MSc Anna Wychowaniec

Multi-analyte fluorescent small molecule probes for investigation of biologically relevant local microenvironment

Cells can be likened to nano-factories, where compartmentalization enables opposing processes to occur simultaneously. Despite years of research, obtaining a detailed real-time view of these reactions and the specific changes in the cellular nanoenvironment remains a challenge. Current methods focus on measuring changes within a whole region (e.g. organelle) or require disturbing the whole system, resulting in significant information loss (e.g. with isolation of chosen macromolecules and *in vitro* tests). Acquiring a more detailed picture at the nano scale will change the way we understand physiological and pathological processes, leading us closer to molecular-level understanding and precision treatments.

The intracellular environment undergoes multiple changes concurrently. Observation of these changes is largely impossible without use of specialized tools and techniques as most components of cellular environments do not intrinsically generate detectable signal. One such method providing excellent resolution and causing minimal disturbance to cell homeostasis is fluorescence imaging. In particular, environment-sensitive fluorogenic tools have great potential in monitoring otherwise non-observable changes. Fluorogenic probes become fluorescent only upon interaction with a chosen analyte, allowing for the possibility of more reliable imaging without the need for their removal (so called “no-wash” staining).

To gain information about a specific target (e.g. an enzyme), probes can be directed using a covalently attached sensing group. One of the most promising labelling methods described in the literature is known as affinity-driven (or affinity-based) labelling. These probes consist of three components: a reversible ligand selective to the target, a reactive group (prone to attack of a chosen amino acid on the target'), and a reporter part (fluorophore). These probes offer selective fluorescent labelling of target proteins without the need for genetic modification, allowing potential observation of natural target activity. In theory, these probes could provide information about analytes in the target’s microenvironment, enabling real-time, *in cellulo* observations using advanced imaging (e.g. super-resolution microscopy). However, additional analyte detection around specific targets has not yet been achieved with affinity-driven probes.

The main objective of the current work was to design, synthesize and validate two fluorogenic environment-sensitive fluorophores and transform one of them into an affinity-based labelling probe for hCAII to possibly detect a chosen analyte (pH changes). To develop such probes, a comprehensive literature review was performed to identify two suitable fluorescent scaffolds: 4-sulfonamide benzo[*c*][1,2,5]oxadiazole and 1,8-naphthalimide. Synthesis of the final probes were based on established literature methods, and the selected scaffolds were synthesized and furnished to successfully produce two probes: SOLpH1 and SOLpH2 (confirmed by ^{1}H NMR, ^{13}C NMR and HRMS methods). These probes were designed with environment-sensitive properties, making them fluorogenic and sensitive to physicochemical changes in their closest microenvironment (pH changes and/or polarity changes). Their fluorogenic nature was directly related with their pH-sensing properties. Both molecules bear

a piperazine ring, with one lone pair on the nitrogen atom not engaged in the π-conjugated aromatic systems. At high pH the probes should not emit fluorescence or fluoresce poorly due to PeT quenching. Once the pH of microenvironment decreases, the nitrogen becomes protonated and an increase in fluorescence is observed.

The probes' emissions and sensitivity to polarity and viscosity was measured in presence of a set of biologically relevant analytes. Basic photophysical properties (fluorescence quantum yield, extinction coefficient, brightness) were determined for both probes at two different pH values (4.0 and 7.5) with use of standards with known properties. Colocalization experiments with mitochondria and lysosomes trackers in two different cell lines (healthy HEK293T and cancerous A549) were conducted. The last step was intracellular pH-detection in HEK293T cell line. The SOLpH1 probe was then introduced into an affinity-labeling one, SOLpH1-Tos. The probe SOLpH1-Tos was incubated with its potential target, hCAII (48 hours), the mixture was digested with trypsin and the obtained peptides were analyzed to identify possible labeling locations. Last but not least, pH-sensing properties and enzymatic activity of SOLpH1-hCAII labeled protein were studied. The conducted experiments confirmed the expected properties of the novel fluorophore structures. Both probes showed pH-sensing properties with an increase of emission between pH=4.0 and pH=8.0 (14-fold increase SOLpH1 (exc/em 420/600 nm) and 13-fold increase for SOLpH2 (exc/em 390/530 nm). Their calculated pK_a were respectively 6.4 (SOLpH1) and 6.5 (SOLpH2), making them both suitable for *in cellulo* applications. SOLpH1 exhibited larger Stokes shift changes between the two most polarity-extreme solvents/solutions, and showed higher emission intensity as polarity of the solvent decreased. SOLpH2, on the other hand, behaved in the opposite manner. While both SOLpH1 and SOLpH2 respond to viscosity changes, in more viscous environments, SOLpH1 displays a 12.5-fold emission intensity increase, while SOLpH2 presented fluctuating emission intensity with changing viscosity. Finally, colocalization experiments did not suggest any preference of the probes to localize in mitochondria or lysosomes. Furthermore, both probes were successfully able to monitor intracellular pH-changes.

Careful analyses of the obtained data resulted in the selection of SOLpH1 for further development towards an affinity-based labeling probe, SOLpH1-Tos. SOLpH1-Tos consists of a short ethylene glycol linker, tosylate/tosyl reactive group, cadaverine linker and reversible inhibitor for hCAII, benzenesulfonamide. The *in vitro* labeling experiment of human carbonic anhydrase II with the probe SOLpH1-Tos was successful, and analysis of resulting data confirmed a reaction occurring between SOLpH1-Tos and an amino acid in close proximity to the active site of the enzyme (histidine His64 at the entrance of the protein's active site, as opposed to His67 in the source publication). SOLpH1-Tos is, to the best of our knowledge, the first pH-sensitive probe for affinity-driven labeling of a protein target without the need of genetically encoded tag use. This novel probe displays utility molecule in pH-based experiments, and once attached on human carbonic anhydrase II, could provide insight into minor pH changes in immediate proximity to the active site of the enzyme. Such local pH changes may be critical for protein function, potentially crucial to physiological and pathological processes.