Analysis and characterization modified in vitro transcribed RNA using LC-MS/MS method

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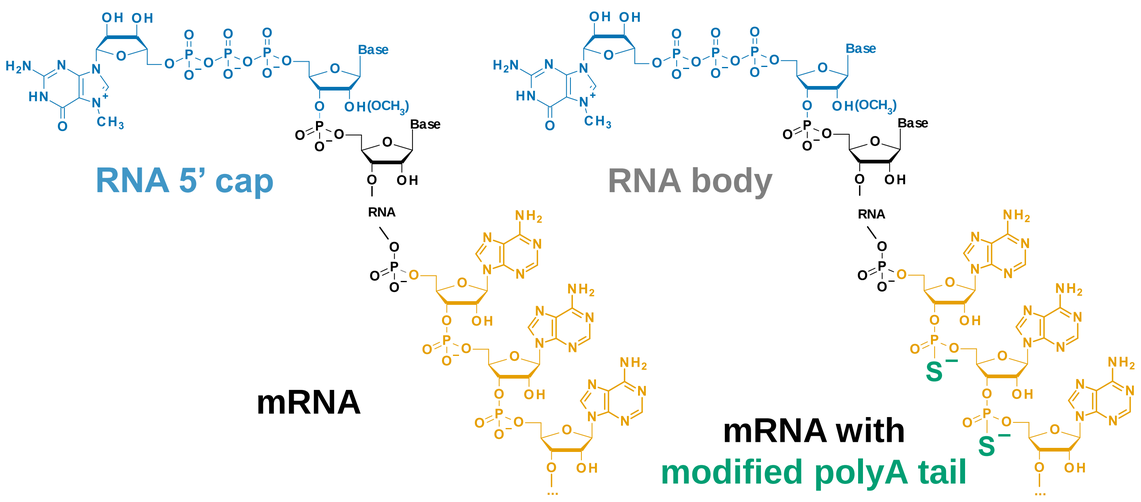
Introduction

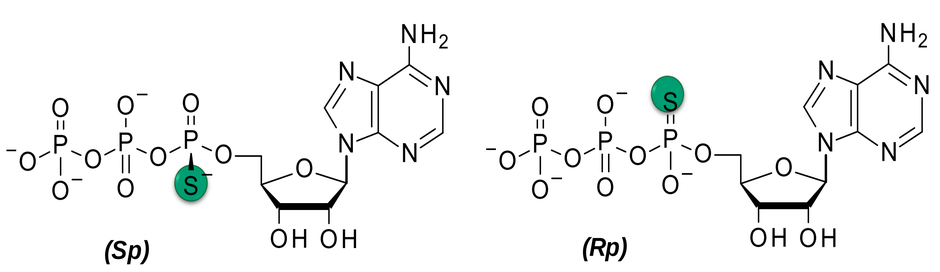
Messenger RNA (mRNA) is a promising therapeutic agent with potential uses in cancer therapy, gene therapy, and cell reprogramming. The stability of mRNA largely depends on the 3'- end and 5'- end structures, which are targets for enzymatic degradation. Chemical modifications in those regions can be introduced into *in vitro* transcribed mRNAs in order to confer on them resistance to degradation and increase their translation efficiency, thereby increasing their therapeutic potential.



Aim of the project

The project is aimed at the development of LC-MS/MS method for quantitative analysis of modified nucleotides introduced to RNA

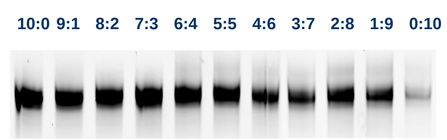
Examples of modified nucleotides introduced to RNA



Methods

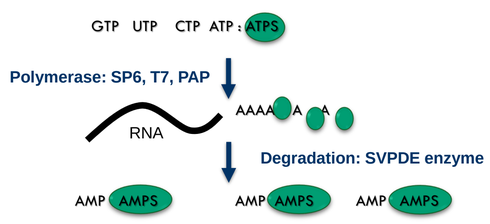
In vitro transcription

- RNA was obtained in in vitro transcription using different ratio of ATP and ATPS



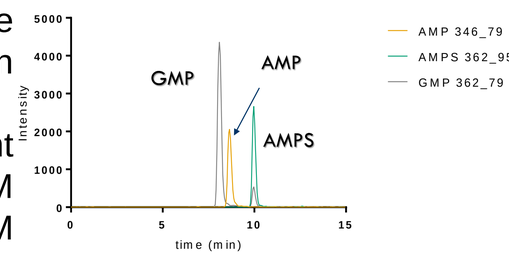
RNA synthesis and degradation

- Modified RNA was enzymatically degraded to single nucleotides
- Obtained nucleotides were resolved and quantified by LC-MS/MS
- We use ESI-QQQ (Qtrap-3200, Sciex) coupled with Agilent 1290 Infinity



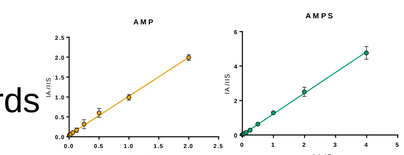
MRM chromatograms

- We applied ion pair chromatography (**N,N-Dimethylhexylamine**) and **C-18** column (Eclipse XDB, Agilent) to separate RNA degradation products
- We use 0,700 ml/min flow rate, linear gradient 15min 50% mobile phase B; phase A: 10mM DMHA pH 5.0, phase B: 50% acetonitrile, 10mM DMHA pH 5.0



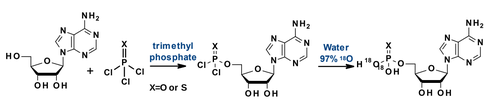
Calibration curves of AMP and AMPS with their internal standards

- We prepared calibration curves for each of analyzed nucleotides using the synthetized isotope-labeled standards
- Concentrations range: 0,015 – 4 μM



Synthesis of ^{18}O labeled AMP, AMPS

- We synthesized RNA degradation products labelled with heavy oxygen (^{18}O) within the phosphate and used them as internal standards



LC MS/MS analysis

$$\frac{I_A}{I_{IS}} = a\frac{C_A}{C_{IS}}$$

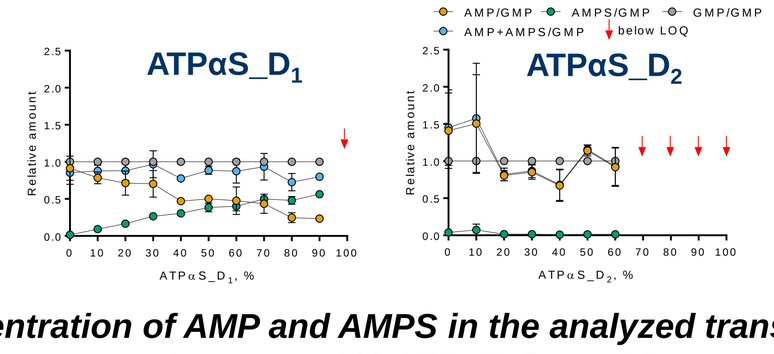
I_A- intensity of analyze,
I_{IS}- intensity of internal standard,
C_A- concentration of analyze,
C_{IS}- concentration of internal standard

Concentration of AMP and AMPS
↓
AMP : AMPS ratio
↓
Effectiveness of introduction of ATP and ATPS

Results

Effectiveness of introduction of modified nucleotides into RNA

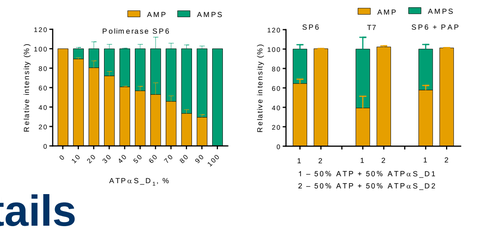
- We proposed the quantitative method that enabled determination of frequency of incorporation of ATPαS_D1 into in vitro transcribed RNA using polymerase SP6.



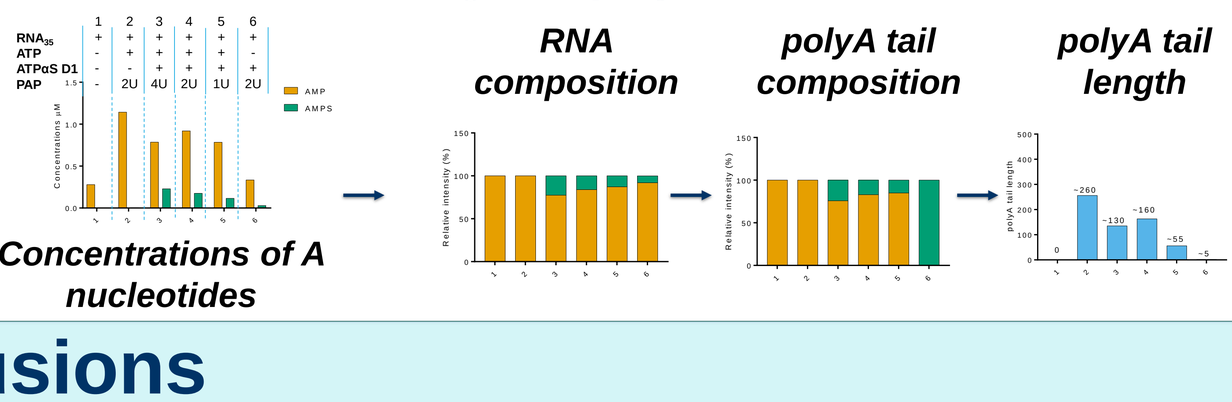
Concentration of AMP and AMPS in the analyzed transcript as a function of % ATPαS_D_1 and D_2

Comparison of substrate selectivity of different RNA polymerases

- We characterize various popular RNA polymerases (such as SP6, T7, PAP) with regard to their selectivity towards ATP and ATP derivatives (including stereochemical preferences).



Length of polyA tails



Concentrations of A nucleotides

Conclusions

- We proposed a quantitative method that enables determination of frequency of incorporation of thiophosphate modification into IVT RNAs
- The procedure will allow for the structural characterization of modified mRNA and help elucidate structure-activity relationship for these molecules
- The research will contribute to the development of the methodology of the quantitative determination of synthetic modifications introduced into RNA and those occurring naturally
- The proposed procedure could contribute to popularization of the application of low resolution equipment in the analysis of large molecules such as nucleic acids
- This research will be first step in the formulation of general procedures and protocols for the determination of modified and unmodified nucleotides within mRNAs, including mRNAs introduced into cells to exert therapeutic effects

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