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The ULTIMATE Reagent: A Universal Photo-Cleavable & Clickable Reagent for the Regiospecific and Reversible End Labeling of Any Nucleic Acid

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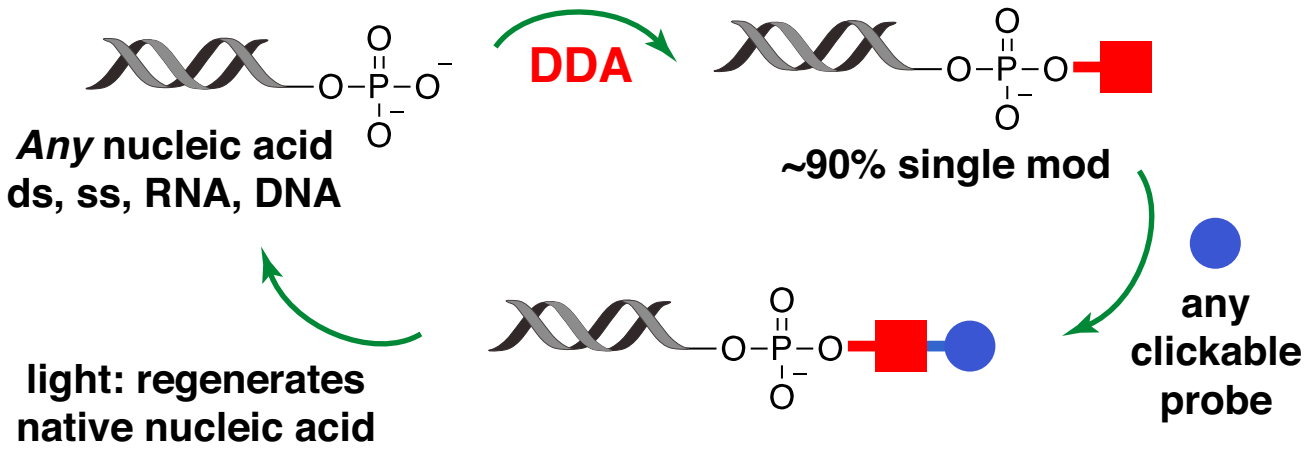
KEYWORDS: RNA, DNA, nucleic acids, end-labeling, photocleavage

ABSTRACT: There is a need for methods to chemically incorporate photocleavable labels into synthetic and biologically sourced nucleic acids in a chemically defined and reversible manner. We have previously demonstrated that the light cleaved diazo-DMNPE group (diazo di-methoxy nitro phenyl ethyl) has a remarkable regiospecificity for modifying terminally phosphorylated siRNA. Building on this observation, we have identified conditions by which a diazo-DMNPE reagent that we designed (diazo-DMNPE-azide or DDA) is able to singly modify *any* nucleic acid (RNA, DNA, single stranded, double stranded, 3' or 5' phosphate). It can then be modified with any clickable reagent to incorporate arbitrary labels such as fluorophores into the nucleic acid. Finally, native nucleic acid can be regenerated directly through photolysis of the reagent. Use of the described approach should allow for the tagging of any nucleic acid, from any source, natural or unnatural, while allowing for the light-induced regeneration of native nucleic acid.

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TOC Text: We have created a method whereby any terminally phosphorylated nucleic acid, whether single stranded or double stranded, and whether DNA, RNA or heteroduplex, can be modified. This modification can then be linked to any clickable probe. Finally, the modification can be cleanly removed using light to release native nucleic acid.

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INTRODUCTION

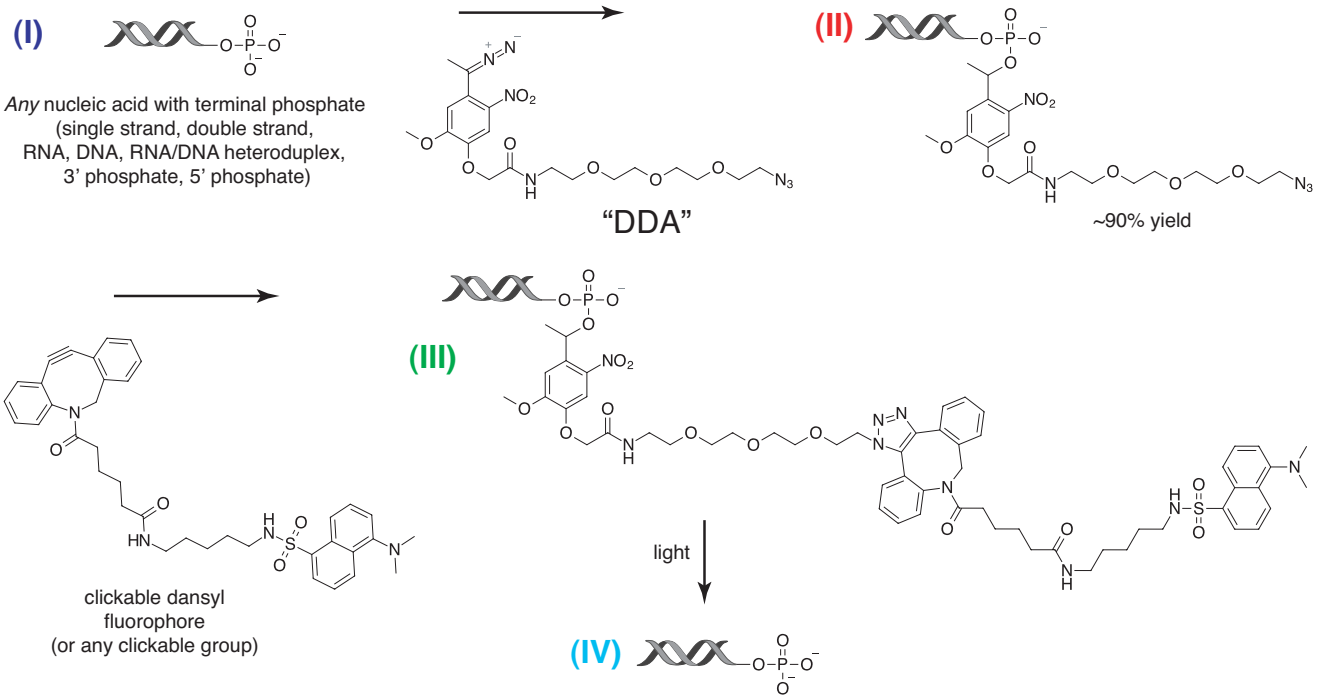
The convenient introduction of labels into nucleic acids is a central technique for a wide range of applications, including detection, capture, quantification, and delivery [1] Stimuli-responsive nucleic acid tags in particular, provide control and reversibility for understanding and manipulating biology.[2] In an early example of this Okamoto and coworkers used Bhc-diazo to post-synthetically modify the backbone of mRNA to control its activity with light.[3] Although nucleic acids are routinely modified *during* solid phase synthesis, only a few existing methods allow modification of nucleic acids post-synthetically.[1g, 1h, 4] Furthermore, few of these post-synthetic methods enable site-specific modifications or allow removal of tags with an external stimulus.[1e, 1o, 5]

For existing techniques there is no easily applicable and general way to specifically, and importantly *reversibly*, introduce chemical labels into naturally occurring nucleic acids, or synthetic nucleic acids once solid phase synthesis is completed. In this work, we describe a new method of efficiently end labeling any nucleic acid, which introduces an azide containing group that can then be linked with any 'clickable' label. In addition, this group can be cleanly removed through photolysis, yielding native nucleic acid.

This approach is based on our earlier observation that the diazo form of the dimethyl nitro phenyl ethyl group (diazo-DMNPE) has a remarkable regiospecificity in siRNA for terminal phosphates over internal phosphates or nucleobases.[6] We observed this during experiments to modify siRNA and dsRNA with photocleavable groups to bring RNA interference under the control of light. We demonstrated that diazo-DMNPE would incorporate very poorly into siRNA without terminal phosphates, but would install four DMNPE groups into siRNA with four terminal phosphates. This was further confirmed using model nucleotides and MS/MS analysis of the products.[6a] These showed conclusively that the diazo-DMNPE group reacted rapidly with terminal phosphates, and could be cleanly installed into an siRNA, wherever a terminal phosphate was present.

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Scheme 1. Complete reaction cycle of nucleic acid using DDA: alkylation, click reaction, photolysis. Phosphorylated nucleic acid (I) reacts with DDA to form adduct (II). This adduct is reacted with a clickable group to form (III). This product can then be photolyzed to reform the starting nucleic acid (IV).

We recently created a version of DMNPE with a PEG linker terminated with an azide group (diazo-DMNPE-azide or DDA) to allow insulin to be linked to an insoluble resin, and then released with light (from what we call a photoactivated depot or PAD).[7] We subsequently realized that this reagent can also be used to label nucleic acids using the regiospecificity of DMNPE that we previously discovered. This reagent can be used to modify any nucleic acid that contains a terminal phosphate. It can then be reacted with any clickable label. And finally, the native nucleic acid can be released from the label using light. This reaction cycle is summarized in Scheme 1. In the work described in this article, we identify universal conditions by which this reagent singly modifies *any* nucleic acid, not just siRNA. Specifically, we demonstrate the installation of a single DDA into a wide range of nucleic acids, including dsRNA, dsDNA, ssRNA, ssDNA, and RNA/DNA heteroduplex. We show this works

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efficiently to allow both 3' and 5' terminal phosphate modification. In addition, we demonstrate that the DDA-modified nucleic acids can be linked to reactive labeling moieties, via a click reaction. Finally, we show that the original native nucleic acid can be regenerated through photolysis of the link between the reagent and the phosphate group. This reagent exhibits most of the needed properties of a universal cleavable nucleic acid labeling reagent and we have therefore named it the ULTIMATE reagent (for Universal Light-cleaved TerminI-Modifying And TagablE reagent).

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RESULTS AND DISCUSSION

DDA was prepared as previously described.[7] Briefly, the precursor carboxylic acid was condensed with an amino-PEG-azide.[8] This was then converted to the hydrazone using hydrazine, and finally to the diazo form via oxidation with MnO_2. Our aim was to find conditions that allowed labeling of any type of nucleic acid, so we examined and optimized conditions using a specific 20-mer sequence that contained an equal mixture of all four bases randomly distributed. We examined this sequence in the form of duplexes (RNA/RNA, RNA/DNA, DNA/RNA, DNA/DNA) and single strands (RNA, DNA). With each of the species, we created three sub-species which incorporated a single phosphate on the 5' terminus, the 3' terminus or incorporated no terminal phosphate. This gave a total of 18 different nucleic acid species examined. The purpose of examining a non-phophorylated species was to confirm our previous observation that the terminal phosphate was required for reaction in all examined species.

The modification was performed by reaction of the DDA with the individual nucleic acid species. We found that previously described conditions that worked effectively with siRNA did not work for all nucleic acid species, in particular single stranded species. Specifically, we observed over-reaction with single stranded species using our previous conditions. We have previously shown that higher modifications are due to reactions at nucleobase sites. This makes sense as the nucleobases are more exposed in single strands than double strands. Our aim was to have a single set of conditions that can be universally applied to all nucleic acids, so we optimized conditions, examining the level of modification in screening experiments using mass spectrometer signals. While direct MS signals can't be used to determine absolute levels of different species, they are effective in examining relative changes in the synthesis of different species. The ultimate yields of reactions with different nucleic acid species was determined by HPLC and MS analysis.

The factors optimized include the buffer constituents, pH, presence and concentration of $MgCl_2$, the presence and concentration of DMSO and the mole ratio of DDA to nucleic acid species. These

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optimization experiments are detailed in the supporting information (figure S5-S19). The results from representative studies are shown in Figure 1.

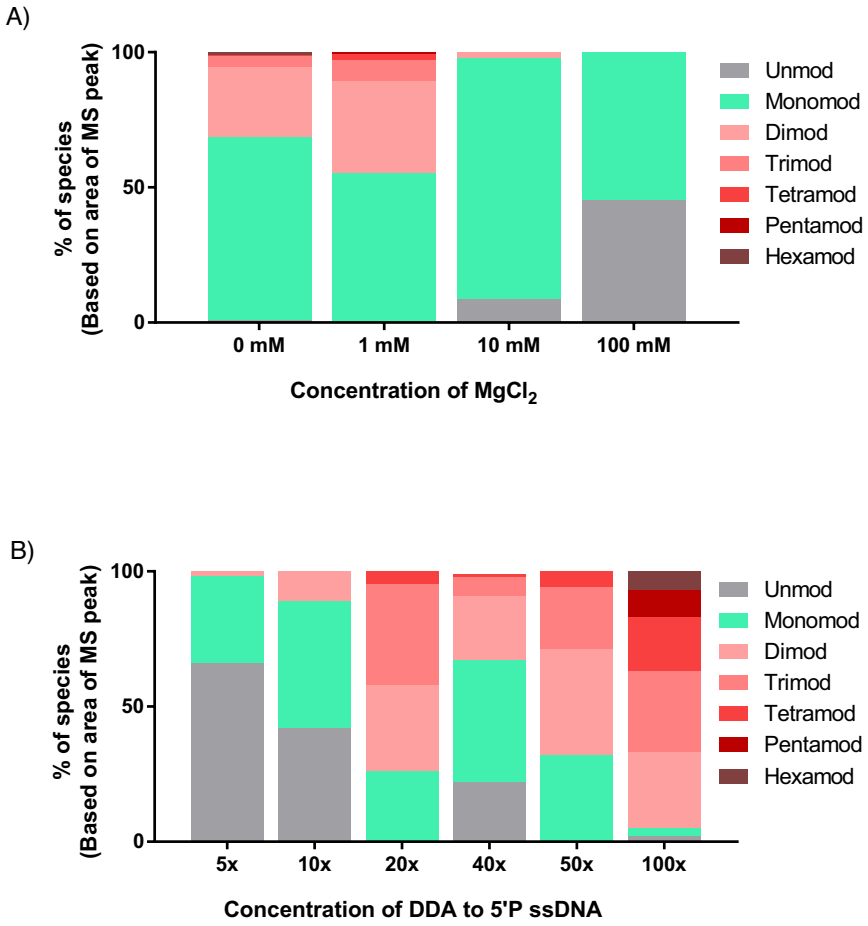


Figure 1. Example optimization experiments. A) Effect of changing concentrations of $MgCl_2$ during the reaction of 10 mM DDA with ssDNA containing 3' phosphate oligonucleotide (250 μM). Solution conditions were 10 mM Tris-HCl, 1 mM EDTA, pH 7.6, 25% DMSO. 10 mM $MgCl_2$ was found to be optimum for single modification. B) Different level of modifications observed in the MS for various concentrations of DDA reacted with 25 µM of ssDNA oligonucleotide, containing 5' phosphate in 25% DMSO/water, reacted for 24 h.

In figure 1a we examined the effect of $MgCl_2$ concentration on the levels of modification of 3' phosphorylated single strand DNA. We found that low $MgCl_2$ allowed multiple modifications in single stranded species and higher $MgCl_2$ suppressed them. The optimum of 10 mM $MgCl_2$ was incorporated

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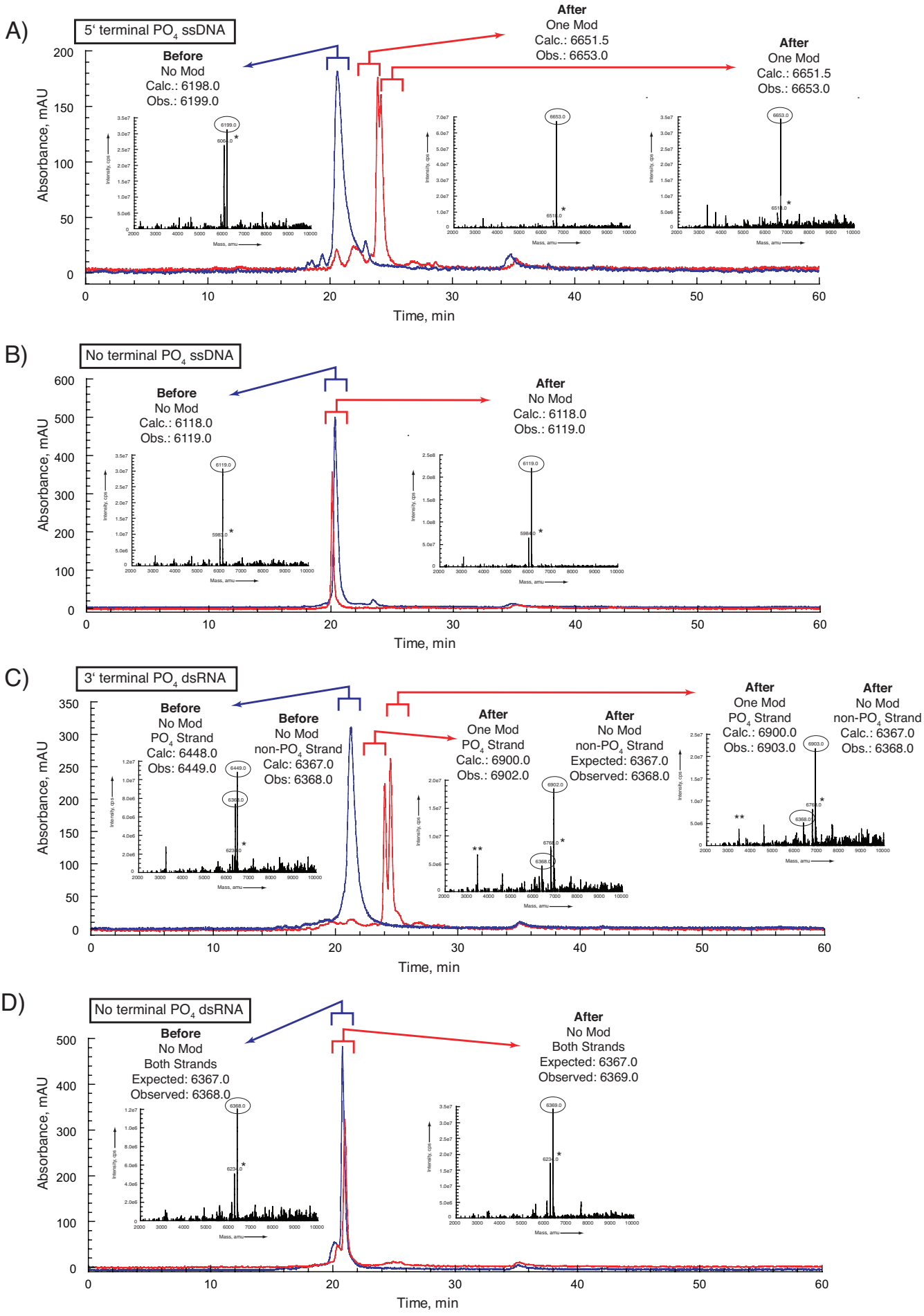
in the final reaction conditions. In figure 1b, we examined the effect of DDA stoichiometry on the levels of modification under early, unoptimized conditions.

Through this process of optimization, we identified conditions that resulted in 80-97% singly modified nucleic acid, independent of the nucleic acid species. The final conditions are 10mM tris-HCL, pH 6, 1mM EDTA, 10mM $MgCl_2$, 200μM nucleic acid, 20% v/v DMSO and DDA (3mM with single stranded nucleic acids, 5.3mM with double stranded). Using the optimized conditions, we analyzed the reaction of all 18 species with DDA in greater detail using HPLC and ESI-MS, as previously described.[9] Two representative reactions are depicted in figure 2, specifically 5' phosphorylated single stranded DNA, and 3' phosphorylated double stranded RNA. With both of these examples, the reaction of the identical non-phosphorylated species is also shown, to illustrate the requirement of the terminal phosphate for reaction. These results are typical of all 18 nucleic acid species, which are detailed in supporting information. For example, in figure 2A, the reaction of 5' phosphorylated ssDNA, we observe a single major HPLC peak of the starting material that has a molecular weight consistent with the unmodified single strand (blue chromatogram). Upon reaction with DDA, we observe near-quantitative consumption of the starting material peak, and conversion to a tight cluster of two, nearly identical height peaks (red chromatogram). These both give masses that are consistent with the mass of the singly modified product. We interpret these two peaks as being the diastereomers formed upon alkylation of the terminal phosphate. We have previously observed similar diastereomers in examining the reaction of DMNPE with model nucleotides.[6a]

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Figure 2. Examples of specific reaction of DDA with nucleic acid terminal phosphates. A/B) Reaction of single stranded DNA with (A) and without (B) a 5' terminal phosphate. C/D) Reaction of double stranded RNA with (C) and without (D) a 3' terminal phosphate. Blue traces show the nucleic acid before reaction, and red traces after. Inset are the mass spectra of each of the indicated regions. Asterisks * indicate commonly observed -135 depurination peaks. As described in the text, double peaks are likely due to diasteriomers that form during the modification reaction. All HPLCs monitored at 260nm.

In figure 2B, the paired, non-phosphorylated ssDNA species shows no consumption of the starting material, and no significant formation of singly modified species. This is because the terminal phosphate group is required for modification. This identical pattern of reactivity is illustrated in figure 2C and 2D which details the reaction of double stranded RNA with and without a 3' terminal phosphate. Indeed, all 18 species examined show the same pattern, summarized in Table 1. They show the reaction of DDA with nucleic acids to form exclusively a singly modified species, but only when there is a terminal phosphate present. This product is formed in 80-97% yield, with higher yields being found with duplex reactants. In the absence of this terminal phosphate, we observe little to no modification, consistent with the terminal phosphate being required for reaction. This is also illustrated in the MS spectra of the terminally phosphorylated duplexes which typically show each of the two strands as separate masses. In these, upon reaction, only the phosphorylated strand shows modification by DDA.

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Nucleic Acid	Location of Terminal Phosphate	Major Species Observed in MS	Observed Mass of Major Species	Expected Mass of Major Species	Major Species Proportion of Total (% by HPLC)
ssRNA	5'	RNA+DDA	6903.0	6900.3	88.8
	3'	RNA+DDA	6903.0	6900.3	83.5
	None	RNA	6369.0	6366.8	96.5
ssDNA	5'	RNA+DDA	6653.0	6651.5	80.6
	3'	RNA+DDA	6653.0	6651.5	79.7
	None	RNA	6119.0	6118.0	100.
RNA/RNA	5'	**RNA**+DDA/RNA	6903.0/6370.0	6900.3/6367.8	93.5
	3'	**RNA**+DDA/RNA	6903.0/6369.0	6900.3/6367.8	96.3
	None	RNA/RNA	6369.0	6367.8/6367.8	96.6
RNA/DNA	5'	**RNA**+DDA/DNA	6903.0/6120.0	6900.3/6118.0	92.1
	3'	**RNA**+DDA/DNA	6904.0/6120.0	6900.3/6118.0	96.7
	None	RNA/DNA	6370.0/6119.0	6367.8/6118.0	100.
DNA/RNA	5'	**DNA**+DDA/RNA	6653.0/6369.0	6651.5/6367.8	95.0
	3'	**DNA**+DDA/RNA	6653.0/6369.1	6651.5/6367.8	92.1
	None	DNA/RNA	6119.0/6370.0	6118.0/6366.8	100.
DNA/DNA	5'	**DNA**+DDA/DNA	6653.0/6120.0	6651.5/6118.0	95.2
	3'	**DNA**+DDA/DNA	6653.0/6119.0	6651.5/6118.0	97.4
	None	DNA/DNA	6120.0	6118.0/6118.0	100.

Table 1. Summary of DDA reactions with 18 nucleic acid species: ssDNA, ssRNA, RNA/RNA, DNA/DNA, RNA/DNA and DNA/RNA all with either a 5' or 3' terminal phosphate or no modification. All sequences are 5'ACT GAT ACG TGT GCA CTC AG 3' except for RNA strands which contain U instead of T. The terminal phosphate is on either the 5' or 3' end of this strand. In double stranded species, the second strand is the exact complement, 5' CTG AGT GCA CAC GTA TCA GT 3', except for RNA strands which contain U instead of T. Bolding indicates the strand that contains the terminal phosphates in duplexes. DDA = diazo-DMNPE-azide.

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In addition, we took one of the nucleic acid species, 3' phosphorylated dsRNA, through a complete cycle of 1) DDA modification 2) click reaction with a fluorophore and 3) photolysis, regenerating native dsRNA, to demonstrate the efficiency of these transformations. These results are shown in figure 3. Again we see a single peak on the HPLC trace of the starting nucleic acid. Upon reaction, we see the doublet of singly modified diastereomers and no other products. This species was then reacted with a clickable fluorophore (dansyl-cadaverine modified DBCO). The DBCO moiety is a strained cyclo-octyne which allows for copper free click reactions.[10] The product gives a broad peak on HPLC with a mass that is consistent with the expected click product, indicating fluorophore attached to the oligonucleotidenucleotide. Finally, upon irradiation of this specie with a 365nm light source, the peak for the modified nucleic acid is consumed, and a peak with a retention time (and molecular weight) of the starting material is generated quantitatively.

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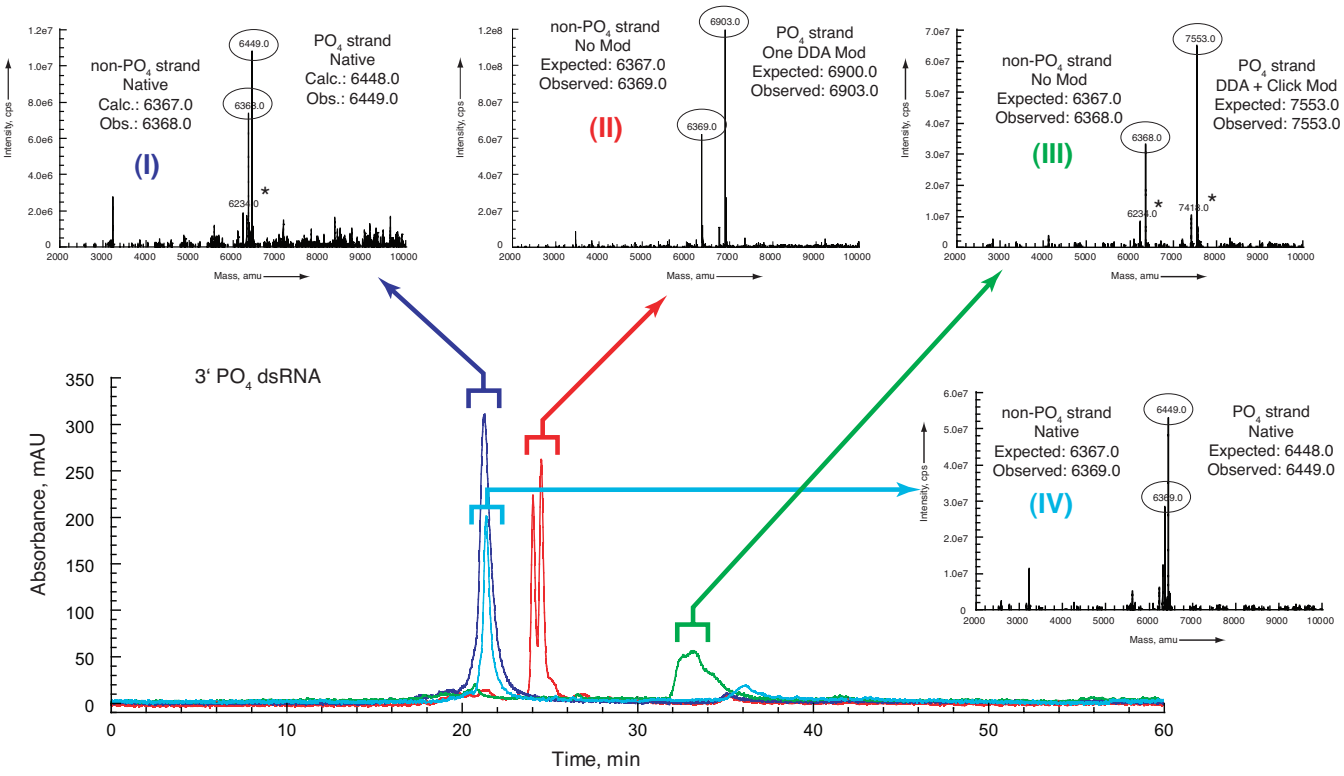


Figure 3. Complete reaction cycle of 3' phosphorylated, double stranded RNA. RNA (I) reacted with DDA to form (II). A dansyl derivative is clicked onto the DDA-modified RNA, to form (III). Photolysis regenerates native RNA (IV). Asterisks * indicate commonly observed -135 depurination peaks. All HPLCs monitored at 260nm.

Conclusions

In this work, we demonstrate a new method for incorporating any label into a defined terminal position in any nucleic acid. While there exist methods for labeling nucleic acids specifically, they typically depend on solid phase synthesis, so there is no way of doing this labeling on naturally occurring nucleic acids (e.g. pools of isolated mRNA, miRNA etc.) With our approach, even naturally occurring nucleic acids can potentially be primed through phosphorylation with polynucleotide kinase, for example. In labeling applications, the label itself has the potential to interfere with or confound the

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nature of the action of the nucleic acid of interest. With our approach, after the label has been specifically incorporated, it can be easily removed using light, generating native nucleic acid. As such it should enable a wide range of studies with nucleic acids, especially where pools of natural nucleic acids would benefit from visualization and isolation.

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MATERIALS and METHODS

Synthesis of dansylcadaverine-DBCO

DBCO acid (1 eq., 31.8 μmols, 10.6 mg), dansylcadaverine (2 eq., 63.6 μmols, 21.3 mg) and 1-hydroxybenzotrizole hydrate (2 eq., 63.6 μmols, 9.7 mg) were dissolved in DMF (97.1 μL). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.7 eq., 54.1 μmols, 10.4 mg) was then added to the reaction mixture and then stirred for 4 days. DBCO-DC amide product was purified using acid/base extraction. The product was purified by partitioning the reaction mixture between ethyl acetate (10 mL) and saturated sodium chloride (10 mL). The ethyl acetate layer was washed twice with saturated sodium chloride solution and the combined aqueous layers were washed once with ethyl acetate. The combined organic layers were washed with saturated sodium bicarbonate solution, 1N HCl and again with saturated sodium bicarbonate solution. The organic layer was then dried with magnesium sulfate and evaporated to give an off-white solid. Yield 17.5 mg (84.6%), TLC (EtOAc:MeOH, 75:25 v/v): RF = 0.55; 1H-NMR (400 MHz, DMSO-d6): δ 8.56 p.p.m. (d, J= 8.4 Hz, 1H), 8.42 (d, J= 8.4 Hz, 1H), 8.22 (d, J= 7.2 Hz, 1H), 7.64-7.54 (m, 3H), 7.49-7.47 (m, 4H), 7.37-7.30 (m, 3H), 7.28-7.26 (m, 2H), 6.75-6.71 (m, 1H), 5.11 (d, J= 14 Hz, 1H), 3.62 (d, J= 13.6 Hz, 1H), 2.94-2.83 (m, 2H), 2.87 (s, 6H), 2.26-2.20 (m, 2H), 1.87-1.79 (m, 4H), 1.39-1.16 (m, 10H); 13C-NMR (100 MHz, Acetone-d6): δ 172.8, 172.4, 153.2, 152.8, 149.6, 137.4, 133.4, 130.7, 130.6, 130.5, 130.3, 129.7, 129.6, 128.8, 128.8, 128.6, 128.4, 127.7, 126.0, 124.2, 124.0, 123.0, 120.5, 116.0, 115.3, 109.0, 55.8, 45.6, 43.7, 43.6, 39.2, 36.3, 35.1, 25.8, 29.8, 25.5, 24.4; UV/vis (DMSO): λmax (ελ): 291 nm, 310 nm ; MS (m/z): [M+H]+ calcd for C38H42N4O4S, 651.3; found, 651.2; Reversed phase HPLC-MS (flow rate 0.4 mL/min, runtime 30 min, injection volume 25 μL) solvent A (0.1% formic acid in H2O), solvent B (0.1% formic acid in ACN), gradient 0% B to 50% B over 5 min, 50% B to 100% B over 22 min, Isocratic 100% B for 2 min, 100% B to 0% B over 1 min, C8 Hypersil column (5μm, 100 x 4.6 mm, Varian): Retention time (min) 17.64; Reversed phase HPLC-MS (flow rate 0.4 mL/min, runtime 30 min, injection volume 25 μL)

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solvent A (0.1% trifluoroacetic acid in H2O), solvent B (0.1% trifluoroacetic acid in ACN), gradient 0% B to 100% B over 20 min, isocratic 100% B for 6 min, 100% B to 0% B over 2 min, isocratic 0% B for 2 min, C8 Hypersil column (5 μm, 100 x 4.6 mm, Varian): Retention time (min) 14.66; ESI-MS (m/z): [M+H]+ calcd for $C_{38}H_{42}N_4O_4S$, 651.3; found, 651.5;

Annealing of strands

Annealing of duplexes was performed by heating both the strands at 400 μM in RNase free water at 85°C for 15 min followed by slow cooling for about 2 hours.

Caging of oligonucleotides

Caging of oligonucleotides was performed by following this optimized protocol. Briefly, 2 µL of 100 mM Tris-HCl buffer at pH=6.0, containing 10 mM EDTA and 100 mM magnesium chloride was added to both single-stranded oligonucleotides and annealed double-stranded oligonucleotides (400 µM, 10 µL) and mixed by shaking. For single stranded oligonucleotides, 15 mM DMNPE-diazo in DMSO (4 µL) was added to each sample whereas for double stranded oligonucleotide, 26.5 mM of DMNPE-diazo in DMSO (4 µL) was added. Each sample was allowed to react for 1 h by shaking gently and followed by ethanol precipitation.

Precipitation of nucleic acids

Briefly, caging reactions were terminated by addition of ammonium acetate (9 M) to the mixture to achieve a final concentration of 3 M ammonium acetate. Samples were vortexed for 15 min. Added 2 μL of glycogen (20 mg/mL) and 3 volumes of ethanol and stored at -20oC for overnight. Centrifuged at 17,400 g for 30 min and washed twice with 75% ethanol. Pellet was dried in air and re-dissolved in RNase free water (100 μL). 10 μL of this sample was used for preparing samples for MS-analysis

ESI-MS infusion analysis of caged oligonucleotides

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ESI-MS analysis of all the oligonucleotides was performed by following earlier established protocol in our lab with some slight modifications. Briefly, caged dsRNA or siRNA solution stock (10 μL, ~35 μM) was diluted by addition of RNase free water (25 μL) and then triethylamine (TEA, 1 μL) and acetonitrile (ACN, 35 μL) were added to the solution to achieve a final concentration of 5 μM in a mixture of 35:35:1 (H2O:ACN:TEA). Samples were analyzed by mass spec (2000 QTrap, Applied Biosciences) by infusing (10-20 μL/min) in a negative ion mode with enhanced multiple charge (EMC) scan type. Finally, EMC spectra were deconvoluted using Bayesian protein reconstruct tool in the Bioanalyst software (AB Sciex).

HPLC analysis of samples

Caged oligonucleotides were purified by reversed phase HPLC using a Microsorb-MV 100-5 C8 column (250 x 4.6 mm, Varian). Solvent-A was 0.1 M triethylammonium acetate buffer (pH 7.0) and solvent-B was 50% ACN in solvent A. HPLC grade water (Fisher) was used to prepare the buffer. Flow rate was 1.0 mL/min. Gradient was 0% B to 40% B over 45 minutes and then increased to 100% B at 50 min and maintained at 100% B until 60 min. 50 μL injections (~50 μM) of DMNPE-azide caged oligonucleotide samples were used for purification. Collected fractions were further dried by Speed-Vac to completely evaporate the buffer. Samples were reconstituted in RNase free water and prepared for ESI-MS by following the earlier described procedure. Percentage yield of each species was determined by calculating the ratio of area of representative peak to the summation of areas represented by all the species.

Click reaction

Caged oligonucleotide (2.5 nmols, 40 μL) in RNase free water was treated with DBCO-DC (20 nmols, 40 μL) in DMSO with a final total volume of 80 μL 50% DMSO/water. The solution was stirred at 37°C for overnight. After the reaction clicked ssRNA was precipitated following standard ammonium acetate/glycogen/ethanol precipitation. Finally, ssRNA was dissolved in 40 μL of RNase free water and

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ESI-MS was performed on 10 μL of this sample by following general ESI-MS sample preparation. Majority of the desired product was formed as confirmed by ESI-MS analysis

Photolysis

Precipitated clicked oligonucleotide (~200 μM in RNase free water, 10 μL) was placed in a flat bottom glass vial insert 100 μL, [diameter 3.4 mm (inner), 4.5 mm (outer), height 30.5 mm (Agilent, 5183-2090)] It was then irradiated from the bottom for 10 minutes using a Nichia 200 mW 365 nm LED source at a distance of ~1 mm. After the irradiation, sample was prepared for ESI-MS analysis as described earlier.

Supporting Information

Complete materials and methods, including NMR, HPLC and MS characterization of all intermediates. In addition, HPLC/MS characterization of all 18 nucleic acid species reactions describe and the detailed results of optimization experiments.

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Abbreviations

DDA, diazo-DMNPE-azide; DMNPE, di-methoxy nitro phenyl ethyl;

Funding Sources

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