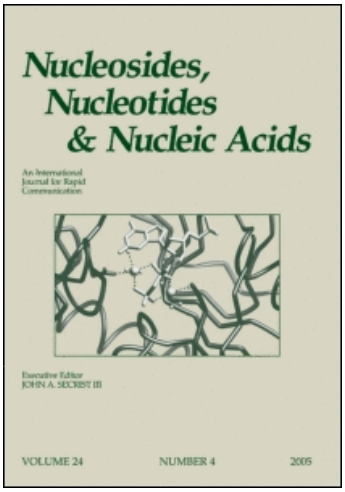

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SYNTHESIS AND STABILITY OF NOVEL TERMINAL PHOSPHATE-LABELED NUCLEOTIDES

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□ *Novel compounds consisting of a nucleotide triphosphate labeled with a PEG linker and various terminal groups attached to the γ-phosphate of the nucleotide were constructed for use in efforts to produce a new class of DNA sequencer. The stability of these novel compounds was investigated to determine their utility as sequencing reagents. Hydrolysis rate constants were measured for both the natural nucleoside triphosphate dATP and novel dATP derivatives. The γ-labeled dATP was approximately 20-fold more stable to hydrolysis than dATP.*

Keywords PEG: Phosphate-labeled nucleotide; Single-molecule sequencing

Single-molecule DNA sequencing is an area of research that has been explored for decades in an attempt to produce a high-throughput, low cost sequencing methodology. Several different avenues of research on single-molecule DNA sequencing are being explored, including sequencing by synthesis, labeling each base and detecting the label after incorporation[1,2]; sequencing by degradation, producing a fully labeled DNA strand that is later degraded to identify each label[3–5]; sequencing through nanopores, using a current to move unlabeled DNA through a nanometer-sized pore and detecting alterations in the current corresponding to the sequence of the DNA[6–9]; and sequencing by direct detection, using scanning tunneling microscopy or other forms of microscopy to directly probe a DNA strand and determine the sequence.[10,11] Multiple groups, including ourselves, are focusing on using a modified form of sequencing by synthesis to achieve single-molecule sequencing.[12,13] Rather than utilizing nucleotide triphosphates labeled directly on the base for sequence identification, we

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are investigating the use of terminal phosphate-labeled nucleotides in our single-molecule sequencing experiments.[14]

Using nucleotides that contain a fluorescent moiety attached to the terminal phosphate offers a distinct advantage over directly labeling the nucleotide base. As the terminal phosphate labeled nucleotide is incorporated into the growing DNA strand, the phosphodiester bond formation between the 3′-hydroxyl terminus of the DNA strand and the α-phosphate of the incoming deoxyribonucleoside triphosphate releases the labeled pyrophosphate and results in the formation of an unmodified DNA strand. This DNA extension reaction provides an opportunity to visualize and identify the incoming nucleotide, either directly before or after phosphodiester bond formation and release of the labeled pyrophosphate. The release of the labeling moiety and subsequent formation of an unmodified DNA strand provides an opportunity to generate long DNA strands and thus long sequencing reads. Long sequencing reads from base-labeled DNA, on the other hand, are difficult to generate, with the high label content having the tendency to alter the properties of the DNA strand, resulting in insolubility of the highly labeled strand.[15]

Novel chemistry was needed to generate labeled nucleotides for use in our single-molecule sequencing efforts, including the new synthesis of the linker and dye complexes attached to a nucleotide triphosphate. For these synthesis and stability studies, dATP conjugates were constructed with polyethylene glycol (PEG) linkers, with and without the fluorophore tetramethylrhodamine (TAMRA), attached to the γ-phosphate position. Hydrolysis rates were calculated for both natural nucleotide triphosphate dATP, and the novel dATP derivitized compounds. The stability of the γ-phosphate-labeled dATP was increased by approximately 20-fold over that of the natural nucleotide due to the addition of the PEG linker. Magnesium was also discovered to have a stabilizing effect on the new terminal phosphate labeled compounds. This stabilizing effect may be due to a reduction in the metal ion promoted hydrolysis of the β-γ phosphoanhydride of the modified dATP, because of the addition of the PEG linker to the γ-phosphate of the nucleotide.

METHODS

Instrumentation

All HPLC analyses were conducted using the Agilent 1100 system (Foster City, CA). All preparative work was done using the Varian PrepStar system (Varian, Palo Alto, CA). LC/MS and MS analysis was done using an Agilent 1100 system coupled to an Agilent SLP ion trap, using electrospray ionization for the ion source. UV-Vis spectra were obtained on an HP8453

spectrophotometer. NMR spectra were obtained on a Bruker Avance DRX system, 500 mHz with a TXI cryoprobe (Bruker BioSpin, Ettlingen, Germany). Dephosphorylation rates were calculated using data collected over time by ion-exchange chromatography and analyzed using an in-house iterative kinetics program.

PEG8-diamine (2)

BOC-amino PEG8 amine (2 g) (**1**), purchased from PolyPure (Oslo, Norway), was added to a trifluoroacetic acid/chloroform solution (1:1 v/v, 20 mL total). Reaction was stirred at room temperature for 2 hours. $NaHCO_3$ (10 mL, 10 mM) was slowly added to the reaction. The aqueous layer was separated from the organic layer and retained. Chloroform was added and two extractions (20 mL each) were performed. To the aqueous layer, ethyl ether (20 mL) was added and the extraction was performed twice. The aqueous layer was dried in vacuo. The final product was a clear oily residue, and weighed approximately 1 g. Yield was estimated at less than 100% due to possible salts and water still present in sample. ^{1}H NMR, D2O: δ 3.675 (t, 4H), 3.61 (m, 24H), 3.1 (t, 4H). ESI MS [M + 1] calculated 369.3. Observed [M + 1] 369.2.

dATP-PEG8-amine (4)

13.3 mg dATP (**3**) from Sigma (24.8 μmol, 1 equiv) and 190.6 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) from Aldrich (992 μmol, 40 equiv) were added together in 500 mM MES buffer, pH 5.8, resulting in concentrations of 79 mM and 3.2 M, respectively. The reaction proceeded at room temperature for 10 minutes. A PEG8-diamine (**2**) solution (750 mM, 186 μmol, 7.5 equiv) in 500 mM MES buffer, pH adjusted to 6, with 5 M KOH was added to the dATP/EDC (248 μL). The reaction proceeded at room temperature overnight. The large excess of reagents and overnight reaction converted the entire amount of starting material to desired product. The product was first purified on a HiPrep DEAE 20 mL column from Amersham with buffers A and B. Buffer A: 10 mM phosphate + 20% acetonitrile (ACN) (Fisher Scientific) and buffer B: buffer A containing 1 M NaCl. LC conditions: 0–10 minutes 0% B, 10–15 minutes 0–100% B, 15–20 minutes 100% B at a flow rate of 10 mL/minutes. The free PEG eluted from the column in void volume. The nucleotide conjugate product eluted as a broad peak at 5–10 minutes. This broad peak was collected and dried in vacuo. The product was further purified on a 30 × 250-mm 10-μm Inertsil ODS-3 column from Varian, with a new buffer system A and B, where buffer A was 100 mM triethylaminoammonium acetate (TEAAc) (Glen Research) (pH 6.6) with 4% ACN, and buffer B was 100 mM

TEAAc (pH 6.6) with 80% acetonitrile. LC conditions: 0–5 minutes 0–15% B, 5–10 minutes 15–20% B, 10–20 minutes 20–100% B with a flow rate of 20 mL/minutes. The product that eluted at 12 minutes was collected and then dried in vacuo. Yield averages 30% after purification steps. ^{1}H NMR D2O: δ 8.52(s,1H), 8.27 (s, 1H), 6.5 (t, 1H), 4.3 (t, 1H), 4.2 (m, 1H), 4.1 (m, 1H), 3.8(m, 29H overlaid with contaminant), 3.2 (t, 2H + contaminant), 3.1 (m, 2H+ contam.), 2.8 (m, 1H), 2.6 (m, 1H). Contaminants present in sample were TEAAc and glycerol. This particular sample was used only for NMR; no further synthesis was performed with this particular lot. ESI-MS [M+1] calculated 842.25, observed [M+1] 842.3.

dATP-PEG8-TAMRA (6)

dATP-PEG8-amine (**4**) was reconstituted in 20–50 μL water, depending on the scale of the reaction. Product was then quantitated using an extinction coefficient of 15,300 cm^{-1} M^{-1} at 260 nm. The extinction coefficient that was used was the extinction coefficient for ATP. No experiments were conducted to verify the extinction coefficient of the desired product. dATP-PEG8-amine (**4**) (95 nmol, 5 μL, 1 eq), 50 mM phosphate buffer, pH 8 (29 μL), and 5 (6)TAMRA-X SE (**5**) purchased from Molecular Probes (140 nmol, 1.2 eq, 9 μL at 10 mg/mL in DMF) were added together. The reaction proceeded at room temperature for 2 hours. Purification of desired product was carried out using a 30 × 250-mm 10-μm Inertsil ODS-3 column from Varian. Buffer A: 100 mM TEAAc, pH 6.6–6.8, 4% ACN. Buffer B: 100 mM TEAAc, pH 6.6–6.8, 80% ACN. LC conditions: 0–2 minutes 0% B, 2–10 minutes 30–38% B, 10–15 minutes 38–50% B, 15–20 minutes 50–100% B at 20 mL/minutes. Desired product eluted as two peaks due to the two isomers present. The products eluted at 10 and 12.6 minutes were collected and then dried in vacuo. Yield for reaction was 60% with combined isomers. ESI-MS calculated [M + 1] 1367.5. Observed [M + 1] 1367.5.

dATP-PEG8-Ac (7)

570 nmol of dATP-PEG8-amine (**4**) was reconstituted in 0.5 M MES, pH 6, buffer to a concentration of 25 mM (22.89 μL, 1 eq). EDC purchased from Aldrich (57 μmol, 100 eq, 17.8 μL) was dissolved to a final concentration of 3.2 M using same buffer. Glacial acetic acid solution from VWR was made at a 1:50 dilution (57 μmol, 10 eq, 16.3 μL). The EDC and acetic acid were added together. The dATP-PEG8-amine (**4**) was added to the EDC/acetic acid solution. Reaction proceeded at room temperature for 30 minutes. LC was used to check the status of the reaction. No starting material was present after 30 minutes.

Product was purified using 30 × 250-mm 10-μm Inertsil ODS-3 column from Varian. Buffer A: 100 mM TEAAc, pH 6.6–6.8, 4% ACN. Buffer B:

100 mM TEAAc, pH 6.6–6.8, 80% ACN. Gradient set at 0–5 minutes 0–15% B, 5–10 minutes 15–20% B, 10–20 minutes 20–100% B at 20 mL/minutes. The product eluted at 12.6 minutes and was collected and then dried in vacuo. Reaction yielded 30% desired product after purification. ESI-MS calculated [M − 1] 882.2. Observed [M − 1] 882.9.

PP_i-PEG8-TAMRA (8)

Snake venom phosphodiesterase I (USB) (32 units/mL) stored in a solution of 100 mM Tris–Cl, pH 8.9, 100 mM NaCl, 150 mM MgCl2, 50% glycerol, was diluted 1:10 in a solution of 100 mM HEPES, pH 7.4, buffer.

dATP-PEG8-TAMRA (**6**) was reconstituted in water at a concentration between 1 and 8 mM. dATP-PEG8-TAMRA, 15 μL, (**6**) was added to 85 μL of the 10% phosphodiesterase I solution (PDA I) (USB). The reaction proceeded at 37°C for 1 hour. Complete conversion of starting material to desired product occurred under these conditions. Product was first purified using a SWIFT mololithic DEAE 10 × 100-mm column from Teledyne-ISCO. Buffer A: 20% aq ACN. Buffer B: 1 M LiCl, 20% aq ACN. Gradient set at 0–5 minutes 0% B 5–20 minutes 0–37.5% B 20–21 minutes 37.5–100% B 21–31 minutes 100% B at 1 mL/minutes. Product eluted at 15.7 minutes, which is the same time as starting material. This purification was done to eliminate any PEG8-TAMRA degradation product. Product was dried in vacuo. A second purification was done on a 10 × 150-mm C18 5-μm Polaris column from Varian. Buffer A: 100 mM TEAAc, pH 6.6–6.8, 4% ACN. Buffer B: 100 mM TEAAc, pH 6.6–6.8, 80% ACN. LC conditions: 0–20% B for 0–2 minutes, then 2–25 minutes 20–100% B, 3 mL/minutes. The product eluted as a broad peak at 7.85 minutes and was collected and then dried in vacuo. Calculated yield after purification was 23%. ESI-MS calculated [M + 1] 1054.4. Observed [M + 1] 1054.6.

P_i-PEG8-TAMRA (9)

PP_i-PEG8-TAMRA (**8**) made as before, except the buffer used for dilution was 20 mM Tris-Cl, pH 8.0, 10 mM MgCl2 (final concentrations). The PDA was heat killed at 65°C for 15 minutes. PP_i-PEG8-TAMRA (**8**) product does not need to be isolated for this experiment. 1 μL of Shrimp Alkaline Phosphatase (SAP, USB) stock enzyme solution was added to 25 μL of the PP_i-PEG8-TAMRA (**8**) reaction. The reaction proceeded for 5 minutes at 37°C. Product was first purified using a SWIFT mololithic DEAE 10 × 100-mm column from Teledyne-ISCO. Buffer A: 20% aq ACN. Buffer B: 1 M LiCl, 20% aq ACN. LC conditions: 0–5 minutes 0% B 5–20 minutes 0–37.5% B, 20–21 minutes 37.5–100% B, 21–31 minutes 100% B, 1 mL/minutes. The compound eluted at 10.8 minuutes and was collected and dried in vacuo. A second purification was done on a 4.6 × 250-mm 5-μm Inertsil

ODS-3 column from Varian. Buffer A: 100 mM TEAAc, pH 6.6–6.8, 4% ACN. Buffer B: 100 mM TEAAc,pH 6.6–6.8, 80% ACN. LC conditions: 0–5 minutes 0–15% B 5–10 minutes 15–20% B 10–20 minutes 20–100% B, 1 mL/minutes. The product eluted at 16.6 minutes and was collected and then dried in vacuo. ESI-MS calculated [M + 1] 974.4 observed [M + 1] 974.6.

Stability Analysis

Nucleotides and labeled phosphates were prepared as described above for use as starting compounds in the hydrolysis studies. Figure 1 shows both the starting compounds and the hydrolysis products relevant to this study. The starting compounds were dATP (**3**), dATP-PEG8-TAMRA (**6**), dATP-PEG8-Ac (**7**), PP_i-PEG8-TAMRA (**8**), and P_i-PEG8-TAMRA (**9**). Hydrolysis experiments were performed in the same buffer and at the same temperature as will be used for single-molecule sequencing. Samples contained: 20 mM Tris–Cl, pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, and 200 μM nucleotide or labeled phosphate compound. Incubation was at 74°C for up to 630 hours, and 10-μL aliquots were withdrawn for analysis at various time points. Different HPLC columns were used for different starting compounds to best resolve the hydrolysis products. The HPLC instrument was an Agilent 1100 with a UV-Vis detector. An observation wavelength of 260 nm was used for compounds that did not contain a fluorophore, while TAMRA containing compounds were observed at a wavelength of 540 nm. Chromatogram peaks were quantified by the HPLC software as the percentage of total absorbance summed across all of the peaks. Chemical structures were assigned based on HPLC of appropriate standards and/or mass spectrometry. Chromatography conditions were as follows:

1) *Ion exchange*. Samples were run on a 10 × 100-mm Teledyne-ISCO, SWIFT DEAE column at 1 mL/minutes using a gradient of buffer B in buffer A: 0–5 minutes of 0% B, 5–20 minutes of 0–37.5% B, 20–21 minutes of 37.5–100% B, 21–31 minutes of 100% B, where buffer A is 20% acetonitrile in water, and buffer B is 1 M LiCl, 20% acetonitrile in water. An alternative method used just for P_i-PEG8-TAMRA (**9**) was an isocratic elution using 3% buffer B at 1 mL/minutes.
2) *Reverse phase*. Samples were run on a 4.6 × 250-mm Varian Inertsil ODS-3 (5 μm) column at 1 mL/minutes using a gradient of buffer B in buffer A: 0–5 minutes of 0–15% B, 5–10 minutes of 15–20% B, 10–20 minutes of 20–100% B, where buffer A is 100 mM TEAAc, pH 6.6–6.8, 4% acetonitrile, and buffer B is 100 mM TEAAc, pH 6.6–6.8, 80% acetonitrile.

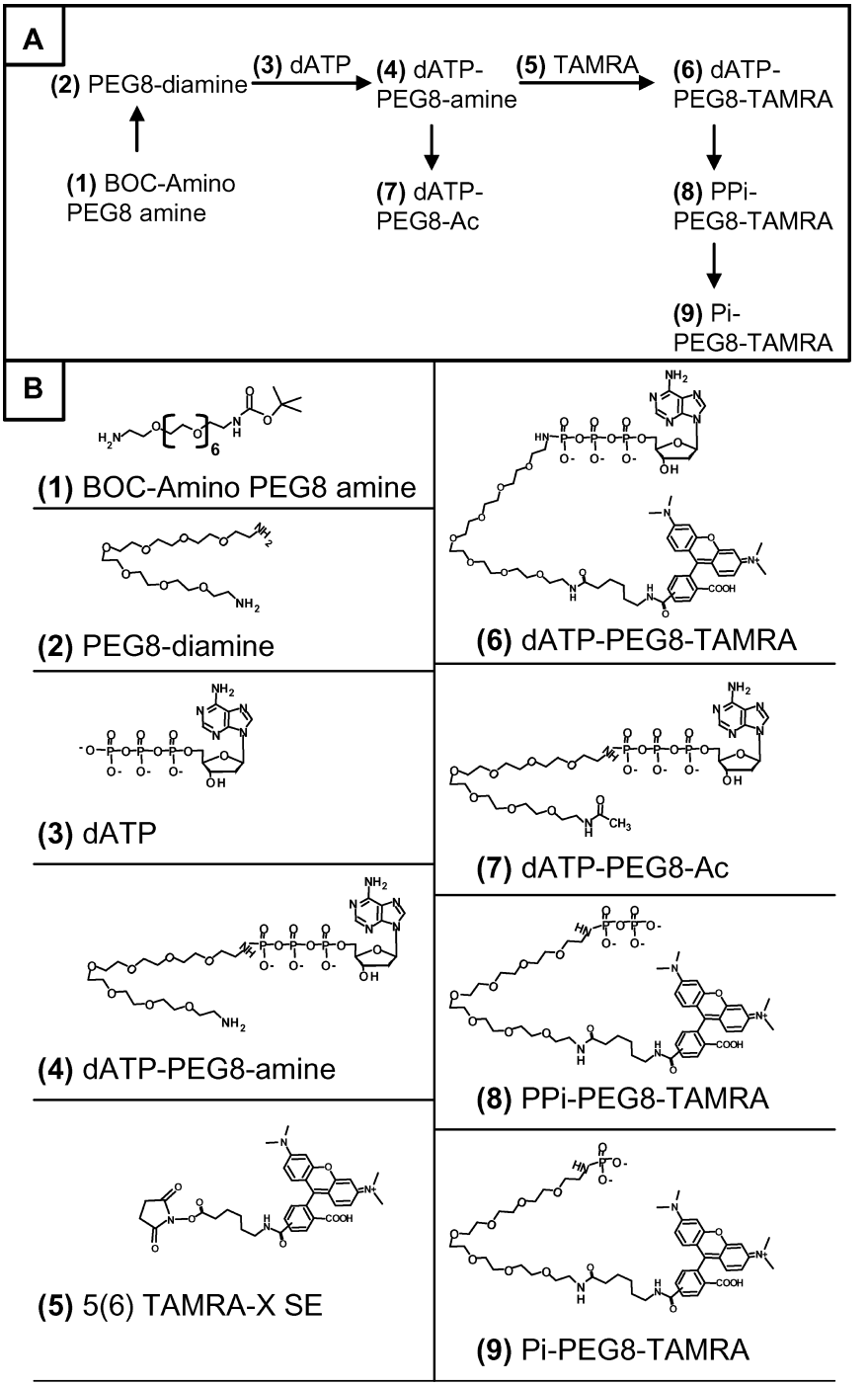

FIGURE 1 (A) Synthesis scheme for generating γ-phosphate fluorescently labeled dATP and additional related compounds for use in stability studies. (B) Structures of compounds relative to this study. Compounds **3**, **6**, **7**, **8,** and **9** were synthesized as starting materials for hydrolysis experiments.

3) *Reverse phase.* Samples were run on a 4.6 × 100-mm Varian Polaris (3 μm) column at 1.5 mL/minutes using a gradient of buffer B in buffer A: 0–2 minutes of 20% B, 2–25 minutes of 20–100% B, 25–30 minutes of 100% B, where buffers A and B are the same as specified above for the Inertsil column.

RESULTS AND DISCUSSION

Terminal Phosphate-labeled Nucleotide Synthesis

Our single-molecule sequencing strategy requires the utilization of terminal phosphate-labeled nucleotides.[14] The ability to attach a labeling moiety to the terminal phosphate of a nucleotide provides an opportunity to generate long, unmodified, DNA strands and thus long sequencing reads, due to the release of the pyrophosphate upon dNTP incorporation.

A strategy for attaching a fluorescent dye to nucleotides was devised, in which a PEG8 linker was used to connect the fluorescent moiety to the γ-phosphate of the nucleotide (Figure 1). PEG8 was chosen as the linker between the fluorophore and nucleotide, presumably providing enough length to extend the fluorophore out of the polymerase active site and into the surrounding solvent area. Extending the bulky fluorophore outside of the nucleotide binding pocket should help facilitate the incorporation of the modified nucleotide compounds.

During the synthesis of the fluorophore-labeled nucleotides, the nucleotide was first coupled to the PEG8 linker using an EDC reaction that takes place at the γ-phosphate. Weak anion exchange chromatography was used in addition to reverse-phase purification to remove any free PEG8 and to purify the desired product. Control experiments, however, later determined that excess PEG8 contaminating the PEG8-labeled nucleotide would have no effect on either DNA polymerase activity or on nucleotide hydrolysis rates (data not shown). Nucleotide conjugate synthesis continued following PEG8-labeled nucleotide coupling, with the addition of the TAMRA fluorophore. The fluorophore was directly attached to the PEG-nucleotide under normal conditions for NHS-ester coupling.

Aside from generating the labeled nucleotides, we were also ultimately interested in the stability of these new compounds under conditions that would be used with single-molecule sequencing. To more fully investigate the hydrolysis kinetics of these labeled nucleotides, we also generated and purified additional compounds for use in the stability studies. Snake venom phosphodiesterase I was used to generate labeled PP_i (PP_i-PEG8-TAMRA) (**8**), and HPLC chromatography was used to isolate the desired product for use in our stability studies. Snake venom phosphodiesterase I binds nucleotide triphosphates and cleaves the α-β phosphoanhydride bond[16]; fortunately, the PEG8 linker on the γ-phosphate did not prevent

the enzymatic cleavage, allowing for the isolation of the desired PP_i-PEG-TAMRA product. Labeled P_i (P_i-PEG8-TAMRA) (**9**) was synthesized for the nucleotide hydrolysis studies by alkaline phosphatase treatment of PP_i-PEG-TAMRA (**8**). Snake venom phosphodiesterase I cleaved the β-γ phosphoanhydride bond, but could not cleave the phosphoramidate (P-N) bond linking the γ-phosphate to the linker. Snake venom phosphodiesterase I treatment thus provided an easy route to the preparation of P_i-PEG-TAMRA (**9**) from PP_i-PEG-TAMRA (**8**).

Stability Studies

Stability studies of our labeled nucleotide conjugate as well as the conjugate by-products were performed to help determine the utility of this type of labeled nucleotide with the scheme of our single-molecule sequencing effort. The stability studies were performed at a temperature of 74°C, in a buffer solution containing Tris–Cl, pH 8.8 (at 20°C), KCl, $MgSO_4$, and Triton X-100, comparable to the conditions that would be used during DNA synthesis using a thermostable DNA polymerase.

Hydrolysis rate constants of compounds **3**, **6**, **7**, **8**, and **9** were determined using HPLC analysis and iterative curve-fitting software developed in-house for chemical kinetics analysis (unpublished) (Figure 2). For each starting compound, a stability calculation series was conducted. Ion-exchange chromatography was used to follow the progress of the hydrolysis reactions, with reaction timepoints analyzed to determine the relative percent of both the starting product and all hydrolyzed products (Figure 3). The chemical structures of the hydrolyzed products were assigned based on HPLC of appropriate standards and/or mass spectrometry. The area of each individual product peak was quantified using the Agilent 1100 HPLC software as a percentage of the total area under all peaks. Hydrolysis rate constants were estimated using the relative quantitation data collected for up to 630 hours for each of the compounds tested. The kinetics determination software iteratively fits curves to the collected data, converging the forward and reverse rate constants that are being tested. For a simple hydrolysis of dATP to dADP and then to dAMP, a total of 6 rate constants are included in the calculation. Using such kinetic pathways, the hydrolysis rates for dATP as well as the novel dATP conjugates were calculated (Figure 2).

Analysis of the data indicates that the hydrolysis of the γ-phosphate PEG8-linker labeled nucleotides (**7**) were approximately 20-fold slower than that of the natural dATPs (**3**) (Figure 2F), and the nucleotides labeled with the PEG8 linker and the TAMRA moiety (**6**) were hydrolyzed at a rate approximately 14-fold slower than the natural dATPs (**3**) (Figures 2A and B). The increased stability of the γ-labeled nucleotide may be due to a reduction in the metal ion promoted hydrolysis of the β-γ

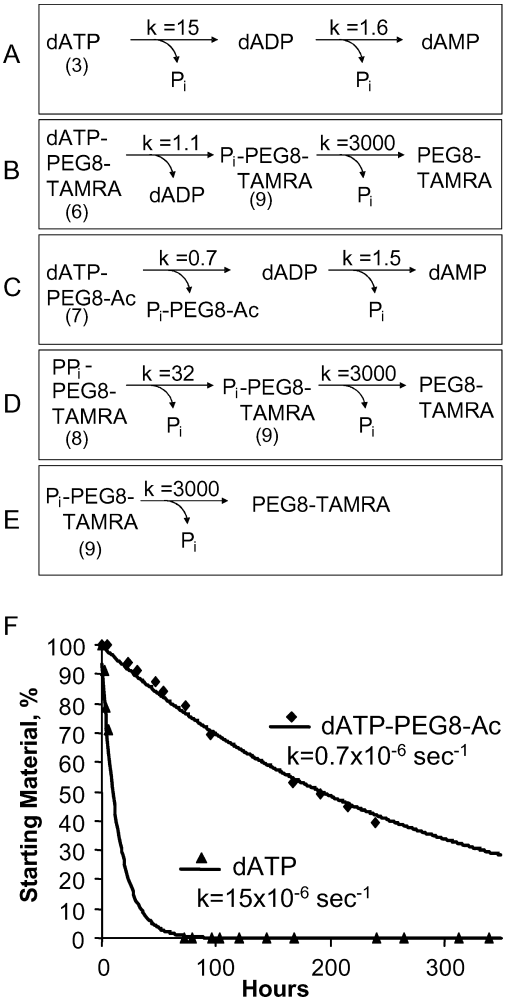

FIGURE 2 Estimated hydrolysis rate constants for starting compounds **3**, **6**, **7**, **8**, and **9**. (A–E) Hydrolysis rates determined at 74°C by HPLC analysis as described in Methods. Rate constants are indicated as the value listed $\times 10^{-6}$ s^{-1}. For example, "$k = 15$" in (A) indicates a hydrolysis rate of 15×10^{-6} s^{-1}, the exponential decay constant for hydrolysis of dATP to dADP. (F) Stability estimation measurements of dATP and dATP-PEG8-Ac are shown. A direct comparison of dATP and dATP-PEG8-Ac shows that the triphosphate is stabilized approximately 20-fold by the addition of the PEG8 linker to the γ-phosphate. Experimentally determined data points are shown as points on the graph, with fitted curves shown as solid lines. Rates for dATP hydrolyzing to dADP + P_i and for dATP-PEG8-Ac hydrolyzing to dADP + P_i-PEG8-Ac are indicated.

phosphoanhydride. Research on metal ion-assisted interactions that facilitate the dephosphorylation of nucleotides has shown that metal ions, such as Mg^{2+}, can catalyze an intermolecular attack at the γ-phosphate.[17–19] All of our stability studies were conducted in buffer solutions that contain $MgSO_4$, and under conditions that would be present during single-molecule sequencing experiments. Therefore, the Mg^{2+} metal in the buffer may be con-

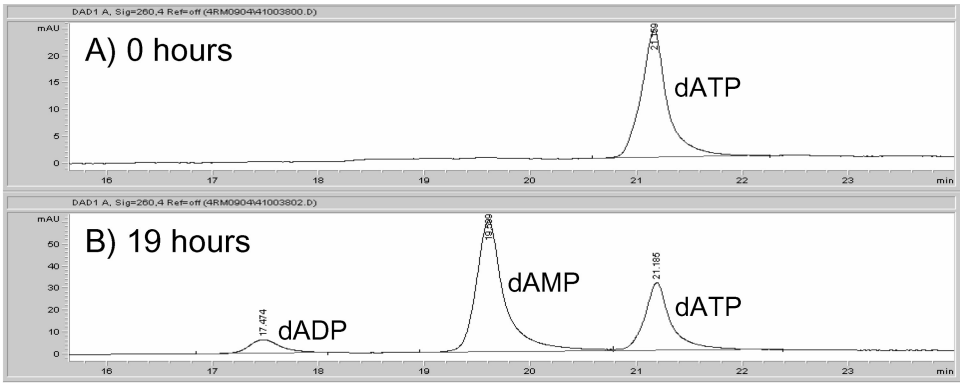

FIGURE 3 Stability timepoint chromatography analysis example. dATP hydrolyzes to dADP and dAMP after 19 hours incubation under buffer and temperature conditions specified in the Methods section. Analysis was by ion-exchange chromatography. Each individual peak area was quantified by the HPLC software (HP 1100) as a percentage of the summed peak areas.

tributing to the hydrolysis rates for the natural dATP (**3**). The γ-labeled nucleotides, on the other hand, may be better protected from the metal coordinated attack and removal of the γ-phosphate. Following the removal of the labeled γ-phosphate from the nucleotide, the rate of hydrolysis for the remaining dADP going to dAMP is nearly identical to that of the corresponding reaction occurring in the natural dATP stability study (Figure 2A, C) ($k = 1.6 \times 10^{-6}$ s^{-1} versus 1.5×10^{-6} s^{-1}).

Further stability studies were conducted to better assess the affect of Mg^{2+} on the hydrolysis rate of our γ-phosphate labeled nucleotide compounds (**4**). Stability time course experiments on dATP-PEG8-amine were performed at 74°C, in either our standard buffer solution (20 mM Tris-Cl, pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100), or the same buffer solution without $MgSO_4$. The results show that Mg^{2+} has a stabilizing effect on the γ-phosphate labeled nucleotide, reducing the hydrolysis rate of the compound by approximately 13-fold over the rate observed in buffer containing no Mg^{2+} (Figure 4). These results appear contradictory to research on the hydrolysis of the natural nucleotide, dATP, which shows that the addition of divalent metals actually increases the rate of dephosphorylation.[17,20,21] The stabilizing effect of the Mg^{2+} on the γ-phosphate labeled nucleotides may be due to a combination of charge shielding by the divalent metal and the large reduction in metal ion promoted hydrolysis. For natural nucleotides, the metal ion promoted dephosphorylation may overwhelm any stabilization due to charge shielding, thus resulting in an increase in the overall hydrolysis rate for dATP.

These results show that we can successfully generate γ-phosphate labeled nucleotides that are more stable than unlabeled dNTP's under the conditions utilized in our single-molecule sequencing platform. From

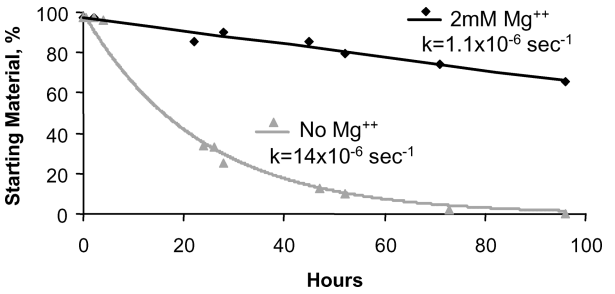

FIGURE 4 Direct comparison of dATP-PEG8-amine with or without the addition of Mg^{2+} to the buffer solution shows that the triphosphate is stabilized approximately 13-fold by the addition of the metal. Experimentally determined data points are shown as points on the graph, with fitted curves shown as solid lines. Rates for dATP-PEG8-amine hydrolyzing to dADP + Pi-PEG8-amine are indicated.

these initial results, we will proceed with generating multiple nucleotides that contain a variety of fluorescent moieties for use in single-molecule sequencing.

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