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Direct and Selective Modification of RNA – An Open Challenge in Nucleic Acid Chemistry

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Abstract: We present the state-of-the-art in direct RNA modification as well as the challenges that hold back further development of RNA mechanistic probes and medicines. Solid-phase synthesis has revolutionized the synthesis of short DNAs and RNAs. Many open questions in RNA biology are with large long-non-coding RNAs or mRNAs and there is also interest in developing these big RNAs as medicines. Techniques for direct modification will become more important in the coming years and we give a current snapshot of the field here, with a bias towards our own contributions.

Keywords: Bioconjugation · Bioorthogonal reporters · Nucleic acid therapeutics · RNA chemistry · RNA therapeutics

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1. Why Is RNA Modification Important and What Challenges Are Unresolved?

Solid-phase oligonucleotide synthesis (SPOS) has underpinned extraordinary developments in nucleic acids and protein research. Coupled with ligation techniques or assembly reactions SPOS enables access to any DNA, including DNA bearing non-canonical bases. RNA, however, poses a greater challenge; its chemical instability and constant threat of ribonuclease contamination make many nucleic acid chemists wary of RNA synthesis. Another big problem is that RNA synthesis cannot be primed or amplified the way DNA synthesis can. Although SPOS is an invaluable technology it has shortcomings; for example, the synthetic linearity imposes a size limitation, and non-canonical phosphoramidite building blocks are challenging to synthesize, particularly for RNA. Whenever large RNAs with internal alterations are needed, fragments from transcription are combined with synthetic fragments through splint-mediated ligation, a tedious and often low-yielding process. Nevertheless, precisely tagged or modified RNAs are crucial in the study of RNA biochemistry and biology;[1] for example as mechanistic probes or spectroscopic labels. Biophysical tags typically confer some observable property that reports on the occurrence, structure, or binding interactions of nucleic acids. While most modified nucleic acids are made by SPOS, direct modification would be more efficient; given the functional group redundancy of large RNAs, however, such a strategy faces a daunting chemoselectivity problem. Enzymes are Nature's solution to achieving selective RNA modification and here we will outline chemical strategies that co-opt and expand on Nature's approach. In 2015 we reviewed the literature on direct catalytic RNA modification,[2] the work described here focusses on the newest developments in this rapidly evolving field. In the interest of space we focus here on techniques for direct RNA modification because such techniques could be used to modify RNAs obtained from transcription. We do not cover the pioneering synthetic work where a functional handle is installed through solid-phase synthesis or polymerization[3] and is then later reacted through selective chemistry. This approach is crucial in modern DNA and RNA chemistry, but is now an established technology. Still in the early stages of development are direct modification techniques and we focus exclusively on those here. We have not compiled a comprehensive review, but rather tried to give an overview of the field; we apologize to researchers whose work we have not included.

A final important point that frames our interest in RNA modification is the potential of RNA medicines. All of the nucleic acid drugs on the market are chemical analogues of RNA and they need to be made through solid-phase synthesis.[4] The next class of nucleic acid medicines will likely be derived from mRNAs. For diseases that stem from missing, haploinsufficient, or malfunctioning proteins, cellular delivery of mRNA offers the possibility of providing the cell with fully functioning copies.[5] Although selective delivery is an enormous challenge, if this could be accomplished mRNA offers several advantages over genomic therapies or protein replacement. Genetic medicines are permanent, with

long-term effects that are hard to predict. Genome integrating viruses often cause cancer, and this remains a concern with genomic medicines. Delivering proteins intracellularly is an even greater challenge than nucleic acids and externally delivered proteins typically elicit an immune response. Delivering mRNA means the protein produced will be post-translationally modified in a way that is recognized as 'self', and the effect is catalytic, but transient (*i.e.* one mRNA can create many protein copies, but with time the mRNA will degrade). Despite the potential advantages of mRNA as a medicine, it is a large unstable molecule. As this field develops chemistry will play a role in tailoring the pharmacokinetic properties of mRNAs, but total chemical synthesis is not an option. Instead transcription must be the starting point of any mRNA synthesis. Chemical modifications have to be introduced either as a part of transcription, or through selective post-synthetic direct modification.

2. Chemoenzymatic RNA Modification

Studying or reprogramming biopolymers at the molecular level typically requires precise chemical modification. Chemical modification or synthetic labeling of RNA is challenging, expensive, and time consuming.[6] Modifying large RNAs like long non-coding RNAs or mRNAs is an open – and important – challenge.[7] Nature has evolved many enzymes that interact with DNA or RNA specifically, and expanding on Nature's system might offer a mild and selective path for modifying DNA or RNA. Natural proteins find their target DNA or RNA sequence and act either by simply binding or modifying it covalently. DNA methyltransferases (MTases) are one of such group of enzymes, which interact with a specific sequence in DNA or RNA and label it with a methyl group. MTases incorporate the methyl group of its cofactor *S*-Adenosyl-L-methionine (Adomet, Fig. 1) by catalyzing the nucleophilic attack of cytosine (C), adenine (A), or guanine (G) on to the electrophilic methyl group. Despite the high specificity of MTases the methyl group is a poor reporter unless radioactively labeled with ^{14}C or tritium, and therefore a number of researchers have examined the tolerance of MTases to Adomet analogues (Fig. 1).[8] Detailed accounts of the background and development of this technique has been published,[2,7] so here we will only try and give an overview of the different Adomet analogues that have been used (Fig. 1) and a representative example (Fig. 2A).

The most recent innovations relating to methyltransferase repurposing comes from the Rentmeister group. She has explored human trimethylguanosinesynthases, which convert standard m^7G caps in eukaryotic mRNA to the 2,2,7-trimethylguanosine, or trimethylguanosinesynthases from *Giardia lamblia* (GlaTgs), which normally install a single methyl group at the N2 of m^7GDP, m^7GTP, and m^7GpppA using Adomet or its analogues (Fig. 2A). The enzyme's tolerance to modified analogues was exploited to add unique functionality such as terminal alkynes to the 5'-cap structure of mRNAs *in vitro* and in cell lysates. Protein and substrate engineering have since further improved the efficiency and ease of use of the method.[9] A recent extension of this technique has identified a highly permissive capping methyltransferase (Ecm1) from the microsporidian parasite *Encephalitozoo cuniculi*, which accepts diverse Adomet substrates.[10] Through access to chemical modifications in the RNA cap these researchers were able to tune translation efficiency in live cells. A shortcoming of all the strategies that rely on Adomet substrate analogues is the need to synthesize the substrates and the challenge of getting these into cells. An exciting advance is the use of methionine analogues that are *in situ* biosynthesized to the requisite Adomet analogues directly in cells.[11] This innovation should reduce the technical entry barrier for exploiting this technology.

In a series of recent papers the Devaraj lab has developed a chemoenzymatic technique that exploits a transglycosylase enzyme normally responsible for installing the non-canonical nucleobase preQ1 (Fig. 2B). *Escherichia coli* tRNA guanine transglycosylase (TGT) tolerates a variety of modifications of the preQ1 nucleobase at the C7 position. The enzyme has a specific 17-nucleotide hairpin recognition motif that signals the installation of preQ1; normally this occurs in tRNA, but Devaraj and coworkers have shown that this motif can lead to RNA labelling in diverse RNAs as long as the stem-loop is conserved.[12] In a conceptually similar way the agmantine post-transcriptional modification of tRNA has been exploited for the transfer of clickable probes to the cognate cytosine of the $tRNA^{Ile2}$-agmatidine synthetase (Tias, Fig. 2C).[13]

3. Ribozymes and Deoxyribozymes

Although DNA and RNA have less functional group diversity than proteins, their predictable and evolvable binding properties have underpinned the creation of DNA and RNA-based catalysts (often termed DNAzymes for DNA and ribozymes for RNA). The Silverman lab has been a pioneer in developing DNAzymes for cleavage, ligation, or modification reac-

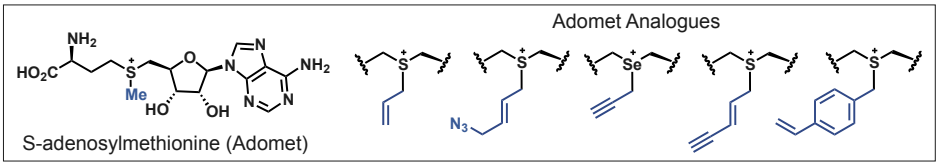


Fig. 1. *S*-adenosylmethionine (Adomet) analogues with a variety of chemical groups are tolerated by certain methyltransferases (MTases).

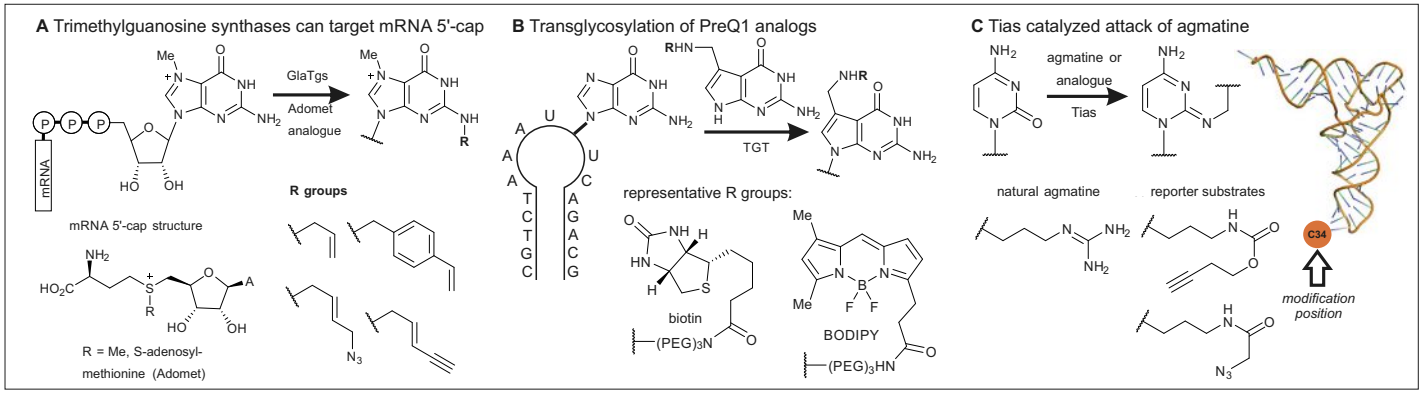


Fig. 2. Different chemoenzymatic approaches to RNA labelling.

tions of nucleic acids. Their first approach was to create a deoxyribozyme-catalyzed RNA labelling method that resembles certain aspects of natural RNA splicing.[14] Here a tagging RNA is held in close proximity to the RNA target through complementarity on the DNAzyme (Fig. 3A). The tagging RNA can be pre-labelled with any type of biophysical probe through amide formation with activated esters. It also bears a 5'-triphosphate, making it primed for reactions with hydroxyl groups. The 2'-hydroxyl of an internal adenosine then attacks the 5'-triphosphate of the tagging RNA to create a branched RNA bearing the reporter group. While an important proof-of-concept, this approach suffers from the complexity of the tagging RNA. A technique from the Höbartner lab has eliminated many of the shortcomings of the first generation RNA tagging DNAzymes.[15] Building from earlier work,[16] they identified a DNAzyme that can catalyze the coupling of various guanosine triphosphates to the 2'-hydroxyl of an internal adenosine of a target strand (Fig. 3B). Using a mononucleotide is advantageous because the tagging unit is much smaller and there is no pendant oligoribonucleotide at the branch site. A key discovery was that terbium(III) accelerates the rate of the ligation reaction. Using terbium-assisted DNAzymes, they could achieve multiple labelling of large complex RNAs with a variety of biophysical reporters in high yields. A feature that bodes well for the implementation of the Höbartner approach is its simplicity: aside from the target RNAs all components can be purchased.

Chemical modification of RNA through guided Watson-Crick base-pairing with DNA reagents that bear transferable alkyl groups has also been accomplished, providing access to large sequence-specifically modified DNAs and RNAs.[17] A disadvantage of these approaches is the synthetic investment needed to create the DNA-linked reagents.

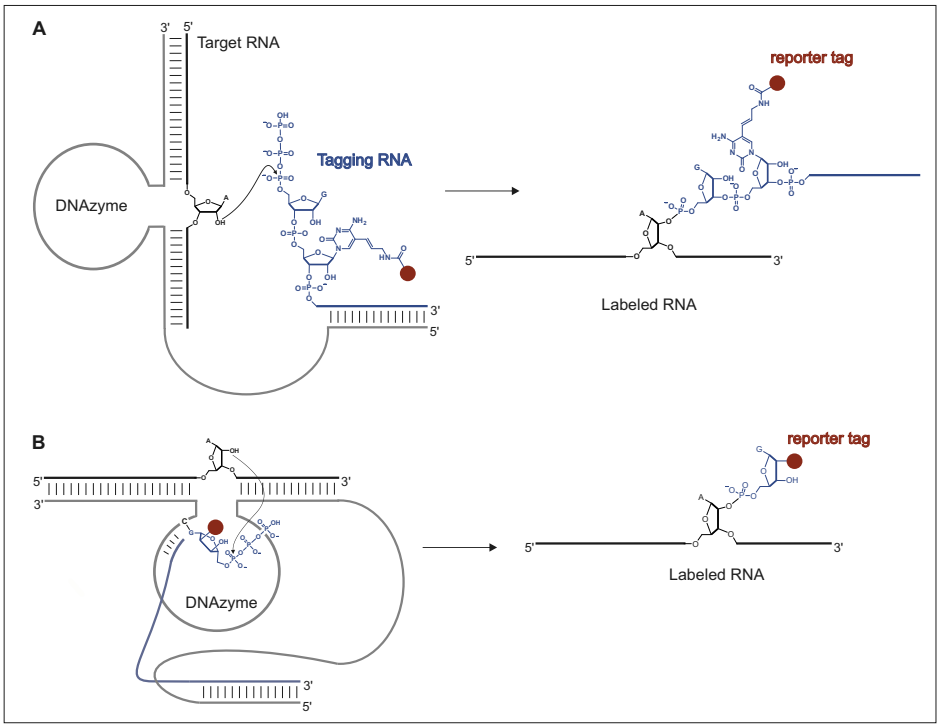
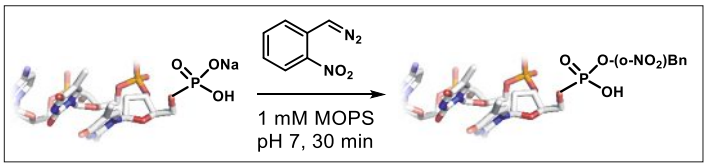


Fig. 3. A: A DNAzyme catalyzes the transfer of an 8-17 nucleotide tagging RNA bearing a reporter tag; B: A DNAzyme can couple a guanine mononucleotide bearing a reporter tag.

Fig. 4. Alkyl diazo compounds alkylate the phosphate group under aqueous conditions.



4. End Functionalization of RNA

Numerous natural base modifications have been identified in the various families of RNAs and many more are likely to be discovered.[18] End modifications play an important role in RNA stability (particularly mRNA) and so methods to target the ends of an RNA are needed. Typically these modifications have been incorporated through co-transcription, ligation, or chemical synthesis.[19] Recent work from a number of labs[20] has shown that alkyldiazo compounds react with phosphate groups to create the corresponding phosphate esters (Fig. 4), in a reaction reminiscent of diazomethane esterification of carboxylic acids.[21] The reactions are surprisingly selective and yet broadly active across many types of biological phosphates. Phosphate ester formation with diazo compounds is easy to implement, high-yielding, and the requisite phosphate group can be installed through a simple phosphorylation. The key to the selectivity is likely the need for a proton to activate the diazo group for alkylation.[20e] The phosphate group is uniquely reactive because it has an ionizable proton (pK_a of approximately 6.2) that can perform the essential protonation of the diazo reagent.

Selective and high-yielding 3' functionalization is underdeveloped. The classical method is periodate oxidation of the diol on the 3'-end of RNA, which gives a dialdehyde that can be permanently coupled with amines or hydrazides through a reductive amination.[6,22] Although this protocol degrades some of the RNA and gives oxidation byproducts, it is nevertheless reliable and widely used. Recently chemoenzymatic[23] and ribozyme[24] approaches have been developed. The chemoenzymatic approach uses a tailing reaction with modified NTPs; since it is untemplated, however, this reaction will deliver heterogeneous 3'-ends unless a chain terminating NTP is employed. The ribozyme approach[24] uses an RNA-based RNA polymerase and the reaction is controlled by a template strand. By using a single NTP the reaction is halted with high fidelity after a single addition as long as the next series of bases in the template are all mismatches for the NTP. This feature means that commercial NTP analogues are viable substrates; since the RNA catalyst itself is made by *in vitro* transcription, this method promises to an important and practical innovation in selective 3'-end labelling of RNA.

5. Organometallic Catalysis for RNA Modification

Unnatural catalysts – such as organometallic systems – could offer reaction manifolds inaccessible to enzymes, ribozymes, or DNAzymes. For small oligonucleotides the efficiency and reliability of solid-phase synthesis is hard to beat, but for large RNAs post-transcriptional chemical modification will be crucial. While using organometallic chemistry for protein modification is a vibrant research area,[25] the same is not true for nucleic acids. My group has been developing organometallic catalysts for post-synthetic modification of DNA and RNA and we have found that metal carbenes derived from diazo compounds can alkylate DNA and RNA

chemoselectively.[26] An important recent finding is that changing the substitution of the diazo compound can completely switch the site selectivity in nucleobase alkylation. In particular, we have shown that copper-carbenes generated from diazo compounds can alkylate the O^6G in DNA with high chemoselectivity.[27] We have recently extended this method to include the selective alkylation of the O^6G in various RNAs,[28] including large mRNAs (Fig. 5). A key innovation in extending to RNA was the development of a new N-heterocyclic carbene ligand for copper that was stable under aqueous conditions and did not lead to RNA degradation (see bottom of Fig. 5). We are currently working to make this reaction sequence specific.

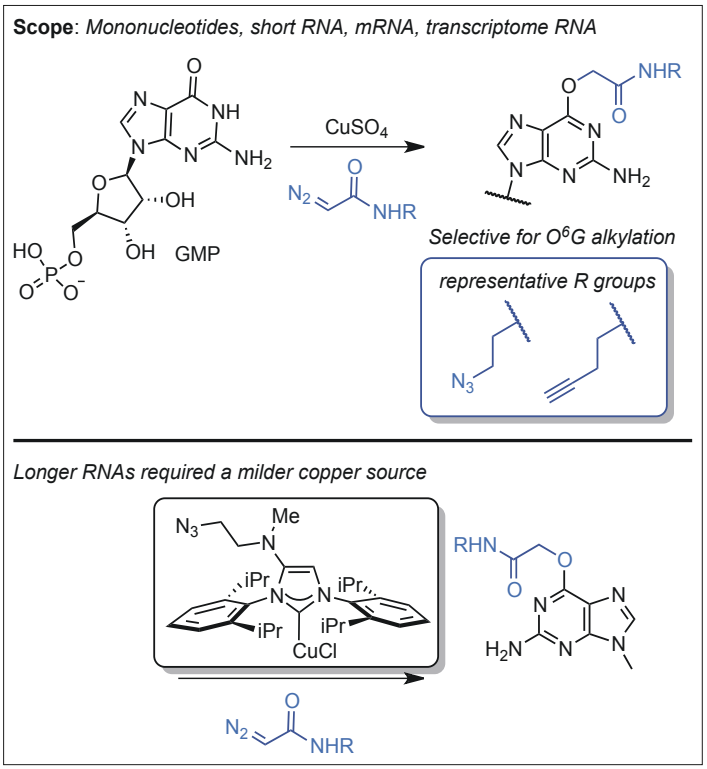


Fig. 5. Diazoacetamides alkylate diverse RNA substrates with a single type of alkylation. Top: mononucleotide alkylation proceeds effectively with $CuSO_4$, targets the O^6G and is insensitive to the 2'-hydroxyl of RNA; Bottom: Larger RNAs undergo some degradation with $CuSO_4$, so an N-heterocyclic carbene ligand that is less Lewis acidic than $CuSO_4$ was developed.

6. Conclusion

Just as solid-phase synthesis was a crucial component of the molecular biology revolution, the preparation of large modified RNAs with new or augmented functions will be essential for studying their biology and using them as medicines. Here we have given an overview of chemical strategies toward direct RNA modification, but it is clear that significant challenges need to be overcome before large chemically tailored RNAs are widely available to non-experts. At the moment *in vitro* end modification is powerful and many forms of RNA can be labelled in high yields with current technologies. The greatest open challenge is new approaches to post-synthetic internal modifications of large RNAs.

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