## Abstract

This doctoral thesis focuses on the enzymatic synthesis of base-modified RNA probes with diverse functional groups, including reactive cross-linking, hydrophobic and fluorescent moieties, or affinity tags. The construction of nucleobase-modified oligonucleotides is accomplished either through conventional *in vitro* transcription with T7 RNA polymerase or by an innovative approach leveraging engineered mutant DNA polymerases and primer extension reaction (PEX).

In the first section of the thesis, a novel ribonucleoside triphosphate building block with reactive chloroacetamide functionality was synthesised using an aqueous Pd-catalysed Sonogashira cross-coupling reaction, directly applied on iodinated nucleotide. The chloroacetamide modified triphosphate was then tested as a putative substrate for T7 RNA polymerase in *in vitro* transcription reaction, aiming to construct RNA probes with one or multiple reactive groups. The selectivity of chloroacetamide-modified RNA for thiol-, or cysteine-, and histidine-containing (bio)molecules was demonstrated by model bioconjugation reactions and cross-linking experiments with three RNA-binding proteins of diverse structures and functions. The efficient formation of RNA-protein covalent adducts was confirmed by western blot or gel, and mass spectrometry analyses conducted under denaturing conditions. Identification of the RNA-binding sites and targeted amino acids residues within the model proteins was performed through proteomic analysis of cleavage products. The effectiveness and potential applicability of the novel modified RNA probe was further demonstrated by cross-linking with extracted cellular proteins, where sequence specific protein recognised and cross-linked only to its cognate modified RNA probe.

In the second part of the thesis, a novel methodology was developed for constructing base-modified RNA oligonucleotides with varying lengths and number of unnatural nucleotides utilising engineered thermostable DNA polymerases and PEX. Modified nucleotides, featuring diverse functional groups attached to position 5- of pyrimidines or position 7- of 7-deazapurines, were investigated as suitable substrates for two engineered polymerases – TGK and SFM4-3. Both mutant polymerases demonstrated efficiency in incorporating either one or four modified nucleotides into a single RNA strand. The increased activity of TGK polymerase was showcased by synthesising fully modified RNA probes, wherein all four canonical nucleotides were replaced with modified counterparts bearing distinct functional groups. This surpassed the performance of the conventional T7 RNA polymerase *in vitro* transcription. Conversely, SFM4-3 polymerase failed in achieving this level of activity. Efficient elongation was also demonstrated with a DNA primer, facilitating the construction of intriguing DNA-RNA hybrids. Moreover, a straightforward methodology utilising selective 2'-deoxyuridine cleavage was developed to enable the removal of DNA primer from the synthesised part of RNA, facilitating the generation of hypermodified RNA probes where each nucleobase is modified. Additionally, a method for more challenging the selective labelling of RNA at specific positions, utilising combination of single nucleotide incorporation (SNI) and PEX, was successfully developed.

RNA probes decorated with two distinct fluorophores at internal positions were employed for fluorescence structural studies. Importantly, this approach was also applied to the synthesis of region-modified or point-modified messenger RNAs, revealing for the first time, that a single 5-methylcytidine modification within protein-coding region significantly enhanced protein production in both *in vivo* and *in cellulo* experiments when compared to its natural or fully modified mRNA counterparts.