Analytical Biochemistry 449 (2014) 118–128

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Effective and site-specific phosphoramidation reaction for universally labeling nucleic acids

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ARTICLE INFO

Article history:
Received 1 November 2013
Received in revised form 3 December 2013
Accepted 15 December 2013
Available online 19 December 2013

Keywords:
Nucleic acid
Labeling
Postsynthesis
Site specific
Phosphoramidation

ABSTRACT

Here we report efficient and selective postsynthesis labeling strategies, based on an advanced phosphoramidation reaction, for nucleic acids of either synthetic or enzyme-catalyzed origin. The reactions provided phosphorimidazolide intermediates of DNA or RNA which, whether reacted in one pot (one-step) or purified (two-step), were directly or indirectly phosphoramidated with label molecules. The acquired fluorophore-labeled nucleic acids, prepared from the phosphoramidation reactions, demonstrated labeling efficacy by their F/N ratio values (number of fluorophores per molecule of nucleic acid) of 0.02–1.2 which are comparable or better than conventional postsynthesis fluorescent labeling methods for DNA and RNA. Yet, PCR and UV melting studies of the one-step phosphoramidation-prepared FITC-labeled DNA indicated that the reaction might facilitate nonspecific hybridization in nucleic acids. Intrinsic hybridization specificity of nucleic acids was, however, conserved in the two-step phosphoramidation reaction. The reaction of site-specific labeling nucleic acids at the 5′-end was supported by fluorescence quenching and UV melting studies of fluorophore-labeled DNA. The two-step phosphoramidation-based, effective, and site-specific labeling method has the potential to expedite critical research including visualization, quantification, structural determination, localization, and distribution of nucleic acids *in vivo* and *in vitro*.

Site-specific labeling with fluorescent dyes, affinity tags, or radioisotope probes is critical to biomolecule detection and quantification. Such labeling has contributed to the rapid progress of biomolecule investigation for fundamental research and clinical applications. One of the major achievements of labeling techniques for biomolecules is the ability to selectively tag nucleic acids with fluorophores. For example, site-specific labeling of oligonucleotides with fluorophores enables high throughput and automatic analysis, and drastically improves the efficiency of genome-wide DNA sequencing projects, as evidenced by the completion of human genome sequencing in 2003. In addition, labeling biomolecules with fluorescent dye-quencher pairs allows measuring of real-time distances between or within biomolecules via fluorescence resonance energy transfer (FRET) and, for decades, has provided a plethora of essential structural biology information [1–3].

Many useful labeling methods have been developed and exploited for detection [4–8] and quantification [9–17] of DNA/RNA. To attain site-specific labeling, nucleic acids such as oligonucleotides are introduced with desirable chemical modifications at positions in specific nucleotides during solid-phase oligonucloetide synthesis by phosphoramidite chemistry [18]. Unfortunately, the efficacy and harsh reaction conditions of solid-phase chemistry have limited the length of the nucleic acids synthesized and the diversity of their incorporated chemical functionality. Consequently, postsynthesis site-specific labeling of nucleic acids is a reasonable approach for complementing the shortcomings of solid-phase oligonucleotide synthesis. Such a labeling strategy is

Abbreviations: ATP-C8-NH_2, 8-(6-aminohexyl)-amino-ATP; DABCYL, 4-(dimethylaminoazo)benzene-4-carboxyl; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDANS, 5-(2-aminoethylamino)-1-naphthalenesulfonic acid sodium salt; EPPS, 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid; FITC, fluorescein isothiocyanate; F/N ratio, the number of fluorophore molecules per molecule of nucleic acid; FRET, fluorescence resonance energy transfer; LRBE, Lissamine rhodamine B ethylenediamine; NHS, *N*-hydroxysuccimide; PCR, polymerase chain reaction; POCs, peptide-oligonucleotide conjugates; qPCR, quantitative PCR; SAv, streptavidin; TdT, terminal deoxynucleotidyl transferase; T_m, melting temperature; urea-PAGE, urea polyacrylamide gel electrophoresis.

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http://dx.doi.org/10.1016/j.ab.2013.12.021

amenable to any size of nucleic acids and generally performs under mild reaction conditions to facilitate introduction of various labile chemical functionalities. Effective postsynthesis site-specific labeling of nucleic acids is also advantageous as the only feasible labeling approach for nucleic acids isolated from living organisms. It is not surprising therefore that many useful postsynthesis site-specific labeling methods for nucleic acids have been explored and innovated. Selective nucleic acid labeling methods include popular *N*-hydroxysuccimide (NHS) ester chemistry for acylation reactions [19] and the recently emerged cyclo-addition reactions based on click chemistry [20–22] and inverse electron-demand Diels–Alder reactions [21,23]. However, none of these postsynthesis site-specific labeling methods are able to indiscriminately integrate tag molecules to nucleic acids, whether they are DNA/RNA or prepared by chemical or biochemical reactions.

Here we reported an effective universal and selective labeling method for nucleic acids, of either synthetic or enzyme-catalyzed origin, to prepare fluorescent-labeled nucleic acids under mild conditions in water. The labeling strategies are based on the phosphoramidation reaction first described by Orgel and co-workers [24] but recently significantly improved to be more appropriate for nucleic acid conjugation [25,26]. We previously unraveled the potential of aqueous-phase phosphoramidation reactions to covalently link nucleic acids with various molecules including protein, peptide, fluorophore, and affinity tags [25]. In the current study, we exploited the recently optimized advanced phosphoramidation reaction [26] to develop effective and site-specific methods for universally labeling DNA or RNA with fluorophores. Both one-step and two-step phosphoramidation reactions were employed to fluorescently label nucleic acids with large F/N ratio (number of fluorophore molecules per molecule of nucleic acid) values or exclusively fluorescent labeling to the 5′ phosphate in nucleic acids, respectively (Scheme 1). The propensity of the one-step phosphoramidation reaction to have multiple fluorophore labeling in nucleic acids impacted the intrinsic hybridization specificity of the nucleic acids as evidenced by studies of PCR and melting temperature (T_m) measurement. Preservation of intrinsic hybridization specificity in nucleic acids, however, was successfully achieved by the two-step phosphoramidation reaction which precisely labels fluorophores to the 5′ phosphate in nucleic acids as demonstrated by fluorescence quenching and UV melting profile studies. The only requirement of the phosphoramidation-based labeling techniques is a preexisting phosphate moiety at the 5′-end of nucleic acids. Thus, we have established a phosphoramidation reaction for universal and selective labeling of nucleic acids of either synthetic or enzyme-catalyzed origin that will be invaluable in fundamental research and clinical applications.

Materials and methods

All reagent-grade chemicals were purchased from commercial sources (Sigma–Aldrich, Acros, Alfa Aesar, Mallinckrodt Baker, and Life Technologies) except where noted, and were further purified as necessary. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded using either a Varian 200 or 400 MHz spectrometer (Varian, Inc., Palo Alto, CA, USA) at Kaohsiung Medical University, Taiwan (KMU). NMR samples were prepared in D_2O and the chemical shifts of ^{1}H and ^{13}C signals were given in parts per million based on the internal standard of D_2O. ^{31}P signals were reported as parts per million downfield from 85% H_3PO_4. ESI high resolution mass spectra were acquired from Department of Chemistry, National Sun Yat-Sen University (NSYSU), Taiwan, on a Bruker APEX II Fourier-transfer mass spectrometer (FT-MS; Bruker Daltonics Inc., Taiwan). Radio- or fluorophore-labeled nucleic acids were analyzed by urea polyacrylamide gel electrophoresis (urea-PAGE) or streptavidin (SAv) gel shift assay in urea-PAGE, visualized, and quantified by an Amersham Typhoon PhosphorImager (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at KMU. Molecular mass of fluorescent-labeled DNA was measured by an Autoflex III TOF/TOF analyzer (Bruker Daltonics, Taiwan) at KMU [26]. Melting profiles of DNA duplexes and determination of T_m were obtained from a Pharmacia Biotect Ultrospec 4000 UV/visible spectrophotometer (Pharmacia Biotech Inc., Piscataway, NJ, USA) at Department of Chemistry, NSYSU. UV-vis and fluorescence spectra of fluorescent-labeled DNA were recorded on a LAMBDA 650 UV/vis spectrophotometer (PerkinElmer Taiwan, Kaohsiung, Taiwan) and a LS 55 fluorescence spectrometer (PerkinElmer Taiwan, Kaohsiung, Taiwan), respectively. These instruments were located at KMU.

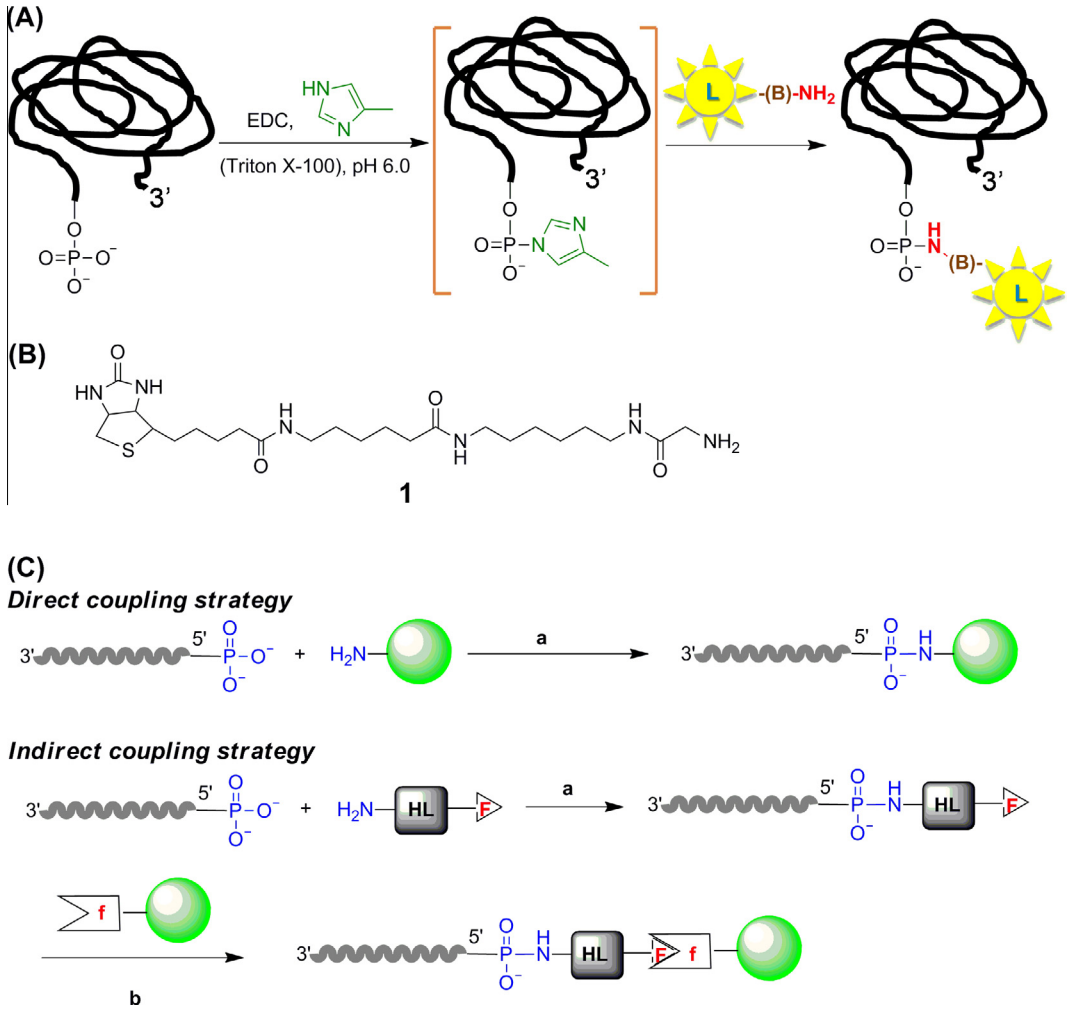
*Synthesis of 8-(6-aminohexyl)-amino-adenosine 5′-triphosphate (**4**)*

*8-Bromo-adenosine 5′-triphosphate (**3**)*

Adenosine 5′-triphosphate disodium salt hydrate (**2**, ATP; 0.6 g, 1 mmol) was dissolved in 1 M sodium acetate buffer (pH 4, 8 mL), followed by adding saturated Br_2-water (2 mL) and reacting at rt with stirring for 12 h [27]. The reaction was stopped by mixing with 36 mL of acetone-ethanol solution (acetone:ethanol = 1:1), shaken vigorously, and then placed in a −80 °C freezer for 1 h. After centrifugation at 4500 rpm for 30 min to separate and remove the supernatant, the remaining insoluble and viscous material of the reaction mixture was lyophilized, redissolved in a limited volume of water, loaded to a DEAE-Sephadex A-25 (GE Healthcare Life Sciences, Taipei, Taiwan) column (20 mL), and eluted by a step gradient of 0–1.0 M triethylammonium bicarbonate buffer (pH 8.0). During the 1.0 M triethylammonium bicarbonate buffer wash, fractions with significant absorbance at 260 nm were pooled and lyophilized to acquire the brown-colored **3** (0.29 g, 50%). ^{1}H NMR (200 MHz) (D_2O) δ: 8.38 (s, 1H, H-2), 6.07 (d, 1H, H-1′), 5.45 (dd, 1H, H-2′), 5.16 (dd, 1H, H-3′), 4.44–4.21 (m, 3H, H-4′ and H-5′). ^{31}P NMR (161.9 MHz) (D_2O) δ: −6.37 to −6.82 (m, P_γ), −9.70 to −10.77 (m, P_α), −21.38 (t, P_β). ESI-MS calculated for $C_{10}H_{15}BrN_5O_{13}P_3$, $[M+H]^+$ 587.0 (calcd.), 588.0 (found).

*8-(6-Aminohexyl)-amino-adenosine 5′-triphosphate (**4**)*

8-Bromo-adenosine 5′-triphosphate (**3**; 0.3 g, 0.5 mmol) was dissolved in an aqueous solution (5 mL) containing sodium carbonate (0.053 g, 0.5 mmol), followed by adding 1,6-hexanediamine (0.581 g, 5 mmol) and reacting at rt for 3 days [28]. The resulting reaction mixture was diluted by adding absolute ethanol (30 mL), followed by vigorously shaking, and then stored in a −80 °C freezer for 1 h. After centrifugation at 4500 rpm for 30 min to separate and remove the supernatant, the remaining insoluble and viscous material of the reaction mixture was lyophilized, redissolved in a limited volume of water, loaded to a DEAE-Sephadex A-25 column (35 mL), and eluted by a step gradient of 0–1.0 M triethylammonium bicarbonate buffer (pH 8.0). Fractions with significant absorbance at 280 nm during the 1.0 M triethylammonium bicarbonate buffer wash were pooled and lyophilized to afford **4** (0.15 g, 46%). ^{1}H NMR (400 MHz) (D_2O) δ: 7.90 (s, 1H, H-2), 5.92 (d, 1H, H-1′), 4.55 (t, 1H, H-2), 4.64 (d, 1H, H-3′), 4.25 (m, 2H, H-5′), 4.19 (m, 1H, H-4′), 3.41–3.30 (m, 2H, $NHCH_2$), 1.58 (m, 4H, $NHCH_2CH_2$), 1.31 (m, 4H, $NHCH_2CH_2CH_2$). ^{13}C NMR (100.67 MHz) (D_2O) δ: 152.3 (C-1), 151.3 (C-3), 149.7 (C-5), 149.0 (C-8), 116.4 (C-6), 86.4 (C-1′), 84.5 (C-3′), 70.5 (C-2′), 69.9 (C-4′), 65.7 (C-5′), 42.3 ($NHCH_2$), 39.4 (CH_2NH_2), 27.9 ($NHCH_2CH_2$), 26.4 ($NHCH_2$ CH_2 CH_2-CH_2CH_2), 25.3 ($NHCH_2CH_2CH_2$), 25.1 ($NHCH_2$ $CH_2CH_2CH_2$). ^{31}P NMR (161.92 MHz) (D_2O) δ: −7.95 (d, P_γ), −11.62 (d, P_α), −22.38 (t, P_β). HRMS (ESI) calculated for $C_{16}H_{31}N_7O_{13}$ P_3, $[M+H]^+$ 621.1193 (calcd.), 621.1185 (found).



Scheme 1. Aqueous-phase phosphoramidation reaction for efficiently and selectively labeling nucleic acids with diverse molecules. (A) The blue-colored "L" stands for labels such as fluorophores, affinity tags and other detectable molecules. The structure inside the orange bracket is a nucleic acid phosphorimidazolide which is not purified in one-step phosphoramidation but is isolated before reacting with nucleophiles in two-step phosphoramidation reactions. The brown-colored "B" in parentheses represents bridging molecules (cross-linkers) required only when indirectly coupling labels with nucleic acids. (B) The structure of **1** for optimizing the aqueous-phase phosphoramidation reactions. (C) Two strategies of exploiting the two-step phosphoramidation reaction for effective and site-specific labeling of nucleic acids with tag molecules. Reaction a, phosphoramidation reaction; b, compatible reactions to couple functional groups F with f in tags and nucleic acids, respectively. HL can be either homobifunctional diamino or heterobifunctional cross-linkers and is ethylenediamine in the current study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nucleic acid preparation and radiolabeling

The single-stranded 3′ primer DNA (5′-TACCCCTTGGGGATAC-CACC-3′) and its complementary DNA (5′-GGTGGTATCCC-CAAGGGGTA-3′) were purchased from Purigo Biotech, Inc., Taiwan, and purified by 20% urea-PAGE. The 5′ GMP-primed TW17 RNA (87-mer; 5′-GGGAUCGUCAGUGCAUUGAGAAGUGCA-GUGUCUUGCGCUGGGUUCGAGCGGUCCGUGGUGCUGGCCCGGUG-GUAUCCCCAAGGGGUA-3′) was prepared as previously described [29]. The TW17 RNA body labeled with ^{32}P and the 3′ primer DNA ^{32}P labeled at the 5′-end were also prepared according to the previously reported procedures [25,29].

Optimized one-step nucleic acid phosphoramidation reaction

The optimized one-step phosphoramidation reaction for RNA was initiated by dissolving the GMP-primed TW17 RNA (0.32 nmol) and EDC (4.17 μmol) in 4 μL of imidazole-urea buffer (0.1 M imidazole, 8 M urea, pH 6.0) and activating at rt for 90 min. To the solution containing the resulting 5′-phosphorimidazolide RNA, 7.5 μL of EPPS-urea buffer (100 mM EPPS, 8 M urea, 2 mM EDTA, pH 8.0) was added and followed by the addition of **1** (1 μL; 187.2 mM in DMF) to have the phosphoramidation reaction at 41 °C for 3 h.

For the single-stranded 3′-primer DNA, the optimized one-step phosphoramidation reaction was performed by dissolving the DNA (2.07 nmol) and EDC (6.52 μmol) in 4 μL of 4(5)-methylimidazole buffer [0.1 M 4(5)-methylimidazole, pH 6.0] and activating at rt for 90 min. Similarly, to the solution containing the resulting 5′-phosphorimidazolide DNA, 1.5 μL of EPPS buffer (100 mM EPPS, 2 mM EDTA, pH 7.5) and a solution of **1** (1 μL; 187.2 mM in DMF) was sequentially added to proceed the phosphoramidation reaction at 55 °C for 3 h.

All acquired nucleic acid-substrate conjugates were purified twice by ethanol precipitation, analyzed by urea-PAGE (8% for the TW17 RNA and 20% for the single-stranded DNA), visualized, and quantified by an Amersham Typhoon PhosphorImager to determine reaction yield.

One-step phosphoramidation reaction for fluorescent labeling of DNA

Direct coupling

The general method of the direct coupling and labeling DNA with fluorophores by one-step phosphoramidation reaction is

described in the following preparation of DNA- Lissamine rhodamine B ethylenediamine (LRBE; Chart S1) conjugate. The 3′ primer DNA (0.32 nmol) and EDC (4.17 μmol) were dissolved in 4 μL of 4(5)-methylimidazole buffer [0.1 M 4(5)-methylimidazole, pH 6.0] and activated at rt for 90 min, followed by the addition of 1.5 μL EPPS buffer (100 mM EPPS, 2 mM EDTA, pH 7.5) and 1 μL of LRBE (187.2 mM in DMF) to commence the coupling reaction at 55 °C for 3 h in the absence of light. The resulting LRBE-labeled DNA was purified twice by ethanol precipitation and once by 20% urea-PAGE, analyzed by 20% urea-PAGE again, and finally visualized and quantified by an Amersham Typhoon PhosphorImager with the settings of the excitation wavelength at 532 nm and the emission wavelength at 560 nm for rhodamine detection. The F/N ratio value of the 3′ primer DNA-LRBE conjugate was determined by the molar ratio of LRBE (a molar value obtained from A_{532}) to the DNA (a molar value derived from A_{260}).

Indirect coupling

The typical indirect coupling method of labeling DNA with fluorophores by the one-step phosphoramidation reaction is exemplified by the following procedures for DNA tagged with fluorescein isothiocyanate (FITC; Chart S1). The 3′ primer DNA (0.32 nmol) and EDC (4.17 μmol) were dissolved in 4 μL of 4(5)-methylimidazole buffer [0.1 M 4(5)-methylimidazole, pH 6.0] to have the activation reaction at rt for 90 min, followed by the addition of 1.5 μL EPPS buffer (100 mM EPPS, 2 mM EDTA, pH 7.5) and 1 μL of ethylenediamine (187.2 mM in DMF), and allowed to undergo the coupling reaction at 55 °C for 3 h. The resulting ethylenediamine-modified 3′ primer DNA was purified twice by ethanol precipitation, redissolved in carbonate-Triton X-100 buffer (0.1 M, 5% Triton X-100, pH 9.0; 20 μL), and followed by slowly adding 20 μL of FITC (2.57 mM in DMSO) and reacting in the dark at rt for 12 h. The reaction products were precipitated by ethanol and further purified by 20% urea-PAGE to obtain the FITC-labeled 3′ primer DNA, which was analyzed by 20% urea-PAGE again, and visualized and quantified by an Amersham Typhoon PhosphorImager with the settings of the excitation wavelength at 488 nm and the emission wavelength at 525 nm for fluorescein detection. The F/N ratio value of the FITC-labeled 3′ primer DNA was determined by the molar ratio of FITC (a molar value obtained from A_{488}) to the DNA (a molar value derived from A_{260}). The FITC-labeled 3′ primer DNA was directly used in standard PCR to acquire FITC-tagged PCR products.

Two-step phosphoramidation reaction for fluorescent labeling of nucleic acids

Direct coupling

The fluorescent labeling methods for nucleic acids by the direct coupling approach adhered to the previously optimized two-step phosphoramidation reactions [26] but with the following modifications. First, either 5-(2-aminoethylamino)-1-naphthalenesulfonic acid sodium salt (EDANS, Chart S1) or LRBE was substituted for the nucleophile **1** employed in the originally optimized two-step phosphoramidation reaction. In addition, the phosphoramidation reaction and subsequent workup were carried out in the dark to avoid fluorophore decay. Analysis of LRBE-labeled nucleic acid followed the same procedures of the one-step phosphoramidation reaction described above. The EDANS-labeled 3′ primer DNA was also analyzed by 20% urea-PAGE, and visualized and quantified by an Amersham Typhoon PhosphorImager with the settings of the excitation wavelength at 335 nm and the emission wavelength at 473 nm for EDANS detection. The F/N ratio value of the 3′ primer DNA-EDANS conjugate was determined by the molar ratio of EDANS (a molar value obtained from A_{335}) to the DNA (a molar value derived from A_{260}).

Indirect coupling

In the indirect coupling strategy, the diamino linker ethylenediamine was introduced to nucleic acids according to a previously reported optimized two-step phosphoramidation reaction for synthesis of nucleic acid-cystamine conjugates [26] but with cystamine replaced by ethylenediamine. The acquired diamino-derivatized nucleic acids were labeled with FITC and analyzed by the same procedures as the indirect strategy of the one-step phosphoramidation reaction stated above.

Fluorescence quenching experiments

In a typical fluorescence quenching study, a fluorescent-labeled 3′ primer DNA was redissolved in 200 μL of hybridization buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.5) to yield the DNA with a final concentration of 3 μM. Based on published excitation and emission wavelengths for each fluorophore, fluorescence of the solution was measured in triplicate with the excitation slit set at 2.5 nm and averaged. The fluorescent-labeled 3′ primer DNA solution was further added in the complementary 15-mer DNA (5′-T ATC CCC AAG GGG TA-3′) previously labeled with 4-(dimethylaminoazo)benzene-4-carboxyl (DABCYL, Chart S1) at either the 5′-end or the 3′-end (Bioneer, South Korea). The final concentration of the DABCYL-labeled DNA was 15 μM. Hybridization between the fluorophore-labeled 3′ primer DNA and the DABCYL-labeled DNA was attained by heating the DNA mixture solution at 70 °C for 5 min and slowly cooling at rt for 30 min. Fluorescence of the resulting DNA duplex hybrid was also measured in triplicate using the same spectroscopic parameters noted above and averaged. Quenching efficiency was measured by determining change of the emission maxima for each fluorophore and was calculated by dividing the quenched fluorescence by the initial fluorescence, multiplying by 100, and then subtracting from 100 [13].

UV melting profile study of DNA duplexes

The DNA duplex samples (0.4–0.6 μM, 800 μL) for melting experiments were prepared by denaturing either the 3′ primer DNA or the fluorescent-labeled 3′ primer DNA with an equal mole of the complementary 3′ primer DNA in the hybridization buffer noted above at 65 °C for 5 min and gradually annealing at rt for 1 h. Each DNA duplex solution was transferred to a 1-mL quartz cuvette, covered with a Teflon cap, and mounted into the temperature controller of the Pharmacia Biotect Ultrospec 4000 UV/visible spectrophotometer. Changes of A_{260} in DNA samples at specific temperatures were recorded by heating the DNA solution from 25 °C to 95 °C at a heating rate of 0.5 °C min^{-1}. Melting data were plotted as A_{260} with respect to temperature to determine T_m values. The melting experiment for a DNA duplex sample was performed in triplicate; the reported T_m was the mean of triplicate experiments ± SD.

Results

Evaluation of one-step phosphoramidation reaction for effective fluorescent labeling of DNA and RNA

We first studied one-step phosphoramidation reactions to more effectively label nucleic acids with fluorophores and to increase the sensitivity of nucleic acid analysis (Scheme 1A). Our previous work [25] has shown that one-step phosphoramidation reactions would have multiple modifications in nucleic acid conjugates but apparently not affect intrinsic hybridization specificity of nucleic acids. The unique characteristics of multiple labeling and intact intrinsic

hybridization specificity in nucleic acids implicate the potential of one-step phosphoramidation reactions to prepare fluorophore-labeled nucleic acids with large F/N ratio values. Large F/N ratio values are essential for increasing the sensitivity of analytical techniques for nucleic acids such as microarray gene expression analysis and fluorescence *in situ* hybridization [4–6]. These techniques critically depend on effectively labeling of DNA or RNA and preserving distinctive hybridization specificity in nucleic acid probes to identify very limited amounts of nucleic acids in biological samples. In addition, the one-step phosphoramidation reaction provides a facile fluorescent labeling strategy easily applied to any nucleic acids, either from synthetic or biological sources, as long as they are primed in advance with phosphate at the 5′-end.

We initially employed the previously studied one-step nucleic acid phosphoramidation reactions to develop the desired fluorescent labeling method as the reaction provided excellent yields from 60% to quantitative conversion in 0.4–4.5 h [25]. While we optimized the one-step phosphoramidation reaction to be more appropriate for fluorescent labeling of nucleic acids, we were repeatedly baffled by inconsistent product yields by the same reaction every day. We later identified the cause of the inconsistent yield in the one-step phosphoramidation reaction to be the deficiency of a typical SAv gel shift assay. A standard SAv gel shift assay relies on a strong interaction between biotin and SAv to separate biotin-tagged from non-biotin-tagged biomacromolecules [29]. Unfortunately, in the repeated nucleic acid phosphoramidation reactions, SAv-retarded nucleic acids comigrated with polymerized nucleic acids which are by-products of phosphoramidation reactions [30] and are unable to be distinguished from SAv-retarded nucleic acids in the SAv gel shift assay (Fig. S1A and B). The comigration phenomenon in SAv gel shift assay resulted in overestimated yield of the desired biotin-labeled nucleic acids.

We overcome the nucleic acid comigration problem by using a different approach to precisely determine the desired product yield in a one-step phosphoramidation reaction and optimizing the reaction to minimize polymerization of nucleic acids. First, the more accurate determination of phosphoramidation reaction yield was ensured by urea-PAGE analysis, which separates nucleic acids solely based on size and charge, and therefore unambiguously resolves molecules of reactants and phosphoramidation products. Urea-PAGE thus provided a more faithful calculation of product yield for phosphoramidation reactions and was one of the major analysis methods used in this study. In addition, we systematically surveyed and adjusted concentrations of EDC and nucleic acids to significantly suppress the unwanted polymerization of nucleic acids (abou 1%) in the optimal one-step phosphoramidation reaction (Fig. S1C and D). The updated one-step phosphoramidation reaction achieved almost complete conversion for nucleic acid conjugates when reacting nucleic acids with the biotin derivative **1** [25] (yield ≧ 94%; Fig. S1C and D and Scheme 1B).

The optimized one-step phosphoramidation reactions were successfully exploited to fluorescently label DNA through either the zero linker (direct coupling) or the diamino linker (indirect coupling) strategies (Scheme 1C). Both coupling strategies provided effective labeling of DNA with either LRBE (the direct coupling approach) or FITC (the indirect coupling approach assisted by an ethylenediamine linker) as evidenced by their good F/N ratio values (Fig. 1A and Table 1). But, unexpectedly, the more straightforward and direct zero-linker strategy for fluorescent labeling of nucleic acids (requiring phosphoramidation reaction only) did not have a better F/N ratio (0.06, Table 1) than that obtained from the indirect coupling approach (having phosphoramidation reaction followed by thiourea bond formation; F/N = 2.04, Table 1). The outcome is attributed to the far better reactivity of diamines, such as ethylenediamine, than the monoamino nucleophiles including LRBE and **1** to introduce additional primary amino groups in DNA for subsequent thiourea linkage formation [25]. Nevertheless, the direct zero-linker labeling strategy is more efficient because it requires a significantly shorter reaction time compared to indirect coupling labeling (4.5 h vs >19 h).

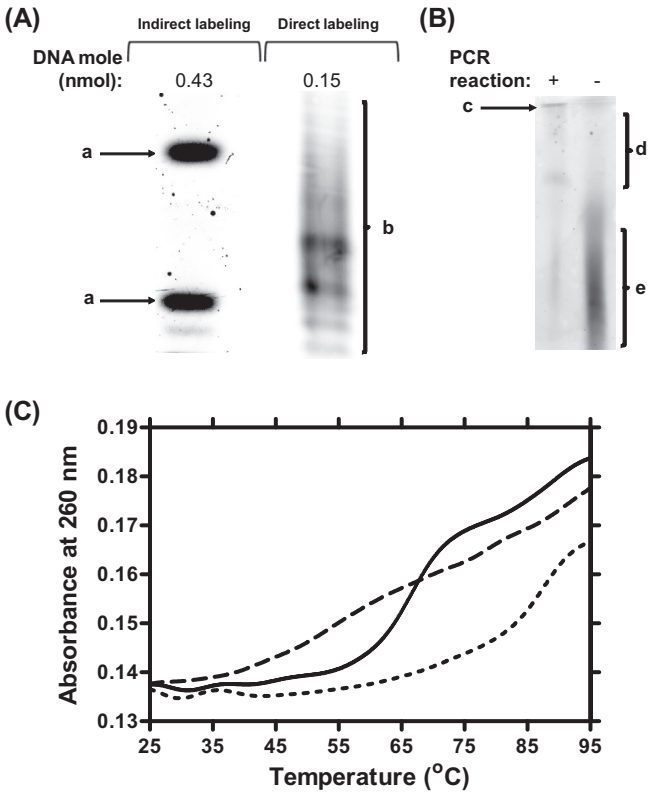


Fig. 1. One-step phosphoramidation reaction to effectively acquire fluorescently labeled nucleic acids but with effects on hybridization specificity in nucleic acids due to multiple conjugation. Fluorescent analysis of one-step phosphoramidation reaction (A) for effectively labeling of DNA with fluorophores and (B) for providing a fluorescently labeled DNA primer later incorporated into PCR products. Samples in (A) were the 20-mer 3′ primer DNA labeled with fluorophores and analyzed by 20% urea PAGE. Biphasic urea PAGE (5% in the top gel and 20% in the bottom gel) was, however, used to analyze samples in (B) in order to have the FITC-labeled both 119-mer PCR product and the 20-mer primer DNA in the same PAGE result. Consequently, the PCR product was stopped on the interface of the two gels. The fluorescent samples were visualized by an Amersham Typhoon PhosphorImager. a, FITC-labeled DNA; b, Lissamine rhodamine B ethylenediamine (LRBE)-labeled DNA; c, the FITC-labeled 119-mer PCR product; d, truncated PCR products; e, the FITC-labeled 3′ primer DNA for PCR. (C) UV melting profiles of the 3′ primer DNA and its LRBE or FITC conjugate prepared by the one-step phosphoramidation reaction. Please see text for the detailed experimental procedures. The UV melting curves for the DNA duplexes are the original 3′ primer DNA (solid line), the FITC-labeled 3′ primer DNA (broken line), and the LRBE-labeled 3′ primer DNA (dotted line).

An F/N ratio value greater than 1 in the optimized one-step DNA phosphoramidation reaction suggests the likelihood of more than one fluorophore molecule labeled to DNA. Indeed, multiple labeling of DNA with fluorophores in a one-step phosphoramidation reaction is visible in Fig. S1D and 1A. The inclination of one-step phosphoramidation reactions to covalently link multiple labels to DNA is consistent with similar reactions studied previously [25]. We wondered whether the added label molecules might have an impact on hybridization specificity in DNA. We thus studied the possibility by including the phosphoramidation-prepared and FITC-labeled single-stranded DNA in standard PCR to understand the correlation of multiple conjugation and intrinsic hybridization specificity in nucleic acids. The PCR results showed that the DNA was an effective primer in the DNA amplification reaction and was successfully incorporated into PCR products (Fig. 1B). We, however, were aware that some PCR products had shorter lengths

Table 1
Phosphoramidation reaction for effective labeling of nucleic acids with fluorophores compared with other conventional postsynthesis fluorescent labeling methods for DNA and RNA.

DNA[a]/RNA[b]	Fluorescent labeling method	F/N ratio	T_m (°C)
DNA	N/A	N/A	67.1 ± 0.7
DNA	One-step phosphoramidation reaction	2.04,[c] 0.06 [d]	81.3 ± 3.2 [d]
DNA	Two-step phosphoramidation reaction	0.02,[c] 0.02, [d] 0.02[e]	66.9 ± 0.7 [c], 65.8 ± 0.5 [d], 66.9 ± 0.5 [e]
RNA	Two-step phosphoramidation reaction	0.16,[c] 0.26 [d]	
DNA	3′-end labeling by TdT [f]	0.69	
DNA	Depurination reaction [8]	0.42 [c]	
RNA	3′-end redox reaction [8]	0.03 [c]	

[a] The 3′ primer DNA.
[b] The TW17 RNA.
[c] Labeled with FITC assisted by the ethylenediamine cross-linker (indirect coupling).
[d] Labeled with Lissamine rhodamine B ethylenediamine (LRBE) directly.
[e] Labeled with EDANS directly.
[f] Activity of terminal deoxynucleotidyl transferase (TdT) to covalently link 8-(6-aminohexyl)-amino-ATP (ATP-C8-NH2; **4**) to the 3′-end of DNA, followed by FITC conjugation through thiourea bond (Ref. [7]).

than expected (d in Fig. 1B) which led to speculation that the one-step phosphoramidation reaction might have produced multiple fluorophore-labled DNA with hybridization specificity different from the original DNA. Ultimately, we determined the UV melting profiles of the original and one-step phosphoramidation-prepared FITC-labeled 3′ primer DNAs to confirm the plausible role of mutiple conjugation on changing the intrinsic hybridization specificity in nucleic acids. If the multiple-labeled 3′ primer DNA prepared by the one-step phosphoramidation reaction does not alter intrinsic hybridization specificity in the DNA, we would expect similar UV melting profiles and the same melting temperatures (T_m) for these 3′ primer DNA duplexes even though that DNA had extensively labeled with fluorophores by the one-step phosphoramidation reaction.

On the contrary, the obtained UV melting profiles substantiated the concern that the one-step phosphoramidation reaction could change the intrinsic hybridization specificity of nucleic acid through multiple conjugation and cause product complexity in PCR (Fig. 1C). The UV melting profiles of the fluorophore-labeled 3′ primer DNA duplexes were significantly different from that of the 3′ primer DNA duplex without structural modification and indicated that T_m and hybridization specificity of the 3′ primer DNA had changed after the one-step phosphoramidation reaction. For example, the determined T_m for the one-step phosphoramidation-prepared LRBE-labeled DNA is 81.3 °C (Table 1) which is 12 °C higher than that of the original 3′ primer DNA. Consequently, increasing labeling extents in nucleic acids might promote nonspecific hybridization and raise T_m in the nucleic acids. The argument is further supported by the UV melting profile of the more extensively labeled one-step phosphoramidation-prepared FITC-3′ primer DNA conjugate (F/N ratio = 2.04). The FITC-labeled DNA showed continuous increases of A_{260} values in the studied temperature range to give a T_m too high to be unambiguously determined (Fig. 1C). We thus concluded that the current format of the one-step phosphoramidation reaction is not appropriate for labeling nucleic acids without compromising the intrinsic hybridization specificity of the nucleic acids, a feature essential to many nucleic acid analyses.

Two-step phosphoramidation reaction for effective and site-specific labeling of nucleic acids with fluorophores

In addition to potentially changing the intrinsic hybridization specificity of nucleic acids, the properties of overmodification and multiple conjugation in the optimized one-step phosphoramidation reaction have the undesired effects when incorporating fluorophores into DNA/RNA for structural or functional study. Fluorescence analysis of nucleic acids by methods such as fluorescence resonance energy transfer (FRET) [2], fluorescence quenching [9,10,13], and quantitative PCR (qPCR) using the TaqMan probes [11,12] and molecular beacons [14,15] all requires fluorophores to be labeled to nucleic acids at predetermined locations to ensure accurate structural or functional information and precise quantitative determination of nucleic acids.

After identifying the serious drawbacks of the one-step phosphoramidation reaction resulting in change of hybridization specificity in nucleic acids which is detrimental to analysis of these molecules, we turned our attention to a two-step phosphoramidation reaction for developing effective and site-specific fluorescent labeling methods of nucleic acids. Past studies have demonstrated that the two-step phosphoramidation reaction labels substrates predominately to the 5′ phosphate in nucleic acids, avoids multiple conjugation of nucleic acids, but generally provides lower labeling yield than that of one-step phosphoramidation format [25]. Fortunately, we recently significantly improved the efficacy of the two-step phosphoramidation reaction to facilitate more effective preparation of nucleic acid conjugates and demonstrated phosphoramidation-prepared peptide-oligonucleotide conjugates (POCs) successfully trafficked into human A549 cells [26]. Similar to the previously reported one-step reaction, the original two-step phosphoramidation reaction also suffered from nucleic acid polymerization to reduce the desired product yields [26]. The improved advanced two-step phosphoramidation, however, overcomes the hurdle and provides optimal and milder reaction conditions to effectively eradicate the problems of polymerization, overmodification, and multiple labeling in nucleic acids that constantly plagues its one-step counterpart (Scheme 1A). We exploited the much improved two-step nucleic acid phosphoramidation reaction to site specifically label nucleic acids with fluorophores while retaining intrinsic hybridization specificity in the nucleic acids.

Congruent with our expectations, the optimized two-step phosphoramidation reaction carried out efficient and selective labeling of DNA and RNA with fluorophores. We first employed the diamino linker (indirect coupling) strategy (Scheme 1C) to successfully label FITC to DNA and RNA and acquired the F/N ratio values of 0.02 and 0.16 for DNA and RNA, respectively (Table 1). In addition, we also performed the zero linker strategy of the two-step phosphoramidation reaction (Scheme 1C) and directly labeled DNA and RNA with LRBE to attain the F/N ratio values of 0.02 for DNA and 0.26 for RNA (Table 1). Moreover, the fluorophore EDANS was coupled to the 3′ primer DNA by employing the zero linker strategy of the two-step phosphoramidation reaction to obtain the labeled DNA with a F/N ratio value of 0.02 (Table 1). The EDANS-labeled DNA is useful for the subsequent fluorescence

quenching study (vide infra). Consistent with the determined F/N ratio values, UV-vis scanning spectra of the fluorescent-labeled nucleic acids prepared from the two-step phosphoramidation reaction showed the characteristic absorption wavelengths for each fluorophore and supported incorporation of fluorophores into the nucleic acids (Fig. S2).

More importantly, the two-step phosphoramidation reaction only labeled a single fluorophore to each nucleic acid molecule and apparently did not have the problem of multiple modification and conjugation in nucleic acids. Urea-PAGE and fluorescence detection analysis demonstrated that, by either the direct or indirect coupling strategy (Scheme 1C), the two-step phosphoramidation reaction only labeled each nucleic acid with a fluorophore and was free from the unwanted multiple modification (Fig. 2A and B). The selectivity and effectiveness of the two-step phosphoramidation reaction for nucleic acid labeling was further demonstrated by urea-PAGE analysis of the phosphoramidation-prepared ^{32}P-labeled LRBE-3′ primer DNA conjugate (Fig. 2C). Single fluorophore labeling in the two-step phosphoramidation-prepared nucleic acid conjugates was further supported by MALDI-TOF spectrometry to acquire molecular mass corresponding to no more than one fluorophore molecule incorporated per nucleic acid (Fig. S3). Moreover, the UV melting profiles of the two-step phosphoramidation-prepared 3′ primer DNA-fluorophore conjugates explicitly showed that the original and fluorophore-labeled 3′ primer DNA had similar melting profiles and essentially identical T_m (Fig. 3 and Table 1). The results strongly supported the ability of the two-step phosphoramidation reactions to fulfill the requirements of effectively labeling nucleic acids with fluorophores while preserving intrinsic hybridization specificity in the nucleic acids.

For comparison, we also labeled DNA and RNA with FITC by three major conventional postsynthesis methods for nucleic acid labeling: 3′-end labeling by terminal deoxynucleotidyl transferase (TdT) in the presence of 8-(6-aminohexyl)-amino-ATP (ATP-C8-NH_2; **4**) [7] and depurination reactions [8] for DNA labeling, and redox reactions for 3′-end labeling of RNA [8]. However, we did not study popular click chemistry [31,32] for fluorescent labeling of nucleic acids because appending an azido or alkynyl group to previously synthesized DNA/RNA is critical to the click reactions but is very challenging to perform in a typical biomedical lab. As a requirement of the TdT labeling method, we developed a facile scheme for synthesis of **4** (Scheme 2). Starting from ATP (**2**), a bromination reaction resulted in synthesis of **3** which was subjected to nucleophilic substitution with hexanediamine to acquire the final product **4** with an overall yield of 25%. Similarly, the FITC-nucleic acid conjugates obtained from the coventional nucleic acid labeling methods were purified by urea-PAGE, and analyzed by urea-PAGE and UV-vis spectrophotometry. The F/N ratio values were 0.75 for the TdT method, 0.42 for the depurination method, and 0.03 for the 3′-end redox method of RNA (Fig. 2A and B, and Table 1).

Among all the postsynthesis fluorescent labeling methods for nucleic acids explored in the current study, the two-step phosphoramidation reaction provided a more practical and universal approach for effective and site-specific labeling of nucleic acids with tags. For fluorescent labeling of DNA, the good F/N ratio values might suggest that the one-step phosphoramidation method is the choice for more sensitive detection of a limited amount of nucleic acids in samples (Table 1). However, as we have indicated above, extensive multiple labeling of nucleic acids with fluorophores changes the intrinsic hybridization specificity in the nucleic acids (Fig. 1). Thus, TdT and depurination methods might be the more desirable choices to label DNA with fluorophores. The TdT method, however, has serious drawbacks because it requires using the expensive enzyme TdT, and enzyme substrates such as **4** or other structural analogs which are typically not commercially available or obtained only from a limited number of vendors at

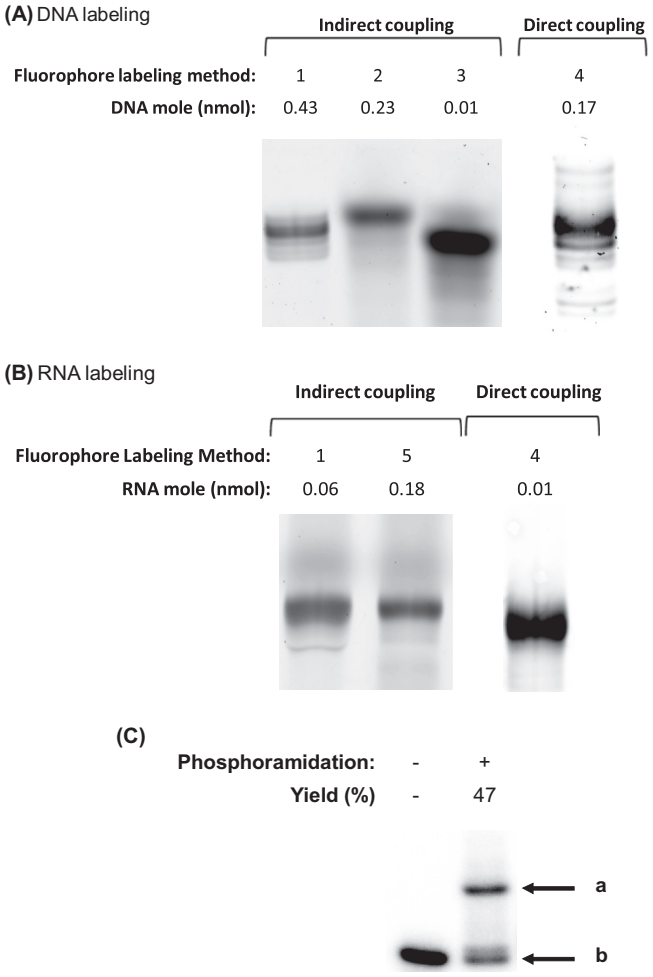


Fig. 2. Two-step phosphoramidation reaction conveying the essential properties of efficiently labeling nucleic acids with fluorophores and devoid multiple fluorophore labeling in nucleic acids. (A) DNA and (B) RNA were labeled with fluorophores by the two-step phosphoramidation reaction or conventional nucleic acid labeling methods for comparison. The studied fluorescent labeling methods of nucleic acids were: 1, indirect coupling with FITC by the two-step phosphoramidation reaction; 2, indirect coupling with FITC by the DNA depurination method [8]; 3, indirect coupling with FITC by the terminal deoxynucleotidyl transferase (TdT) reaction [7]; 4, direct coupling with LRBE by the two-step phosphoramidation reaction; 5, indirect coupling with FITC by the redox reaction [8]. Samples in (A) were the 3′ primer DNA labeled with fluorophores and analyzed by 20% urea-PAGE. The TW17 RNA was fluorescently labeled in (B) and the fluorescent-labeled RNA was analyzed by 8% urea-PAGE. The fluorescent samples were visualized by an Amersham Typhoon PhosphorImager. (C) The two-step phosphoramidation reaction was employed for specifically and selectively tagging the ^{32}P-labeled 3′ primer DNA with LRBE and acquiring the ^{32}P-labeled 3′ primer DNA-LRBE conjugate free from multiple LRBE in the DNA molecule. The LRBE-labeled DNA was purified by ethanol precipitation, analyzed by 20% urea-PAGE, and visualized and quantified by an Amersham Typhoon PhosphorImager. a, the LRBE-labeled 3′ primer DNA; b, the unreacted 3′ primer DNA.

considerable cost. In addition, TdT is also capable of adding more than one nucleotide per DNA which might cause mutiple substrate labeling and change the intrinsic hybridization specificity in DNA. As a result the TdT method is not cost-effective and less reliable for routine or large-scale labeling of DNA. The depurination method for DNA labeling is also problematic because purine bases are removed from DNA to render fluorophore-labeled DNA with impaired or incorrect hybridization specificity and also likely, with mutiple fluorophore conjugation, to change the intrinsic hybridization specificity, unacceptable to general hybridization analysis. Therefore, the two-step phosphoramidation method for labeling DNA with fluorophores stands out as the preferred choice for precise and

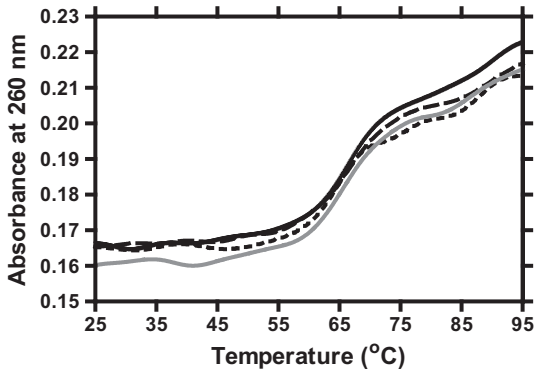


Fig. 3. Two-step phosphoramidation reaction efficiently labeling nucleic acids with fluorophores and safeguarding intrinsic hybridization specificity in the nucleic acids. Evidence of intact intrinsic hybridization specificity in nucleic acids was provided by UV melting profile studies of the 3′ primer DNA and its LRBE, FITC, or EDANS conjugate prepared by the two-step phosphoramidation reaction. Please see text for the detailed experimental procedures. The UV melting curves for the DNA duplexes are the original 3′ primer DNA (black solid line), the FITC-labeled 3′ primer DNA (black broken line), the LRBE-labeled 3′ primer DNA (black dotted line), and the EDANS-labeled 3′ primer DNA (gray solid line).

selective labeling of DNA with probes essential to a myriad of nucleic acid analyses. Conversely, the F/N ratio data clearly indicate that the direct-coupling two-step phosphoramidation method for fluorescent labeling RNA is superior to that of the popular redox reaction method (Table 1). The two-step phosphoramidation reaction is thus well adapted to effectively and site specifically label nucleic acids with various tags including fluorophores. Moreover, the two-step phosphoramidation reaction provides a universal labeling strategy for DNA/RNA either from synthetic or enzyme-catalyzed sources, a significant achievement not attainable by the conventional postsynthesis labeling methods for DNA or RNA.

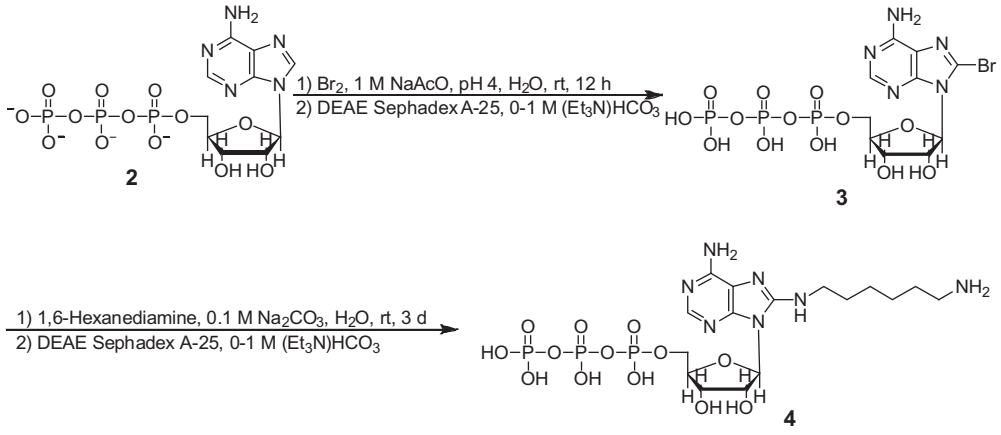
Two-step phosphoramidation reaction to site specifically label the 5′ phosphate in nucleic acids demonstrated by fluorescence quenching

Once we had confirmed that the two-step phosphoramidation reaction could effectively label nucleic acids with fluorophores, but did not label more than one fluorophore to the nucleic acids, we were motivated to demonstrate that the two-step phosphoramidation reaction also had an essential ability to site specifically label fluorophores mainly to the 5′ phosphate in nucleic acids. As emphasized above, achieving site-specific labeling in nucleic acids is crucial to many analytical techniques for nucleic acid detection or quantification and is indispensable when developing novel methods for nucleic acid analysis. The two-step phosphoramidation reaction has the potential to become the preferred method for nucleic acid conjugation and modification only if the reaction can unmistakably deliver both effective coupling of nucleic acids with tag molecules while not affecting intrinsic hybridization specificity, and site-specific labeling of the nucleic acids at the 5′ phosphate with tag molecules.

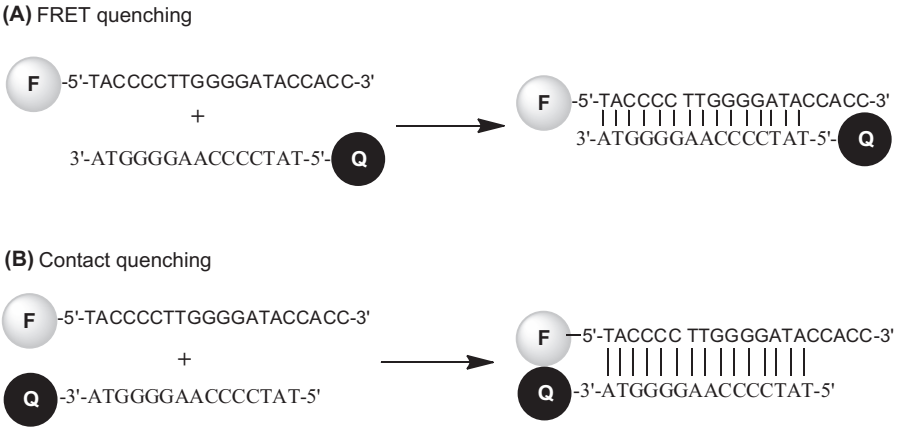
With the aim to affirm site-specific labeling accomplished by the two-step phosphoramidation reaction, we performed a fluorescence quenching study to provide conclusive evidence that the two-step phosphoramidation reaction indeed labels tag molecules such as fluorophores specifically to the 5′ phosphate of nucleic acids. Here we again harnessed the two-step phosphoramidation reaction to prepare fluorophore-labeled DNA duplexes (Scheme 3). According to the proposed site-specific labeling capacity conferred by the reaction, the DNA duplexes would have the appropriate designs to perform FRET and contact quenching of fluorescence by the nucleic acids due to precisely controlled distance between fluorophores and the quencher DABCYL (vide infra). In addition, since the fluorescence quenching properties are fundamental to many homogeneous hybridization analyses of nucleic acids, the two-step phosphoramidation reaction could be employed to develop novel analytical techniques for specific detection and quantification of nucleic acids in crude samples [13].

Three different fluorophores (FITC, LRBE, and EDANS) were selectively and effectively labled to the 5′-end of the 3′ primer DNA (20-mer) by the two-step phosphoramidation reaction as confirmed by MALDI-TOF MS analysis (Fig. S3). The contact quenching constructs were prepared by hybridizing the fluorescent labeling 3′ primer DNA with the 15-mer complementary DNA previously labeled with DABCYL at the 3′-end to render fluorophores and the quencher DABCYL in close proximity. We attained the FRET-quenching constructs by hybridizing the fluorescent labeling 3′ primer DNA with the same 15-mer complementary DNA already labeled with DABCYL at the 5′-end. Here we deliberately separated the quencher and the fluorophores by 15 bp in the FRET-quenching DNA duplexes to limit interferance of contact quenching and more accurately measure the extent of fluorescence quenched by FRET [10]. In addition, with fluorophores accurately appended on the 5′-end of the 3′ primer DNA and very few guanosine nucleotides near the 5′-end of the DNA, the undesired fluorescence quenching by nucleotides [13] would be limited in both fluorescence-quenching constructs.

The quenching efficiency study of fluorophore-quencher pairs in either the FRET quenching or the contact quenching DNA duplex constructs garnered critical information on the DNA duplex structures and strongly supported site-specific fluorescent labeling of the 5′ phosphate in nucleic acids by the two-step phosphoramidation reaction (Fig. 4). In standard FRET quenching, the quenching



Scheme 2. Synthesis of 8-(6-aminohexyl)-amino-adenosine 5′-triphosphate (ATP-C8-NH_2), **4**.



Scheme 3. Construction of the functional (A) FRET and (B) contact quenching DNA duplexes achieved by the two-step phosphoramidation reaction. F, fluorophores; Q, the quencher DABCYL.

efficiency is dependent on distance and relative orientation of a fluorophore-quencher pair, and overlap of the emission spectrum of the fluorophore and the absorption spectrum of the quencher [10,13]. As expected, fluorescence of the conjugated fluorophores (FITC or EDANS) in the studied FRET-quenching DNA duplex constructs was significantly quenched because all the fluorophores have emission spectra overlapping the absorption spectrum of DABCYL (Scheme 3A and Fig. 4). On the other hand, effective contact quenching of fluorescence requires a fluorophores-quencher pair within very close distances so that all the fluorophores are quenched equally by the quencher, irrespective of whether the emission spectrum of a fluorophore overlaps the absorption spectrum of the quencher [9,10,13,16]. Moreover, direct electronic interaction of the fluorophores' excited states with the quencher in the contact quenching mode would perturbate visible absorption properties of the fluorophores-quencher pairs [9,16,17]. Consequently, the visible absorption spectrum of a fluorophores-quencher pair in the contact quenching mode is drastically different from the spectrum when the quencher is too distant to interact with the fluorophore. Consistent with the properties of contact quenching, fluorescent emission of all fluorophores in the studied contact-quenching DNA duplex systems, notably that of the fluorophore LRBE never overlapping with the DABCYL absorption, was effectively quenched by DABCYL (Scheme 3B and Fig. 4). The presence of the close contact construct was further supported by the substantial difference of visible absorption spectra for the DABCYL-labeled and LRBE-labeled DNA before and after complementation to form the contact quenching constructs (Fig. S4). Successful FRET and contact quenching of fluorescence in the studied DNA duplex constructs strongly support that the two-step phosphoramidation reaction specifically labels nucleic acids at the 5′-ends with good efficacy and has no detectable multiple conjugation which effects intrinsic hybridization specificity in the nucleic acids.

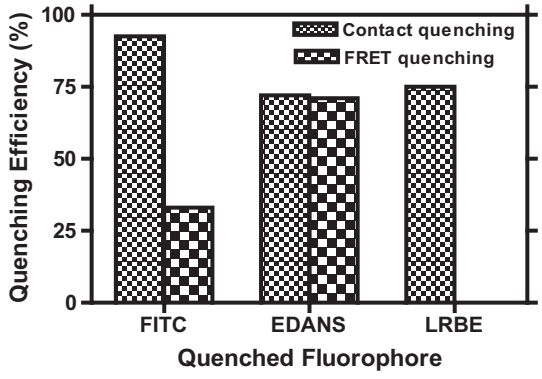


Fig. 4. The two-step phosphoramidation reaction for site-specific labeling of nucleic acids with fluorophores demonstrated by FRET and contact quenching of fluorophore-DABCYL pairs in DNA duplex constructs. Fluorescence intensity for the DNA duplex containing EDANS, FITC, or LRBE was measured at 473, 520, or 590 nm, respectively. Quenching efficiency for each fluorophore-DABCYL pair was determined by dividing the quenched fluorescence by the initial fluorescence at the emission maxima for each fluorophore, multiplying by 100 and then subtracting from 100 [10,13].

Discussion

We have succeeded in exploiting the recently optimized phosphoramidation reaction to develop a universal postsynthesis labeling method capable of effectively and site specifically conjugating nucleic acids with tag molecules. We initially studied and improved a one-step phosphoramidation reaction to take advantage of the high labeling efficiency and product yield (Table 1) for fluorescent labeling of nucleic acids and to establish a simple low-cost labeling approach potentially invaluable for large-scale gene expression studies. Unfortunately, the optimized one-step phosphoramidation reaction suffered from a change in the intrinsic hybridization specificity of the fluorescently labeled nucleic acids, a change likely caused by the reaction to label multiple fluorophores in the nucleic acids (Fig. 1). However, site-specific labeling of DNA or RNA with fluorophores was effectively achieved by the two-step phosphoramidation method which covalently and selectively linked probe molecules to the 5′-end of nucleic acids (Figs. 2–4 and Table 1). The two-step phosphoramidation reaction thus can label nucleic acids with tags specific to the 5′ phosphate, a critical property essential for preparing nucleic acid constructs for nucleic acid detection and quantification. In addition, the chemical flexibility of the two-step phosphoramidation reaction allows incorporation of novel functionalities into nucleic acids and places the reaction in an excellent position to serve as a versatile platform for conjugating nucleic acids with diverse molecules ranging from proteins and peptides to lower molecular mass tags without comprising intrinsic hybridization specificity in nucleic acids. Broader applications of the phosphoramidation reaction could advance progress on understanding functions and structures of nucleic acids in biological systems.

Even though the one-step phosphoramidation reaction possesses a serious drawback of changing the intrinsic hybridization specificity in labeled nucleic acids, we do not preclude the future possibility that a more comprehensive study of the reaction may

establish a useful nucleic acid labeling strategy with the desired properties of high labeling efficacy and faithful preservation of intrinsic hybridization specificity in nucleic acids. A logical approach to improve the reaction is to reduce the reaction time in the one-step phosphoramidation reaction because previous studies had shown that the reaction could deliver quantitative conjugation of nucleic acids with diamino substrates within 20 min [25]. A longer reaction time would be required for monoamino substrates in the one-step phosphoramidation reaction to attain quantitative conversion due to less positive charge density of the substrates. Diminished positive charge density in monoamino substrates would curtail the strength of the electrostatic force between nucleic acids and monoamino substrates and thereby reduce the rate of product formation. The minimum reaction time for complete consumption of monoamino substrates in the one-step phosphoramidation reaction is likely to be less than the 3 h used in the current study. Moreover, quantitative formation of labeled nucleic acids by a one-step phosphoramidation reaction may not be required and, in fact, could be the major cause of the change in the intrinsic hybridization specificity in nucleic acids. It is perceivable that a shorter reaction time for the one-step phosphoramidation reaction would be more amiable for preserving intrinsic hybridization specificity of nucleic acids and to reach an equilibrium status of labeled nucleic acid production with high yield, less extensive multiple conjugation, and intact intrinsic hybridization specificity in the nucleic acids. In addition, appropriately adjusting reactant and EDC concentrations in the one-step phosphoramidation reaction might also increase the yield of labeled nucleic acids without changing intrinsic hybridization specificity in the nucleic acids. Our ongoing research is exploring different options for further optimizing one-step phosphoramidation in order to achieve high yield of labeled nucleic acids and preserve the intrinsic hybridization specificity in the nucleic acids as the ultimate goal.

The two-step phosphoramidation reaction clearly fulfills our quest for an effective site-specific postsynthesis labeling strategy amenable to DNA and RNA from *in vitro* and *in vivo* souces. As stated, the only requirement for performing the two-step phosphoramidation reaction is to previously append phosphate on the 5′-end of nucleic acids which is easily implemented by standard molecular biology techniques using phosphatase and kinase. Once the phosphate is installed in nucleic acids, the two-step phosphoramidation reaction will specifically label the nucleic acids mainly at the 5′ phosphate by formation of a phosphoramidation bond. Moverover, the two-step phosphoramidation reaction evades the potentially problematic mutiple labeling in nucleic acids and successfully retains intrinsic hybridization specificity of the nucleic acids. As a result, we have validated that the two-step phosphoramidation reaction is truly a universal postsynthesis labeling method for nucleic acids and is well adapted to site-specific labeling of DNA and RNA at the 5′ phosphate with excellent efficacy.

In addition to being a universal site-specific reaction for nucleic acid conjugation and labeling, the two-step phosphoramidation reaction is also a facile approach for covalently linking molecules with diverse functionalities to nucleic acids as demonstrated by the direct and indirect fluorophore labeling strategies explored in the current study (Fig. 2 and Scheme 1C). In the two-step phosphoramidation reaction for fluorescent labeling, amino-containing fluorophores were easily labeled to nucleic acids by the zero-linker direct-coupling strategy in which the fluorophores served as substrates in the nucleic acid phosphoramidation reaction (Scheme 1C). For fluorophores lacking an amino group but carrying amino-reactive functional groups, we harnessed excellent reactivity of diamino substrates in the nucleic acid phosphoramidation reaction and developed the indirect coupling stategy to achieve effective fluorescent labeling of nucleic acids in which diamino molecules played an essential linker role to bridge nucleic acids and fluorophores (Scheme 1C). Similar phosphoramidation reactions can be extended to covalently link molecules other than fluorophores to nucleic acids and to prepare diverse nucleic acid conjugates. Moreover, the indirect coupling strategy based on the two-step phosphoramidation reaction is not restricted to annexing a new amino group to nucleic acids for subsequent conjugation. For example, in our recent study of the two-step phosphoramidation reaction for preparation of POCs, we have demonstrated that the heterobifunctioal cross-linker *N*-(maleimidocarboxyl)succinimide ester could serve a crucial linker role and introduce a maleimide group to nucleic acids for later Michael addition between sulfhydryl-containing peptides and maleimide-containing nucleic acids to attain POC synthesis [26]. In addition, we are actively developing different useful heterobifunctional cross-linkers to further empower the two-step phosphoramidation reaction for more effective and bioorthogonal labeling of nucleic acids critical to studying structures and functions of DNA/RNA *in vivo*. We have already succeeded in employing the two-step phosphoramidation reaction to effectively and efficiently incorporate either alkynyl or azido groups into nucleic acids, and to acquire important functionalized nucleic acids' potential for engaging in bioorthogonal reactions such as copper-free click chemistry [33–35] and Staudinger ligation [36] *in vivo* (Su et al., manuscript in preparation). Detailed accounts of the research will be provided in an upcoming report.

Both of the two-step phosphoramidation-based direct and indirect coupling reactions (Scheme 1C) have the essential characteristics to meet the demand for effective and site-specific labeling of nucleic acids with tag molecules. However, each nucleic acid labeling strategy has distinct practical advantages and disadvantages. The choice of a coupling strategy hinges on the specific demands of a nucleic acid labeling experiment. As discussed above, the indirect coupling strategy (Scheme 1C) can significantly expand arrays of functional groups introduced into the nucleic acid and can easily calibrate nucleic acid reactivity amiable to biocompatible and bioorthognal reactions essential to studying nucleic acid *in vivo*. Moreover, if effectively labeling precious small subtrates to nucleic acids is a key issue, it would be better to employ the indirect coupling strategy for preparing the labeled nucleic acid because the indirect coupling strategy uses a molar excess of substrates (257) less than that of the zero-linker direct coupling approach (585). It is noted that, even though the indirect coupling strategy requires an additional reaction to chemically attach bifunctional cross-linkers (bridging molecules) to nucleic acids as the first step, the direct and indirect coupling strategies provide similar efficacy and F/N ratio values as demonstrated in the current study on fluorescent labeling of nucleic acids (Table 1). The success of the indirect coupling strategy is greatly aided by the use of diamino homobifunctional cross-linkers such as ethylenediamine which generally offers over a 70% yield in the two-step phosphoramidation reaction. The excellent yield of diamino cross-linker-labeled nucleic acids in the two-step phosphoramidation reaction is again due to large positive charge density of diamino substrates which facilitates a strong electrostatic interaction in nucleic acid-diamino substrate pairs and accelerates product formation.

However, if time is a major concern, the direct coupling approach (Scheme 1C) is preferred because, as in the case of one-step phosphoramidation reactions, the zero-linker direct coupling strategy is far more efficient than the indirect coupling method, only requiring 4.5 h of the overall reaction time. As noted, one of the drawbacks of the direct coupling strategy is the required higher molar excess of substrates in the two-step phosphoramidation reaction. However, the time-efficient advantage of the direct coupling method in the two-step phosphoramidation reaction can outweigh the shortcomings of more substrate use and be more competitive than the indirect coupling counterpart. Furthermore,

the unreacted and valuable label molecules may be recovered by chromatographic methods for later reuse.

In summary, we have successfully exploited the two-step phosphoramidation reaction to develop a universal postsynthesis approach capable of effectively and site specifically labeling nucleic acids with tag molecules. Legitimacy of the approach was explicitly demonstrated by effective and site-specific fluorescent labeling of nucleic acids reported in this study. In addition, by either the direct or the indirect coupling strategy, the two-step phosphoramidation reaction is in an excellent position to incorporate any functional group to the 5′ phosphate and to significantly expand attainable functionalities in postsynthesis nucleic acids. Therefore the phosphoramidation reaction is a truly versatile platform for effective site-specific nucleic acid labeling and conjugation which will have broader applications in the study of nucleic acid structures and functions *in vivo* and *in vitro* in the future.

Acknowledgments

We thank Dr. Susan Fetzer for critically reading the manuscript. We also thank Dr. Chi-wi Ong for providing expertise and assistance in determining UV melting profiles of DNA. Finally, we acknowledge technical and instrumental assistance of Center for Research Resources and Development of KMU. This work was supported with Grants from the National Science Council of Taiwan awarded to T.-P.W. (100-2113-M-037-008- and 101-2113-M-037-005-). This work was also supported with a Grant from the Promotion Program for Commercialization of Biotechnology, Ministry of Economic Affairs, Taiwan (10121101033-10103) allocated to T.-P.W. M.H.W. is supported by the undergraduate research program funded by National Science Council of Taiwan (101CFD2100044).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.12.021.

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