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A chemo-enzymatic approach to specifically click-modified RNA†

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The growing interest in single-molecule analysis of RNA calls for programmable enzymatic labeling strategies beyond the horizon of solid-phase synthesized RNAs. Herein we describe an easy and versatile chemo-enzymatic approach to label RNA at its termini or defined internal positions *via* click-chemistry.

After sequencing the human genome, the analysis of RNA-related processes like gene regulation by micro RNAs (miRNAs) as well as their utilization in therapeutic strategies has attracted increasing attention.[1] For more detailed investigations of their mode of action and to overcome the limitations of effective delivery to the targeted cells, powerful/suitable/new RNA-labeling strategies are required.[2]

Hitherto, most of the modifications in RNA are introduced by solid-phase synthesis which is struggling with lower coupling efficiencies and more complex purification protocols in comparison to DNA. Therefore the synthesis of RNA oligonucleotides is more expensive, time-consuming, laborious and usually reaches its limits at sequence lengths longer than 50 nucleotides.[3] Enzymatic methods, *e.g. in vitro*-transcriptions or ligations of short RNA strands, provide an attractive solution to these problems.[4]

Both, solid-phase and enzymatic RNA synthesis allow incorporation of modified (ribo)nucleotides. In cases of incompatibilities due to reactive reagents or a low substrate tolerance, a small and functionalisable group is used instead and is attached with the desired modification post-synthetically. Referring to this, the 1,3-diploar Cu-catalyzed azide–alkyne cycloaddition (CuAAC) has proven to be a powerful method. However this is primarily the case for DNA oligonucleotides because RNA is more sensitive to copper-induced strand-cleavage.[5] Although this effect can be repressed by the addition of copper-stabilizing ligands only a few reports have described modification of RNA by means of CuAAC.[3,6–9]

Using synthetic RNA strands bearing alkyne and azido groups which are suitable for cross-strand and intrastrand CuAAC-ligations, El-Sagheer and Brown have assembled DNA–RNA hybrids ~100 nt in length.[3] Paredes and Das picked up and transferred this idea to *in vitro*-transcribed RNA oligonucleotides that were successfully equipped with azido groups at the 5′-terminus by adding an appropriate azido-transcription starter. Moreover they addressed the 3′-terminus enzymatically with a 3′-azido-2′,3′-dideoxytriphosphate and a poly(A) polymerase. Afterwards they conjugated those oligonucleotides with synthetic RNAs modified with commercially available alkynylated reagents.[6]

For internal labelling nucleoside triphosphates (NTPs) equipped with alkynes are available but are randomly incorporated by RNA polymerases, while the labelling efficiency depends on the chemical nature and the bulkiness of the probe to be incorporated.[4,10] An example for a site-specific internal labeling approach was reported by Winz *et al.*, who installed 2′-azido-nucleoside triphosphates at the 3′-terminus of RNA by using a poly(A) polymerase.[7] After selection of the appropriate enzyme and under optimised conditions, only one nucleotide was attached. The design of this terminal nucleotide enabled a strand prolongation *via* a splinted ligation, shifting the former terminal nucleotide to an internal position. Herein we present a powerful method for chemo-enzymatic synthesis of terminally and internally labelled RNA, without the need for tedious optimisation and splint ligation.

Our intention to detect Dicer-mediated miRNA maturation encouraged us to label its hairpin-shaped precursors (pre-miRNA) at both ends with a fluorophore and a quencher, respectively, to quantify its Dicer-mediated cleavage by measuring the increasing fluorescence over time, which results from the absence of quenching.[11,12] Due to the known limitations of solid-phase synthesis and in order to establish a method that allows a modular conjugation of different labels, we envisioned a technique based on CuAAC in combination with enzymatic methods.

To introduce an alkyne group at the 5′-end of RNA we developed a short and efficient route to novel *O*-propargyl-guanosine-5′-monophosphate. *In vitro* transcriptions in the presence of this starter provide a 5′-terminal alkyne modified RNA molecule that is ready for click-reactions (Fig. 1 and 2).[13,14] A tetrazol-mediated one-pot-phosphoramidite-coupling of TMS-protected propargyl alcohol and the protected 5′-hydroxylguanosine followed by removal of the protecting groups afforded the desired compound **9** (**GMP^{Prg}**) as a

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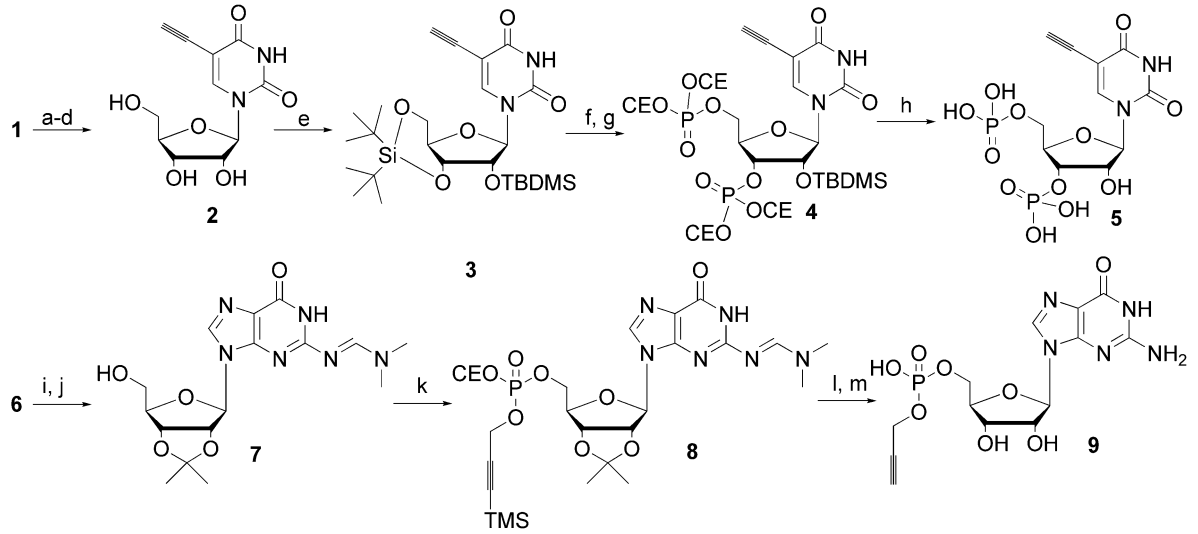

Fig. 1 (a) Ac_2O, 1,4-dioxan; (b) $(NH_4)_2Ce(NO_3)_6$, I_2, MeCN, 80 °C; (c) NEt_3, CuI, $PdCl_2(PPh_3)_2$, DMF; d_1 TBAF, MeCN, d_2 EtOH, NaOH; (e) $tBu_2Si(OTf)_2$, TBDMS-Cl, imidazole; (f) HF·pyridine, THF; (g) $P(OCE)_2(N_iPr_2)$, tetrazole, CH_2Cl_2, *t*BuOOH; h_1 BSA, DBU, pyridine, h_2 NEt_3·3HF; (i) acetone, $HClO_4$; (j) *N*,*N*-di-methylformamide dimethylacetal, DMF; k_1 $P(OCE)(N_iPr_2)_2$, tetrazole, CH_2Cl_2 k_2 tetrazole, 3-trimethylsilyl propargyl alcohol k_3 *t*BuOOH; (l) 7 N NH_3 in MeOH; (m) TFA–H_2O 1 : 1, TEAB buffer.

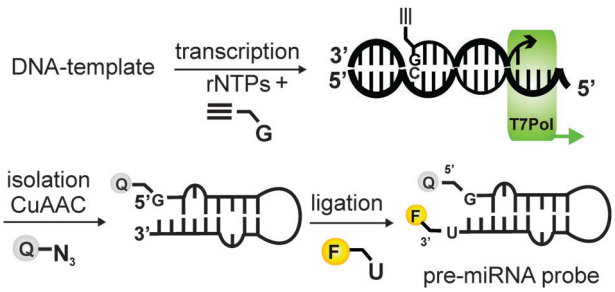

Fig. 2 Illustration of the 5′-, 3′-end labelling methodology.

triethylamine salt in 17% yield over all steps.[15] In the following *in vitro*-transcription with T7 polymerase (T7), the building block **9** competed with unmodified guanosine triphosphate (GTP) for the incorporation at the initiation site of the growing pre-miRNA-transcript. To favour the implementation of **9** without inducing transcription abort we integrated the so-called fed-batch-approach into the manufacturer's protocol.[16] Accordingly we started the *in vitro*-transcription with a 9-fold excess of **GMP^{Prg}** over GTP but added further equivalents of GTP every 15 min until the starting concentration of (A/U/C)TP was reached. After digestion of the DNA template and PCI-extraction the mixture of GTP and **GMP^{Prg}**-primed RNA-transcripts was precipitated with EtOH and used without any further purification steps in a subsequent CuAAC. This reaction was carried out in PUS-buffer with DABCYL-azide **11**, copper sulfate, sodium ascorbate and THPTA as ligand.[17] After 2 h, HPLC purification enabled us to easily separate the DABCYL-conjugated RNA from the GTP-primed transcript. No residues of RNA starting with the initiator **GMP^{Prg}** were detected, indicating that the CuAAC was highly efficient (ESI†). Without any further optimizations for the transcription in terms of salt concentrations, temperature, enzyme mutants *etc.* **11** was linked to the 5′-end of four different DABCYL-labelled pre-miRNAs (pre-let7, pre-miRNA-21, 122, 142,) with overall labeling-efficiencies between 21 and 38%.

For labelling the 3′-terminus we envisioned the use of commercially available T4 RNA ligase 1 (T4) which ligates RNA strands or attaches singular 3′,5′-bisphosphates to the 3′-end.[18,19] Blocking the 3′-hydroxyl group with a phosphate prevents multiple incorporation, however after completion of the ligation, the terminal phosphate can be easily removed by Calf Intestinal Phosphatase (CIP). To address the 3′-terminus we established a synthesis route to alkynylated 3′,5-bisphosphate uridine **5** (**pUp^{Alk}**), which has not been described so far. The synthesis of this building block started from uridine **1** which was modified with an ethinyl group at the C5-position by known procedures (total yield 51%).[20] By using a silyl-based protecting group strategy, the key compound **2** was regioselectively phosphorylated.[21] Removal of the cyanoethyl groups failed first when using methanolic ammonia solution but finally succeeded when switching to bis(trimethylsilyl)acetamide and DBU in pyridine.[22] After silyl deprotection, the azido labelled FAM was conjugated by means of CuAAC (ESI†) and ligated to the ^{DAB}pre-miRNAs with T4 in line with the manufacturer's protocol. The surplus of pUp^{FAM} was separated with centrifugal filters and a final HPLC purification showed no leftovers of non-ligated ^{DAB}pre-miRNAs, suggesting a complete conversion of the substrate. Adding the pre-miRNA processing enzyme Dicer to each of those constructs results in an increase in fluorescence (λ_{em} 520 nm), confirming that these labelled pre-miRNAs are tolerated as substrates (Fig. S4, ESI†). Moreover, all probes were analysed using denatured PAGE. As expected all bands showed fluorescent activity in the absence of SYBR® gold and prove the formation of the full-length product. Molecular masses were confirmed by UHPLC-UV/Vis-MS (see ESI†). The strength of this chemo-enzymatic approach lies in its effectiveness and easy handling as we used standard protocols and commercially available enzymes. Moreover the chosen T4-ligation method does not need to be optimized in regard to multiple single-nucleotide-attachments and does not require a template.

The fluorophore FAM is not the best choice when it comes to experiments in the cell because its maximum of emission lies

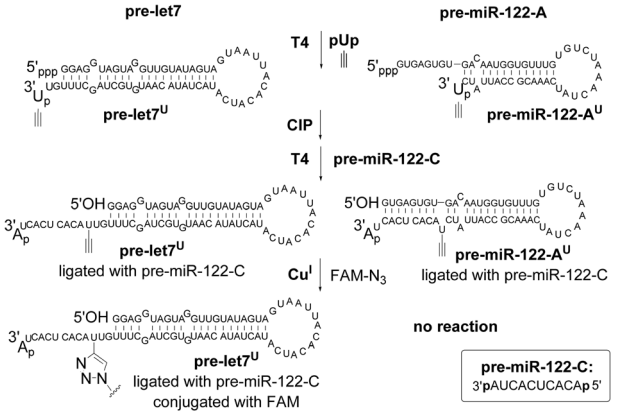

Fig. 3 Internal labelling of extended pre-miRNAs by consecutive ligation–dephosphorylation: left: extended pre-let7, right: extended pre-miR-122A.

in the range of the cellular auto-fluorescence and it is known to be sensitive to photobleaching.[23,24] To tackle this problem we chose Alexa Fluor® 633 as an alternative, however when we started this project there was no azido functionalized Alexa Fluor® 633 purchasable. For this reason, we modified the established strategy to label the biologically highly relevant pre-miR-122. Instead of ligating a single nucleotide to the 3′-end we decided to build up pre-miRNA-122 from two oligonucleotide fragments (Fig. S5a, ESI†). The DABCYL-tagged 5′-end was synthesized according to the aforementioned protocol. The remaining Alexa-RNA strand from the 3′-terminus was obtained from a commercial source and synthesized by solid-phase synthesis. A T4-ligation of both strands, again without any template or splint, led to the full-length product in almost quantitative yield (Fig. S5c, ESI†). The HPLC purified probe was incubated with Dicer and this led to a 5-fold enhancement of fluorescence within 4 hours whereas the treatment with denatured Dicer showed almost no increase.

While several methods for terminal labelling of RNA exist, site-specific internal labelling of RNA is a special challenge.[7,25–27] Our work on the Alexa-labelled pre-miRNA inspired us to extend the scope of our ligation strategy based on the novel building block **pUp^{Alk}**. The 3′-phosphate of the U^{Alk}-elongated 3′end can be removed with CIP thereby creating a new ligation site which could be elongated with a further RNA strand. This concept would provide a smart alternative to the few methods known so far for the site-specific internal labelling of RNAs which require laborious optimizations or template modifications.[7,25–27]

To verify this idea we ligated **pUp^{Alk}** to pre-let7. Instead of HPLC purification we used centrifugal filters to remove any excess of **pUp^{Alk}**. Analytical UHPLC-UV/Vis-MS analysis of the crude product verified a complete conversion of the unlabelled pre-let7 illustrating the effectiveness of this reaction. In the next step phosphate groups were removed and the deblocked 3′-hydroxyl function used for a T4-ligation of a second RNA strand. Finally we carried out a CuAAC together with FAM-N_3. The same procedure was applied to a shortened pre-miRNA-122. Adding the second RNA strand to the U^{Alk}-prolonged RNA provides the natural pre-miRNA-122 enlarged by one internal alkynylated uridine (Fig. 3). A PAGE-analysis is shown in Fig. S6, ESI.† In contrast to the elongated pre-let7 which was successfully labelled with FAM the elongated pre-miRNA-122 showed no labelling. This is possibly due to steric hindrance of the alkyne position caused by duplex formation in the pre-miRNA-122 whereas the alkyne group in pre-let7 is easy accessible. A similar observation was made by Jäschke *et al.* (RNA) and Carell *et al.* (DNA).[7,28]

In summary we described for the first time the synthesis of alkynylated 3′,5′-bisphosphate uridine **5** and *O*-propargyl-guanosin-5′-monophosphate **9**. These building blocks enabled us to establish a chemoenzymatic approach to label RNA at its termini with organic molecules like FAM and Dabcyl bearing an azido group. Additionally, the scope of this methodology was extended to label RNA site specifically at internal positions. In general, this method has proven to be very effective and easy to apply what has been exemplified by the synthesis of several pre-miRNAs probes up to 79 nt in length. The chemoenzymatic approach suggests that much bigger RNA molecules are also feasible.

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