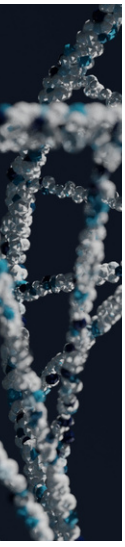
EFFICIENT, SELECTIVE AND SCALABLE COVALENT MODIFICATION OF LONG RNA MOLECULES

BACKGROUND INFORMATION

Virtually all Big-Pharma companies are currently working on mRNA-based therapeutics (e.g.: antiviral vaccines, oncoimmunology, gene supplantation and editing). The main limitations of mRNA therapeutics arise from the complexity of mRNA delivery to the therapeutic target (cell, tissue, organ), inefficiency of subsequent release of the mRNA into the cytoplasm, and its low durability in the cellular environment. Chemically modified and labelled RNA molecules proved to be invaluable tools, that can offer insight into RNA biological functions and transformations. However, chemical modification of RNA is challenging, as it requires high selectivity and efficiency, while maintaining the fragile RNA molecules intact. These problems escalate as the molecular size of the modified RNA increases. As such, post-transcriptional modification of mRNA can be a highly demanding task, even for an experienced biochemist. In order to help the scientific community overcome major limitations of mRNA therapeutics, the market needs efficient, selective and scalable methods for covalent modification of macromolecular RNA.



TECHNOLOGY

We have developed a cost-effective and robust method for site-selective RNA modification that can be used for real-time mRNA monitoring (and more). We can modify both the 5' and 3' ends (a one-pot procedure, orthogonal labeling) of RNA molecules of almost any size. For example, we can synthesize dually labeled mRNA (carrying two different fluorescent dyes) encoding a reporter protein (i.e., green fluorescent protein). When the dually labeled mRNA decays, the 5'- and 3'-end labels dissociate, which can be easily followed by fluorescence microscopy. This makes it possible to observe the dynamic "life" of mRNA in real time: its delivery, expression, accumulation/aggregation and degradation in complex biological environments (i.e. in cells and in vivo), as well as use the dually labeled mRNA as a molecular probe in an in vitro assay.

INVENTION DETAILS

Our chemical method of RNA 3'-end modification is based on the previously unrecognized superior reactivity of N-substituted ethylenediamine (EDA) derivatives in the reductive amination of periodate-oxidized RNA. By using this method, we have been able to obtain fluorescently labeled or biotinylated RNAs of different lengths (from 3 to 2000 nucleotides), containing different chemical structures at their 5' ends (including the m7G cap), in high yields (70–99% by HPLC and gel electrophoresis). The method is scalable (efficient labeling of 0.5–500 μg mRNA in one pot) and combined with label-facilitated HPLC purification yields highly homogeneous products (>95% by HPLC and gel electrophoresis). The combination of 3'-end labeling with 5'-end labeling by click chemistry (SPAAC reaction) provided a one-pot protocol for site-specific RNA bifunctionalization, giving access to two-color fluorescent RNA probes. These probes exhibited fluorescence resonance energy transfer (FRET), allowing real-time monitoring of several RNA-degrading enzymes (RNase A, RNase T1, RNase R, Dcp1/2 and RNase H). The double-labeled mRNAs were efficiently translated in cultured cells and zebrafish embryos, which, in combination with their detectability by fluorescence and the scalability of the synthesis, opens up new avenues for the investigation of mRNA metabolism and the fate of mRNA-based therapeutics.

DEVELOPMENT AND NEXT STEPS

The next steps of development could involve:

1. broadening chemical repertoire: up-to date we developed reagents (EDA derivatives) that can be used for post transcriptional incorporation of fluorescent dyes (carboxyfluorescein, sulfo-cyanine3 and sulfo-cyanine5), biotin, and reactive groups (alkyne, azide, amine) directly to the 3’ end of RNA. The range of modifications explored further (e.g. incorporation of different types of moieties and labels, bioconjugation with peptides, lipids, saccharides, nucleic acids, and nanomaterials). The 3’ end labeling works well with “copper-free” click chemistry (SPAAC reaction), however expansion in this area could be fruitful (e.g. combination with squaramides, NHS esters, iEEDA, photo-click/crosslinking etc.);
2. further biological evaluation (i.e. influence of the labels/modifications on subcellular localization and stress-response induction);
3. ex vivo studies in mammalian models.

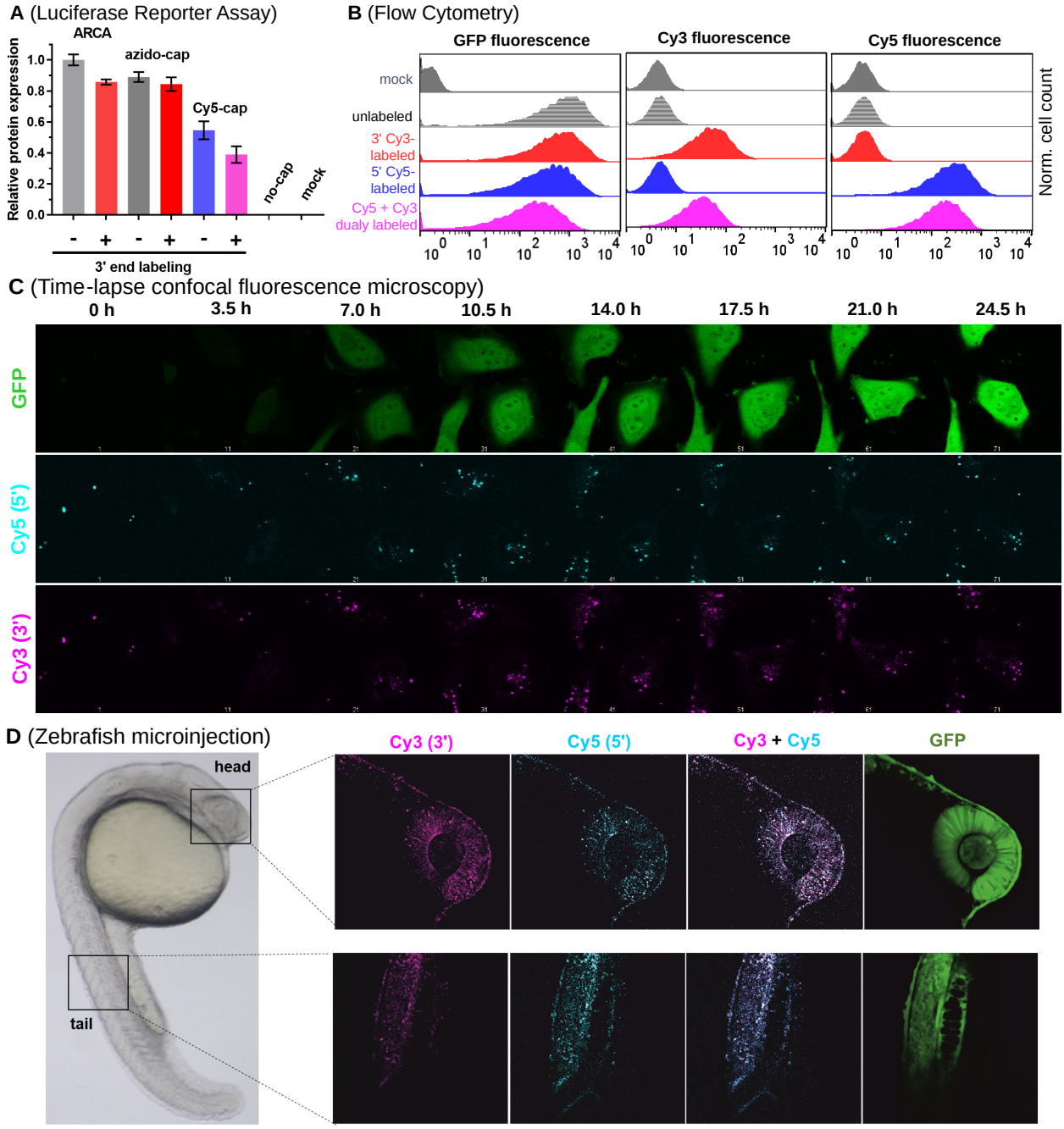
CURRENT SOLUTIONS

		UW technology	Pierce RNA 3' End Biotinylation Kit (ThermoFisher)	5' EndTag DNA/RNA Labeling Kit (Vector laboratories)	HighYield T7 RNA Labeling Kit(s) (Jena Bioscience)
Site of RNA labeling		5’ and/or 3’ end	3’ end	5’ end	Internal, random
Labeling type/strategy		cotranscriptional and/or posttranscriptional (IVT and/or one-pot labeling)	posttranscriptional (IVT and one-pot labeling)	posttranscriptional (IVT and three-step labeling)	cotranscriptional (IVT)
Interference with the process of protein biosynthesis		Correct protein synthesis with good translation yield[1] (50–100% in rel. to unlabeled mRNA)	Correct protein synthesis with low translation yields[2] (~20% in rel. to unlabeled mRNA)	Not suitable for eucaryotic mRNA due to lack of m7G cap at the 5’ end[3]	No/low and incorrect protein synthesis due to random nucleobase modification[4,5]
Labeling yield (for different RNA lengths)	**<100 nt**	85–99%	70–80%	n.d. (up to 99%)[3]	not applicable (cotranscriptional labeling)
	100–1000 nt	75–90%	70–80%	n.d. (up to 99%)[3]	
	>1000 nt	70–85%	n.d. (20–80%)[2,3]	n.d. (up to 99%)[3]	
Labeling principle/procedure		1) IVT with azido-modified initiator (e.g. N3-m7GpppNN), followed by 2) one-pot 5’+3’ chemical labeling or direct one-pot 3’ chemical labeling	Kit uses T4 RNA ligase to conjugate a single nucleotide analog to the 3' terminus of an RNA strand	1) RNA’s 5' triphosphate is dephosphorylated with the alkaline phosphatase; 2) T4 polynucleotide kinase transfers a thiophosphate from ATPγS to the 5' -OH group of RNA; 3) RNA’s 5’-tiophosphate is coupled to a thiol-reactive label (not included).	in vitro transcription (IVT) in presence of NTP analogs
Incubation time and temperature (labeling step(s))		2 h @ 25°C	2–16 h @ 16°C	2x 0.5 h @ 37°C and 0.5 h @ 65°C or 2 h @ 22°C	0.5–4 h @ 37°C
RNA concentration in reaction mixture		10–1000 µM (oligoRNA) 0.2–1 µM (mRNA)	1.7 µM (oligoRNA)	up to 60 µM (oligoRNA)	not applicable (cotranscriptional labeling)
Cost of the 3’ end labeling reagents (UW) or price of a labeling kit (Thermo, Vector, Jena)		< 1 € per reaction (no enzymes involved)	~ 37 € per reaction	~ 23 € per reaction	~17 € per reaction
		10–100 € per gram of mRNA	~6 mln € per gram of oligoRNA	~0.4 mln € per gram of oligoRNA	~0.8 mln € per gram of mRNA*

1. Mamot et al. NAR 2021 (doi.org/10.1093/nar/gkab867);
2. Depaix et al. Chem. Eur. J. 2021 (doi.org/10.1002/chem.202101909);
3. Lai et al. Nat. Comm. 2018 (doi.org/10.1038/s41467-018-06792-z);
4. Baladi et al. JACS 2021 (doi.org/10.1021/jacs.1c00014);
5. Custer & Walter Prot. Sci. 2016 (doi.org/10.1002/pro.3108).

*Assuming IVT yield of ~3 g/l. oligoRNA: noncoding RNA below 100 nt; mRNA: protein-coding RNA (typically 600–2500 nt); n.d.: not determined/provided by the manufacturer.

EXPERIMENTAL DATA



A) Relative expression of Gaussia luciferase after mRNA transfection (HeLa cells) as a function of the presence of 3' Cy3 modification (+/- 3' end labeling) and 5' cap structure (ARCA, azido-cap, Cy5-cap). As a negative control cells were transfected with uncapped mRNA (no-cap) or with MQ water (mock).
B) Flow cytometry readouts after transfection of fluorescent mRNA (3' Cy3-labeled, 5' Cy5-labeled, or Cy5 + Cy3 dually labeled) encoding eGFP. Unlabeled mRNA (ARCA-capped) and mock (transfection with MQ water) served as a positive and negative controls, respectively.
C) Time-lapse microscopy images of HeLa cells transfected with dually labeled mRNA (5' label: Cy5, 3' label: Cy3) encoding eGFP. Time scale set for 0 h at the beginning of recording of the images (1 h after transfection start).
D) Confocal microscopy images of embryos injected with 300 pg of dually labeled mRNA (5' label: Cy5, 3' label: Cy3) encoding eGFP captured at 24 hpf in tail or head sections.

Detailed description and additional data be found at: Mamot et al. NAR 2021 (doi.org/10.1093/nar/gkab867)

IP STATUS AND PUBLICATIONS

N-(2-AMINOETHYL)MORPHOLINE-BASED RNA ANALOGS, METHOD FOR THE PREPARATION AND USE THEREOF, Publication number: 3211579. (Patent proceedings underway in the US, EPO, JP, KR, CA, AU)

- Ethylenediamine derivatives efficiently react with oxidized RNA 3' ends providing access to mono and dually labelled RNA probes for enzymatic assays and in vivo translation, Nucleic Acids Research 2022
- Azido-Functionalized 5' Cap Analogues for the Preparation of Translationally Active mRNAs Suitable for Fluorescent Labeling in Living Cells, Angewandte Chemie International Edition 2017

INVENTOR TEAM

The Jemielity Group is a team of more than 20 scientists at the University of Warsaw dedicated to improving methods for the chemical synthesis of nucleotide analogs, designing new modified nucleotides as tools for molecular biology, and coupling nucleotides to labels, markers, and new materials. Special attention is given to the development of eukaryotic mRNA modifications, including cap analogues, with potential therapeutic applications. In addition to scientific publications in journals such as Nucleic Acids Research, Chemical Science, Nature Communications, Nature Chemical Biology, and the Journal of the American Chemical Society, the team is leading in technology commercialization in Poland.

PATENTS

- **US20100233757** - Synthesis and use of anti-reverse phosphorothioate analogs of the messenger RNA cap – licenced do BioNTech AG in 2010 and applied in several clinical trials
- **US20110092574** - mRNA Cap Analogs - Licensed to BioNTech AG in 2010
- 4 other international patents licenced to ExploRNA Sp. z o.o.

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