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Evaluation of the doctoral thesis:
Reactive modifications of RNA for bioconjugations with proteins and new enzymatic methods for the synthesis of base-modified RNA

PhD candidate: Mgr. Mária Brunderová

The thesis presented by Mgr. Mária Brunderová focused on the development of new strategies for the synthesis of RNAs with different functional groups that allow visualisation, detection and/or reactive cross-linking. This is a timely and highly relevant topic, as RNAs and RNA technologies have recently emerged as highly relevant therapeutic tools. Formally, the thesis is written in clear English. It is logically organised to first provide a comprehensive overview of the advances in RNA synthesis and the introduction of specific ribonucleoside modifications. It provides the necessary background on both solid base and enzymatic approaches and, most importantly, on the practical applications of such synthetic molecules. As a molecular biologist myself, I appreciated the way Maria described the steps of chemical synthesis and different alternatives for the reactive groups. This introductory part also explains the motivation of the thesis to search for a novel ribonucleoside triphosphate building block with reactive chemical groups, which would allow functional studies of RNA-protein interactions, for example. From a biologist's point of view, this would ultimately be a goldmine for a range of studies and the potential to manipulate protein functions in relation to their role in certain diseases.

Maria's PhD work has several interlinked and ambitious aims, which can be summarised in the search for tools to produce chemically modified RNA molecules to study RNA-protein interactions and, possibly in the long term, to enable functional studies in cells.
Based on previous positive experiences with chloroacetamide (CA)-modified DNA in the laboratory, Maria explored the possibility of using the same modification in RNA. She succeeded in using a one-step aqueous Pd-catalysed Sonogashira cross-coupling reaction to produce CA-linked adenosine triphosphate ($rA^{CA}TP$), which was then used as a building block for T7 polymerase in vitro transcription of RNAs containing CA modifications. She then demonstrated the ability of CA-modified RNA to specifically react with Cys and His residues and to form stable RNA-protein conjugates. She performed a series of validation experiments to show that CA labelling of RNAs can be used to capture RNA-binding proteins from complex samples such as cell lysates and to identify RNA-binding regions in these proteins.
Maria's second achievement was to use a thermostable DNA polymerase, previously engineered to synthesise RNA, to produce molecules with multiple rNxMPs incorporated. She used the TGK and SFM4-3 enzymes. This is particularly important as the preparation of RNAs with specific modifications is a bottleneck in many biological and medical studies. The range of molecules that can be produced using this enzymatic approach is astonishing, but the synthesis has a number of drawbacks that need to be addressed.
Maria took the studies a step further by looking at the site-specific incorporation of one or more rNxTPs. Using the engineered DNA polymerase, she was able to produce longer RNAs with various modified rNxMPs and performed several experiments aimed at investigating the function of some of the chemical modifications, e.g. their effect on translation/protein synthesis in vitro.
Throughout this impressive collection of work, Maria maintained an important level of critique, evaluating the pros and cons of her tools. I am particularly impressed by how she has combined her

chemistry skills with additional experimental validation of the functionality of her system, e.g. cross-linking experiments, optimisation of fluorescent labelling to avoid transient isotope labelling, protein binding validations, in vitro translation assays, etc. In summary, this is a high quality thesis, excellent in experimental, intellectual and final written form. Without any doubt, I agree with Maria's ability to defend the thesis and I propose to award her the highest grade.

I have a few questions for discussion:

1. What is the time frame and yield for the production of e.g. 1rA^{CA}MP modified 30nt RNA of a specific sequence? Does the candidate envisage her protocol being used regularly to produce such RNAs for specific biochemical experiments?

2. How stable is an RNA oligonucleotide containing rA^{CA}MP by incubation with a cellular lysate?

3. Can you compare the advantages and disadvantages of using, for example, thioU versus rA^{CA}MP-labelled RNAs to capture specific RNA-binding proteins? Have you tried if endogenous RNA polymerase could accept the rA^{CA}TP?

4. Can TGK polymerase incorporate pseudouridines?

5. Does the presence of multiple rA^{CA}MPs in a longer RNA molecule affect its secondary conformation/base pairing?

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