Faculty of Science
CHARLES UNIVERSITY

Department of
ORGANIC CHEMISTRY

Prague, February 5, 2024

Subject: PhD Thesis Report

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Reviewer: Jiří Míšek, Ph.D.

Thesis Title: Reactive modifications of RNA for bioconjugations with proteins and new enzymatic methods for the synthesis of base-modified RNA

The thesis is split into two main topics. The first deals chloroacetamide (CA) modification of RNA that is introduced through modified 7-deazaadenosine triphosphate and subsequent enzymatic incorporation. Such a reactive modification of RNA is highly important in biomedical research as it allows interrogation of biological binding and functions of RNAs. Despite precedents of utilization of reactive RNA probes, this new approach offers an alternative with a different mode of action. The author started with the successful synthesis of the CA-modified adenosine triphosphate. This building block was then tested in in vitro translation using T7 RNA polymerase. T7 RNA polymerase requires free cysteines for its activity, and modifications with iodoacetamide abolishes this activity. Despite this intrinsic danger, the author was able to use T7 RNAP for single or multiple incorporation of chloroacetamide modification to RNA of various lengths. These constructs were then used for crosslinking studies with various smaller nucleophiles and proteins. These experiments revealed essential features of the RNA probes that can be further utilized for in vitro/in vivo protein-binding studies.

In the next part of the thesis, the author developed a new approach for the enzymatic synthesis of heavily modified RNA. She recombinantly expressed two engineered DNA polymerases known to incorporate nucleoside triphosphates. TGK polymerase showed superior to SFM4-3 and enabled incorporations of diverse chemical modifications of all four bases simultaneously. Furthermore, the author devised a method for DNA primer cleavage and in-situ fluorescent labeling of the synthesized RNA. These approaches were then utilized for advanced studies on the binding of an RNA aptamer and epitranscriptomics.

The author did an incredible amount of work during her PhD studies. This is exemplified by the hefty 252-page long thesis. Given all the unsuccessful experiments that are necessarily associated with such projects, this makes her effort even more impressive. She mastered a broad range of chemical, biochemical, and molecular biology techniques, which makes her a genuinely interdisciplinary scientist. The thesis itself is of highest quality and I undoubtedly recommend this thesis for the defense.

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Questions:

1. On page 65, the PAGE analysis shows that even in the control reaction without ATP, there is still a detectable full-length product. Do you have any explanation for this observation?

2. On page 72, there are structures of small molecules and peptides used for the bioconjugation reactions. The conclusion of the experiments with peptides is that cysteine and histidine do react, whereas lysine and arginine do not. However, I think better controls for lysine and arginine would be needed to make this claim. Would **pept-(+)-H** with lysine or arginine instead of histidine react with the RNA probe?

3. On page 93, there is an SDS-PAGE analysis of CA-RNA probe crosslinking experiments in a HeLa cell lysate. Many proteins get labelled. I believe that that the application of CA-RNA probes in searching for the new RNA-binding proteins is the ultimate goal of the project. For this purpose, it would be useful to see the background labeling with, for instance, CA-modified cyanine 5 probe. Do you have any idea about the specificity of the labeling, and have you proceeded to any untargeted proteomics?

4. On page 94, there is an analysis of RNA crosslinking with HuR protein in a cell lysate showing a band at 250 kDa. This observation is explained by oligomer formation. Why is this behavior not seen with HuR protein itself?

5. Based on the reference 279, K_D of the adenine binding to the adenine aptamer is in the nanomolar/submicromolar range. FRET analysis depicted in Fig. 94 indicates K_D in the millimolar range. Do you have any explanation for this difference?

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