Summary

Enzymatic degradation (decapping) of the cap - a characteristic structure at the 5' end of mRNA - is a key cellular process for regulating gene expression levels. Decapping, which involves the hydrolysis of a phosphate bond located in the bridge connecting the cap structure to the downstream nucleotide sequence in the RNA, results in the exposure of the 5'-monophosphorylated RNA to degradation by exoribonucleases. To date, approximately 20 enzymes capable of cap degradation have been described in the literature, but their substrate preferences have only been roughly estimated. The exact mechanisms of activation of these enzymes are also unknown. It is known that their recruitment requires a number of accessory proteins and other factors, such as divalent cations, which together facilitate the adoption of the appropriate conformation of the active protein and RNA. In addition, numerous reports in the literature indicate the existence of different variants of the canonical cap m^7G structure in non-coding RNA molecules. A complete picture of RNA metabolism forms the basis for understanding cell dynamics and is thus fundamental for the development of new therapeutic approaches. The development of appropriate tools to visualize the course of RNA degradation *in vitro* and *in vivo* seems to be crucial to achieve this goal.

The research carried out within this thesis concerns the synthesis and characterization of RNA structure-based fluorescent probes capable of selectively monitoring the activity of cap-degrading enzymes.

The first phase of my research involved the development of a fluorescence intensity (FLINT) assay that allows high-throughput analysis of the substrate specificity of decapping enzymes. To this end, I synthesized seven natural, di- and trinucleotide analogs of cap using a chemical synthesis approach that combines phosphorus (III) chemistry with phosphorus (V) chemistry. Each compound was subjected to a two-step purification to ensure the highest possible sample quality. In the next step, the prepared cap analogues were cotranscriptionally incorporated into the 70-nucleotide RNA sequence. *In vitro* transcription (IVT) was performed on custom-designed DNA templates encoding the Broccoli aptamer sequence enriched with a polyA fragment. Optimization of the entire process allowed to obtain pure fractions, free from contamination in the form of uncapped RNA and with high final yields. The quality of the RNA was verified by gel electrophoresis. The final RNA, together with its specific fluorogenic ligand, DFHBI-1T, was used to perform a fluorescence assay to determine the substrate preferences of seven selected decapping enzymes from different organisms. This part of the study also included kinetic measurements with the hDcp2 enzyme and cap-1 to determine the kinetic parameters of the reaction. The usefulness of the FLINT assay for finding inhibitors of decapping enzymes was also demonstrated. The determination of the activity range of individual enzymes was used to develop dually labeled fluorescent probes for a single molecular target, which was the focus of the second phase of my research.

I began the synthesis of dually labeled fluorescent probes by preparing trinucleotide cap analogs, containing linkers that enable dye attachment. In this form, the cap analogues were cotranscriptionally inserted into the RNA strand and subsequently labeled with appropriate dyes. The second approach was the labeling of trinucleotides and then the IVT was performed as the next step. Regardless of the strategy adopted, the labeled and capped RNAs were subjected to labeling at the 3' end of the sequence, using either an enzymatic or chemical method. The dyes were chosen to form either fluorophore-quencher or donor-acceptor pairs, allowing observation of the FRET phenomenon. The fluorescent probes were characterized by biophysical methods, and those with the most favorable properties were also characterized for their suitability for monitoring enzymatic reactions with cap-degrading proteins.

The RNA structure-based probes and assays presented in this paper allow rapid, high-throughput analysis of substrate preferences of decapping enzymes. They provide an extremely useful tool for the complete characterization of enzymes, allowing the determination of their substrate specificity and selectivity and the identification of new enzyme inhibitors. This is particularly important in the context of the development of new therapeutic approaches based on RNA molecules, where durability and stability are key elements. The tools I have developed are thus a response to a growing need in an era of intense development in biotechnology and medicine.