Masroor Ahmed Khan

Synthesis of bioluminogenic substrates of firefly and NanoLuc® luciferases and validation of their response to analytes involved in redox homeostasis.

Bioluminescence is a phenomenon that relies on the emission of light resulting from the oxidation of the light-emitting molecule luciferin by its corresponding luciferase enzyme. It is pivotal in biological research due to its high sensitivity, non-invasive nature, and ability to enable real-time monitoring. One application of bioluminescence is the detection of physiologically and pathologically-relevant analytes in through the use of activity-based probes. These bioluminescent probes are engineered to sense specific analytes by masking the functional groups of luciferin with an analyte-targeting moiety, which prevents its reaction with luciferase. When the target analyte is encountered, the masking group is cleaved, freeing the luciferin that can then react with luciferase to generate photons, thereby allowing the quantification of the analyte's presence and activity through the emitted light.

Many diseases involve complex interactions between multiple analytes. Understanding these interactions can inform more effective therapeutic approaches. This requires a simultaneous monitoring of multiple analytes but this approach has been rarely explored for bioluminescent probes. It can be achieved by the use of two separate single-analyte probes, ideally with the same luciferase system to avoid doubling up on the need of introducing two enzymes to the system. Split-luciferin strategy is well suited for that purpose as two separate halves can be caged independently and upon uncaging, undergo a bio-orthogonal reaction at physiological pH to form active D-luciferin. Another way of simultaneous analytes' monitoring is a use of the so-called dual-analyte bioluminescent probes, with each containing two analyte-responsive groups. This eliminates as challenges of the use of two single-analyte probes that can suffer from differences in their pharmacokinetics and co-localization complicating signal interpretation. Nevertheless, up to date, very few such bioluminescent probes have been reported.

The aim of this work was to synthesize and characterise several bioluminescent probes to expand the palette of bioluminescent tools for simultaneous detection of multiple analytes. The primary scaffolds utilized in this research are based on

firefly luciferin and furimazine, a substrate for the small luciferase NanoLuc®. NanoLuc® offers advantages such as brighter luminescence, smaller size, and greater stability compared to traditional luciferases, and it does not require cofactors like ATP or Mg^{2+}, which are needed for firefly luciferase. This work targets analytes related to redox homeostasis, specifically iron, gamma-glutamyltransferase (GGT), and nitroreductase (NTR), as the importance of their interplay in diseases such as cancer is vital and still not fully understood.

First, a split luciferin strategy-compatible probe was developed for the detection of ferrous ions. This probe exhibited responsiveness when tested in vitro and in cell lysates. However, while the expected response could be observed in live cell studies, low intensity and high complexity of response posed challenges in reliable interpretation of the results. Further investigations revealed that certain metal ions, including Fe(II) have the ability to biologically inhibit luciferases, with firefly luciferase being particularly susceptible to this inhibition. This work has therefore contributed to the understanding of challenges associated with the reliable use split luciferin design in real-life detection of analytes in biology and provided tools to ensure their robust validation.

Next, three different nitroreductase-responsive (NTR) probes were developed, compatible with split luciferin as well as NanoLuc systems. In particular, two variants were developed based on 2-cyano-6-hydroxybenzothiazole and D-cysteine. The novel D-cysteine probe, in particular, demonstrated significant activity in vitro, which was validated through spectroscopic and high-performance liquid chromatography (HPLC) kinetic studies. Additionally, the first furimazine-based bioluminescent probe was created that enables potentially more reliable NTR detection taking advantage of the cofactor-independent functionality of the NanoLuc® luciferase. This probe also exhibited a robust response, even at low concentrations of NTR.

Finally, a dual-analyte probe was synthesized based on the aminoluciferin scaffold, which was capable of detecting simultaneous presence of nitroreductase (NTR) and gamma-glutamyltransferase (GGT). This validated the feasibility of using a single molecular probe to monitor multiple analytes simultaneously. Additionally, a D-luciferin variant of this dual-analyte probe was developed, featuring a dual-analyte responsive motif for NTR and GGT as intermediate that can be conjugated

to other fluorophores or bioluminophores. These results expand the available molecular toolset of multi-target bioluminescent probes.

In conclusion, this thesis represents advancements in the field of bioluminescent probes, generating novel tools for the concurrent investigation of multiple analytes within biological systems and shading the light on advantages and limitations of variable probe designs. These probes, therefore, inform future use of such tools in cellular and in vivo applications with higher reliability, which is critical for disease understanding and ultimately development of enhanced therapeutic approaches.