Nucleotide Labelling

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Covalent Chemical 5′-Functionalization of RNA with Diazo Reagents

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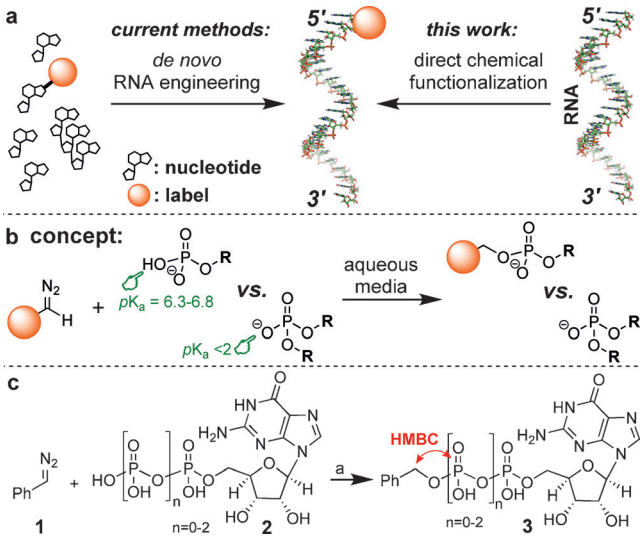
Abstract: *Functionalization of RNA at the 5′-terminus is important for analytical and therapeutic purposes. Currently, these RNAs are synthesized de novo starting with a chemically functionalized 5′-nucleotide, which is incorporated into RNA using chemical synthesis or biochemical techniques. Methods for direct chemical modification of native RNA would provide an attractive alternative but are currently underexplored. Herein, we report that diazo compounds can be used to selectively alkylate the 5′-phosphate of ribo(oligo)nucleotides to give RNA labelled through a native phosphate ester bond. We applied this method to functionalize oligonucleotides with biotin and an orthosteric inhibitor of the eukaryotic initiation factor 4E (eIF4E), an enzyme involved in mRNA recognition. The modified RNA binds to eIF4E, demonstrating the utility of this labelling technique to modulate biological activity of RNA. This method complements existing techniques and may be used to chemically introduce a broad range of functional handles at the 5′-end of RNA.*

Functionalized RNAs find many applications. For instance, fluorophores, radiolabels, and affinity tags are introduced to generate RNA probes for diagnostic and analytic use.[1] Furthermore, structural modification of the 5′-end of therapeutic siRNA and mRNA increases stability, enhances uptake, and can modulate immunogenicity.[2] These 5′-modified RNAs are currently prepared by co-transcription, ligation, or de novo chemical synthesis utilizing chemically modified nucleotides. Direct chemical 5′-functionalization of native RNA in aqueous solution, by reaction with the 5′-phosphate, would be an attractive alternative; however, such methods have so far not been reported. Herein, we disclose the selective alkylation of the RNA 5′-phosphate using diazo reagents, and its application in 5′-biotinylation and the chemical capping of an oligonucleotide.

Challenged with the problem of selectively functionalizing the 5′-end of native RNA, we realized that the 5′-phosphate bears the most acidic proton under physiological conditions, because phosphate diesters will be deprotonated in aqueous solution at pH ≈ 7 [pK_a (phosphate monoesters) = 6.3–6.8; pK_a (phosphate diester) = 1.4–1.7, Scheme 1 b].[3] Analogous to the selective alkylation of carboxylic acids with diazo species, in which an acid-base reaction initially activates both the electrophile as diazonium and the nucleophile as carboxylate,[4] we envisioned alkylation of the 5′-phosphate to be possible using diazo chemistry.



Scheme 1. Phenyl diazomethane alkylates exclusively the terminal 5′-phosphate of nucleotides. Graphic representation of RNA taken from PDB ID: 4RBQ.[5] Reagents and conditions: a) 5 equiv **1**, 1/1/1 MeOH/MeCN/borate buffer (50 mM, pH 6.9), 2–5 h, RT, full conversion; isolated yield: 32% (GMP), 28% (GTP).

Previous studies on labelling of 3′-monophosphates in nucleotides suggested that suitable buffer conditions could be identified that allowed for the desired transformation to occur.[6] To test our hypothesis that terminal phosphates react preferentially over internal phosphates (that is, phosphate monoester vs. diester), we first turned our attention to guanosine mono-, di-, and triphosphate (**2**) as model substrates, and phenyl diazomethane (**1**) as reactive species (Scheme 1 c).[7] As a reaction medium, a mixture of methanol, acetonitrile, and borate buffer was chosen to solubilize both the nucleotides as well as phenyl diazomethane (**1**).[6] Treatment of GMP, GDP, and GTP with 5 equiv of **1** resulted in each case in full conversion to a single product with a molecular mass consistent with the mono-benzyl adduct, based on ion-pairing chromatography/MS analysis (IPC/MS). After purification of the reaction mixtures by ion exchange chromatography, 2D-NMR experiments confirmed that the terminal phosphate had been alkylated exclusively, supporting our hypothesis that the desired selectivity for terminal phosphates over backbone phosphates can be achieved. Next, we turned our attention to a more complex, biologically relevant setting.

Eukaryotic mRNA translation is dependent on the presence of a 7-methyl guanosine cap, which is post-transcriptionally installed by 5′-5′-triphosphate linkage to nascent

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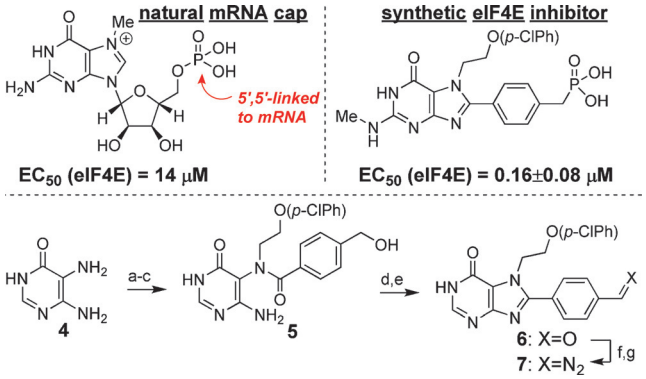
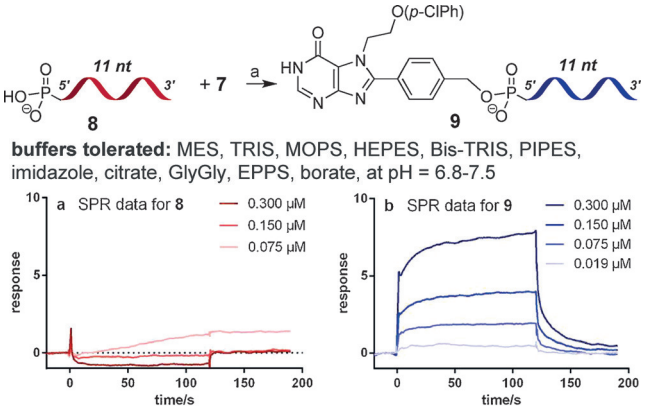


Scheme 2. Synthesis of the diazo-derivative **7** of an orthosteric eIF4E inhibitor as precursor to an artificial mRNA cap. Binding affinities (EC_{50}) of the natural mRNA cap and the synthetic ligand as reported in Ref. 9. Reagents and conditions: a) *p*-chlorophenoxy acetic acid, HATU (1 equiv.), NEt_3 (2 equiv), DMF, RT, 30 min, 60%; b) $LiAlH_4$ (6 equiv), THF, RT, 5 h; c) 4-(hydroxymethyl) benzoic acid (1.2 equiv), NEt_3 (4 equiv), HATU (1.2 equiv), DMF, RT, 5 h, 30% over 2 steps; d) NaO^tBu (5 equiv), ^{i}PrOH, 80 °C, 6 h, 69%; e) MnO_2 (32 equiv), THF/DMF 1/1, RT, 72 h; f) $N_2H_4{\cdot}H_2O$, DMF, 90 °C, 16 h, 97% over 2 steps (*E*/*Z* ca. 1/1); g) MnO_2 (18 equiv.), DMSO, RT, 4 h, 87%.



Scheme 3. Artificially capped oligonucleotide **9**, but not the parent strand **8**, binds to eIF4E by SPR. Reagents and conditions: a) 100 μM **8** (sequence: r(pGA AAA AAA AAA)), 10 mM MES pH 7.0, 30% MeOH, 10% DMSO, **7** (10 + 2 × 40 equiv), 6 h, 80–95% conversion. Conditions for SPR experiment: Biotinylated Avi-eIF4E bound to a Series S Sensor Chip SA at surface densities of 5000–7000 RU; buffer: 1× PBS, 50 mM NaCl, 0.1% Glycerol, 0.1% CHAPS, and 1% DMSO; flow: 30 μL min^{-1}. Data analysis was done using Biacore T200 Evaluation Software. Data points around $t = 0$ were excluded for clarity (buffer effects). In two independent experiments, the K_D (kinetic) for **9** was determined to be 3.81 μM and 3.05 μM.

mRNA (Scheme 2).[8] This cap is essential for recognition of mRNA by the eukaryotic initiation factor 4E (eIF4E) in the first step of mRNA translation. Synthetic eIF4E inhibitors, which bind the cap-binding site on eIF4E, have recently been reported as potential anti-cancer agents,[9] and we hypothesized that these orthosteric inhibitors could be turned into artificial RNA caps, if attached to the 5′-end of RNA using the diazo alkylation reaction.

The diazo-RNA-cap was synthesized in 7 steps (Scheme 2): Diaminopyrimidinone **4** was acylated with *para*-chloro-phenoxy acetic acid, and the resulting amide was reduced with $LiAlH_4$. Acylation with 4-(hydroxymethyl) benzoic acid gave **5** in 21% yield over 3 steps. Amide **5** was cyclized to the corresponding purine using NaO^tBu, and the benzylic alcohol was oxidized using MnO_2 to give aldehyde **6** (69% over 2 steps). Conversion of aldehyde **6** to the desired diazo compound **7** was achieved in 85% yield by condensation with hydrazine followed by oxidation with MnO_2.

With diazo compound **7** in hand, we turned our attention to the 5′-functionalization step (Scheme 3; see the Supporting Information for further details). In preliminary experiments, diazo cap **7** (10 μM) was allowed to react with an undecanucleotide with a 5′-monophosphate in buffered aqueous solution,[10] and the conversion was monitored by IPC/MS. Initial experiments were carried out at pH 5.8, to ensure protonation of the terminal phosphate, and in the presence of 10% DMSO. Under these conditions, addition of 200–500 equiv of **7** resulted in approximately 50% conversion of the starting material to one single, mono-alkylated compound, tentatively assigned as the 5′-alkylated oligonucleotide **9**. Attempts to optimize the reaction by increasing the amounts of organic solvents proved futile. However, at pH 7.0, the mono-adduct was formed in >90%. Notably, dialkylation of **8** was never observed.

To enable further analysis of the reaction product we had to produce **9** on preparative scale by increasing the concentration of the oligonucleotide **8** to at least 100 μM. Unfortunately, the concentration of diazo compound **7** could not be scaled accordingly because of its low solubility, and 100 equiv of **7** gave only poor conversion. We found that the alkylation could be further driven by consecutive addition of **7** (10 equiv + 2 × 40 equiv) and using methanol (30%) to aid solubilization of **7**, providing **9** in 80–95%. Next, we carried out a screen of 20 commonly used buffers and found that the reaction proceeded well in MES, TRIS, TES, MOPS, Bis-TRIS, Gly-Gly, and borate buffers at pH between 6.8 and 7.4. Reaction conditions at pH > 7.4, or using other carboxylate- or phosphate-containing buffers were not tolerated. This sensitivity of the reaction to buffer and pH is in agreement with our mechanistic working hypothesis: At pH > 7.4 the 5′-phosphate will be doubly deprotonated, thereby shutting down its reactivity, and buffers containing carboxylic acids and phosphoric acids effectively quench the diazo reagent.

Next, we sought to demonstrate that **9** was in fact the desired 5′-alkylated species and that selective modification of **8** conferred additional biological function on the oligonucleotide strand. To this end we set up an SPR assay using immobilized eIF4E. We found that oligonucleotide **9**, but not **8**, was recognized by eIF4E (K_D = 3–4 μM). Based on biochemical and crystallographic data,[8] binding of RNA to eIF4E requires the cap to be linked to the 5′-end. Thus, backbone-capped RNA would not be recognized by eIF4E. We had shown in our model studies that the diazo reagent reacts exclusively with the terminal phosphate in nucleotides. The SPR data confirmed that **9** satisfied the structural requirements for binding to eIF4E. Taken together, this data supports our hypotheses that 1) selective 5′-functionalization had occurred, and 2) an eIF4E inhibitor could be turned into an mRNA cap surrogate. These findings suggest that our diazo-labelling method can be used to introduce

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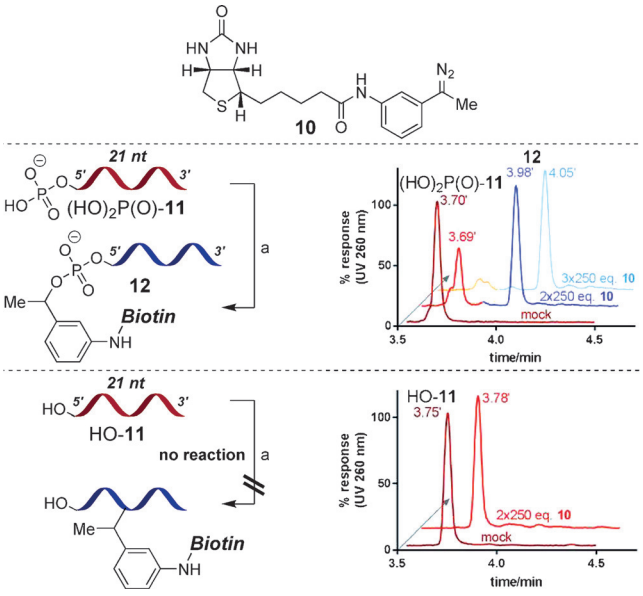
a range of artificial mRNA caps to modulate expression levels or immunogenicity for use in mRNA gene therapy.[2k]

Finally, we set out to further explore the utility of the diazo-functionalization method for installation of a different 5′-label using an oligonucleotide with a primary structure that is representative of native biological RNA. Biotin is commonly used as a label for biomolecules, providing a handle for immobilization, purification, and detection.[11] To see if diazo reagents can be used for 5′-biotinylation of RNA, we prepared known diazo-biotin reagent **10**[12] (Scheme 4) and



Scheme 4. 5′-Biotinylation of RNA **11** is contingent on the 5′-phosphate. Sequence of **11**: r(AACAGCAUAGCAAGUUUAAAU); reagents and conditions: a) 100 μM **11**, 50 mM MES pH 7.0, 10% DMSO, **10** (2–3×250 equiv), 60–90 min, 80–95% conversion to **12**; right: IPC-chromatogram of reaction mixtures of $(HO)_2P(O)$-**11** (top) and HO-**11** (bottom); mock sample did not contain **10**.

reacted it with two 21-nt RNAs with identical, random sequences differing only in the presence and absence of a 5′-phosphate, $(HO)_2P(O)$-**11**, and HO-**11**, respectively. We found that $(HO)_2P(O)$-**11** was selectively converted to a species with the desired mass of the biotinylated product **12** ($R_t = 3.98$–$4.05'$). In contrast, HO-**11** did not react under these conditions. These experiments confirmed that labelling is 1) contingent on the presence of a 5′-phosphate, and 2) not affected by the primary structure of the oligonucleotide, thus showcasing the utility of the method for 5′-selective biotinylation of RNA.

In summary, we showed that diazo reagents can be utilized for selective chemical functionalization of the terminal 5′-phosphate of oligo ribonucleotides through a native phosphate ester bond. This method was applied to modify an oligonucleotide with an orthosteric inhibitor of eIF4E. The chemical modification endowed the oligonucleotide with binding affinity to eIF4E, thus demonstrating the utility of the method to modulate biological functions of RNA. Additionally, we applied the method to 5′-biotinylate RNA and showed that labelling is contingent on the presence of the 5′-phosphate and unaffected by the primary structure of the RNA. This 5′-selective chemical functionalization expands the armamentarium for generation of functionalized RNA for use in diagnostic and therapeutic settings.

Acknowledgements

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