated the universal applications of this approach by effectively conjugating the model peptide tetraglycine (**12**) to RNA, single-stranded DNA, or doubled-stranded DNA with reasonably good yields. The facile procedures of two-step phosphoramidation reactions will alleviate difficulties in preparing POCs in typical biomedical research laboratories and will enable POCs to be used for important therapeutic and scientific applications such as gene therapy, gene silencing, or RNA interference (*24*) (Scheme 2).

Besides the facile approach and suitable yields, the hallmark of two-step phosphoramidation reactions for POC preparation is the general requirements of peptides and nucleic acids involved in reactions. Essentially, any nucleic acids with 5′-phosphate groups and any peptides with no previous modification are suitable reactants in phosphoramidation reactions to prepare desired POCs. A prerequisite of 5′-phosphate groups in nucleic acids ensures that any nucleic acid, ranging from DNA to RNA and natural to artificial nucleotides, will participate in phosphoramidation reactions. Natural or synthetic peptides with no protection groups can follow two-step phosphoramidation reactions to conjugate with nucleic acids to afford POCs. There is no need to protect carbodiimide-sensitivity functionality in peptides prior to coupling reactions. Peptides with any compositions of amino acid residues and no previous modifications can participate in phosphoramidation reactions to obtain POCs. Furthermore, two-step phosphoramidation reactions can be the only method to acquire POCs when the introduction of protection group(s) to peptides severely deteriorates binding affinities between modified peptides and their receptors to inhibit effective uptake of POCs into cells (vide infra). However, two-step phosphoramidation reactions have disadvantages in a lower coupling efficiency than the one-step approach. The disadvantage was demonstrated by the two-step phosphoramidation reactions between **11** and nucleic acids (Figures 1 and 2). We are exploring additional approaches to enhance the half-life of phosphorimidazolide intermediates during two-step phosphoramidation reactions to improve POC yields.

One-step nucleic acid phosphoramidation reactions with higher product yields remain an effective approach to acquire POCs under selected circumstances. As previously noted, the validity of one-step phosphoramidation reactions for POC preparation is warranted only if we can demonstrate that structure-modified peptides preserve the same levels of binding affinity and specificity to their intrinsic receptors. It is thus essential to determining the difference in dissociation constants K_d between protected and unprotected peptides when adsorbed to specific protein receptors before one-step phosphoramidation-prepared POCs can be exploited for in vivo applications and biomedical studies. The assurance of the integrity of hybridization specificity for nucleic acid moieties in one-step phosphoramidation-prepared POCs is especially important. The findings presented on one-step phosphoramidation reactions indicate that these reactions are very reactive to attain higher product yields and multiple conjugations, especially in cases of DNA (Figure 3). Significant multiple conjugations by one-step phosphoramidation reactions were particularly problematic to DNA in which extents of multiple conjugations paralleled a loss of hybridization specificity in DNA (Figure 4 and Supporting Information Figure S1). In essence, one-step nucleic acid phosphoramidation reactions require appropriate controls of the reaction conditions to obtain high product yields and minimize multiple conjugations to retain integrity of hybridization specificity in nucleic acids.

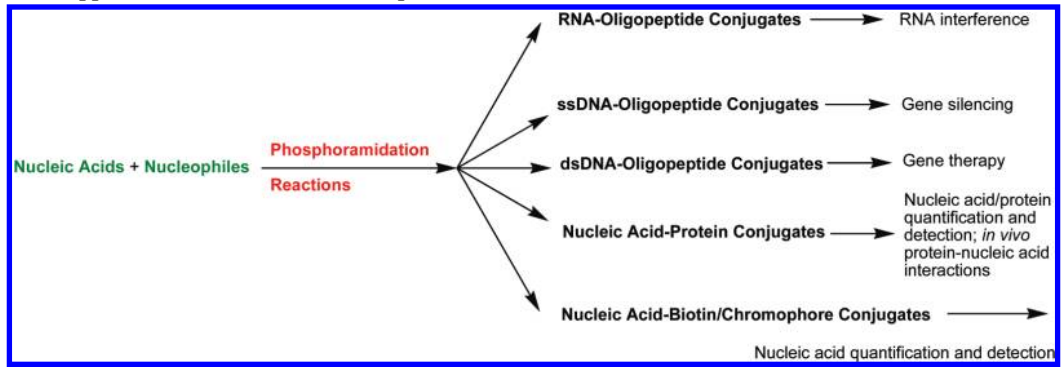
The cause of multiple conjugations in nucleic acids during phosphoramidation reactions can be traced back to the structures of nucleic acids and nucleophiles. The findings presented indicate that DNA molecules have a far greater tendency to experience multiple conjugations during phosphoramidation reactions than RNA. The difference between DNA and RNA is the presence of 2′-hydroxyl group in RNA and an extra methyl group in thymine of DNA when compared to uracil in RNA. 2′-Hydroxyl groups in RNA have no reason to be activated by EDC and react with nucleophiles to contribute multiple conjuga-

Table 1. Yields for Nucleic Acid Conjugates Directly or Indirectly Prepared from Phosphoramidation Reactions in Current Studies

nucleophiles	TW17 RNA	TW17 DNA	3′ primer
11 (one-step phosphoramidation)	70%[a], 79%[b]	23%[a], 80%[b]	69%[a], 60%[b]
11 (two-step phosphoramidation)	40%[a], 52%[b]	38%[a], 32%[b]	30%[a], 21%[b]
12 (two-step phosphoramidation)	44%[c], 40%[d]	39%[c], 42%[d]	27%[c], 34%[d]
1,6-hexanediamine (one-step phosphoramidation)	100%	100%	100%[e]
BSA (one-step phosphoramidation)[f]	8%[g], 44%[h], 45%[i], 26%[j]	5%[g], 5%[h], 2%[i], 4%[j]	N/A[k]
BSA (one-step phosphoramidation)[l]	22%[g], 34%[h], 32%[i], 44%[j]	10%[g], 12%[h], 5%[i], 7%[j]	N/A[k]

[a] In the absence of 6.77 M urea. [b] In the presence of 6.77 M urea. [c] For a 3 h phosphoramidation reaction. [d] For a 16 h phosphoramidation reaction. [e] Under the conditions of 15 min EDC activation and 20 min 1,6-hexanediamine coupling reactions. [f] Using DSS as the linker. [g] For a 2 h coupling reaction with BSA in the absence of 3 M NaCl. [h] For a 16 h coupling reaction with BSA in the absence of 3 M NaCl. [i] For a 2 h coupling reaction with BSA in the presence of 3 M NaCl. [j] For a 16 h coupling reaction with BSA in the presence of 3 M NaCl. [k] Not analyzed. [l] Using DSG as the linker.

Scheme 2. Versatile Applications of Nucleic Acid Phosphoramidation Reactions in Science and Medicine[a]



[a] dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

tions. It is likely that the presence of the thymine base is one reason to confer multiple conjugations in DNA during phosphoramidation reactions. The ability of the extra methyl group in uracil to transform thymine in DNA into a relatively good electrophile in phosphoramidation reactions remains unanswered. The other factor to bestow multiple conjugations in nucleic acids during phosphoramidation reactions is the pK_a of the amine moieties in nucleophiles and the structures of nucleophiles, particularly their lipophilicity and NH_2 functionality contents. According to the results from nucleic acid phosphoramidation reactions with **11**, **12**, ethylenediamine, and 1,6-hexanediamine, the increase of amine pK_a, lipophilicity or NH_2 contents in nucleophiles promotes multiple conjugations in nucleic acids. We thus observed the vigorous activity of 1,6-hexanediamine in phosphoramidation reactions to produce multiple conjugations in nucleic acids (Figure 3). Appropriate designs of nucleophile lipophilicity and NH_2 contents can afford high yields of nucleic acid conjugates while avoiding multiple conjugations in nucleic acids during one-step phosphoramidation reactions.

The problem of multiple conjugations in nucleic acids during phosphoramidation reactions is the negative effect on nucleic acid hybridization specificity manifested by the single-stranded DNA−fluorescein conjugate studies. The decline of nucleic acid hybridization specificity is intimately related to the extent of multiple conjugations in nucleic acid, especially modifications which damage the base-pairing property of thymine. The extents of multiple conjugations may also be a determinant to contribute to lower yields of nucleic acid−BSA conjugates (Figure 6 and Supporting Information Figure S2). These results contradict our earlier assumption that more conjugations in nucleic acids would provide more reactive groups to aid in covalently linking BSA to nucleic acids and thereby increasing yields of nucleic acid−BSA conjugates. In contrast, the extent of multiple conjugations in phosphoramidation-prepared nucleic acid conjugates had the opposite effect on yields of nucleic acid−BSA conjugates (Figure 6 and Supporting Information Figure S2). The TW17 RNA−1,6-hexanediamine conjugates with a minor multiple conjugations offered better coupling efficiency with BSA compared to the same coupling reactions using DNA−1,6-hexanediamine conjugates with more extensive multiple conjugations. The actual cause of higher extents of multiple conjugations in DNA to render low yields of DNA−BSA conjugates is under investigation currently. However, results from these nucleic acid−BSA conjugate preparations suggest that two-step phosphoramidation reaction schemes are a desirable approach to acquire DNA−protein conjugates with better yields.

The controversial role of high concentration of urea in nucleic acid phosphoramidation reaction was intriguing. Urea of 6.77 M was introduced to nucleic acid phosphoramidation reactions to disrupt secondary structures in nucleic acids and to expose 5′-phosphate groups for phosphoramidation reactions. Urea in high concentrations is a common approach in biochemical research to denature nucleic acids because of its typical inertness toward chemical reactions. We thus expected to have higher yields for nucleic acid phosphoramidation reactions in the presence of 6.67 M urea, yet only RNA molecules had better phosphoramidation reaction yields with additions of 6.77 M urea. Furthermore, high concentrations of urea had definite negative effects on two-step DNA phosphoramidation reactions (Figures 1B and 2C,D). The unexpected results can be explained by the ambient nucleophile characteristics in urea (*34*−*36*). The current results suggested that urea plays a dual role in nucleic acid phosphoramidation reactions: one as a surfactant to denature nucleic acids, and the other as a nucleophile to divert phosphoramidation reactions and to reduce product yields. The observed product yield from nucleic acid phosphoramidation reactions is the competition between two counteracting activities. The greater adverse effect of urea in DNA phosphoramidation reactions can be attributed to the presence of a more reactive thymine base in DNA which reacts with urea to decrease product yields. The negative effect of urea in two-step DNA phosphoramidation reactions is further ascribed to the hydrolysis of phosphorimidazolide intermediates of nucleic acids during the workup procedures. We conclude that high concentrations of urea will have beneficial

effects only when applied to RNA phosphoramidation reactions. The substitution of urea with more inert surfactants to increase one-step and two-step nucleic acid phosphoramidation reaction product yields is being undertaken.

In summary, we successfully synthesized a new biotin derivative **11** which was exploited to develop facile one-step and two-step nucleic acid phosphoramidation reactions with excellent yields. One-step phosphoramidation reactions offered better yields of nucleic acid conjugates than two-step phosphoramidation approaches. One-step phosphoramidation reactions were, however, more reactive than two-step phosphoramidation reactions and resulted in multiple conjugations accompanied by deterioration of hybridization specificity in nucleic acids. Multiple conjugations in phosphoramidation-prepared nucleic acid conjugates were most apparent in DNA and nucleophiles contained multiple NH_2 functional groups. Understanding the basic chemistry underlying phosphoramidation reactions allowed an effective application of the reactions to nucleic acid−biotin and nucleic acid−protein conjugate preparations for biomolecule detection and biomedical research (Scheme 2). Most importantly, the phosphoramidation approach provides versatile POCs without scarifying hybridization specificity in nucleic acids. Facile phosphoramidation reactions to achieve effective POC preparation can facilitate therapeutic applications of POCs and fulfill the goals of using POCs for RNA interference, gene silencing, and gene therapy in science and medicine.

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Supporting Information Available: Full details of a reaction scheme, NMR and MS spectra for compounds **2**−**11**, and supporting figures referenced in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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