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

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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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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).