Final Report (630731 PerBioImage)

Introduction: Nature has produced intricate machinery to covalently diversify the structure of proteins after their synthesis in the ribosome. In an attempt to mimic nature, chemists have developed a large set of reactions that enable post-expression modification of proteins at pre-determined sites. These reactions are now used to selectively install particular modifications on recombinant proteins for many biological and therapeutic applications (**Figure 1, top**). Bioorthogonal site-selective chemistry may also be used to label specific proteins in living cells which allows uptake and intracellular trafficking to be tracked as well as for physiological parameters to be measured optically (**Figure 1, bottom**).

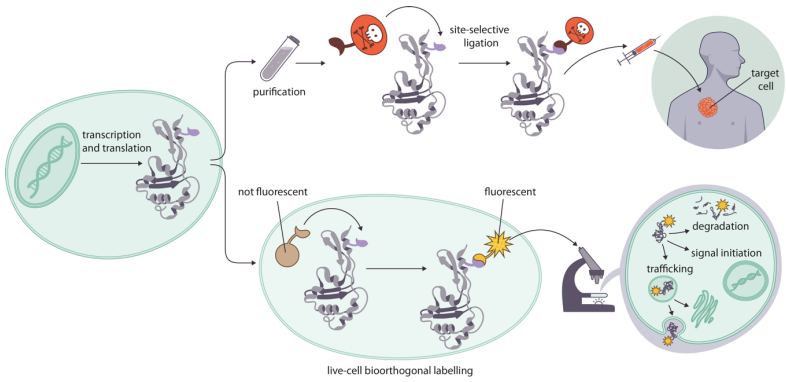

Figure 1. Conceptual strategy to achieve site-selective protein modification using biocompatible chemical reactions *in vitro* and *in vivo*. Typically, site-selective chemical modification of proteins can be achieved either on a natural amino acid side chain with unique reactivity (for example, cysteine or lysine) (top) or on a non-canonical amino acid featuring a side chain with a functional group that is normally not present in living systems but can be introduced using chemical or genetic encoding procedures (for example, azide, alkyne, alkene and tetrazine) (bottom).

Results & discussion: Bioorthogonal chemistry is now permitting to site-selectively install modifications on individual proteins in complex biological mixtures, including in living organisms (Nature Chem 2016, 103). In this project, I have explored a conceptually novel bioorthogonal approach that combines the use of small, stable handles that may be labelled upon chemical activation in a temporal controlled manner. The initial approach was focused on a new photo triggered [2+2] cycloaddition bioorthogonal ligation between two alkene containing partners as well as the known inverse electron-demand Diels-Alder cycloaddition (IEDDA) reaction between the minimal alkene-tag and a tetrazine. First, we developed a method for the site-specifically installation of the unstrained *S*-allyl cysteine (Sac) amino acid into proteins (Angew Chem Int Ed 2016,14683). We found that the photo [2+2] cycloaddition reaction between Sac and an alkene moiety (maleimide) allows controlled labelling of Sac-tagged proteins in the test tube, but the use of such approach in live cells failed because of selectivity issues (unpublished). On the contrary, we have demonstrated that unstrained S-allyl handles precisely installed at predefined cysteine residues within the sequence of a protein are suitable chemical handles for IEDDA reactions with

tetrazine dyes. This strategy allowed for selective labelling of proteins in live cells using a pre-targeting approach (Angew Chem Int Ed 2016,14683). The easy site-specific installation and the small size of the allyl handle, which is potentially less disruptive compared to non-canonical amino acids bearing bulky strained alkenes, is likely to be of a general benefit for other sensitive protein systems including antibodies used in pre-targeting approaches. As such, we believe that the simple site-specific labelling strategy disclosed here, which enables bioorthogonal live-cell imaging, will find significant use in the biological community, allowing imaging of specific targets with minimal effects on their intrinsic properties.

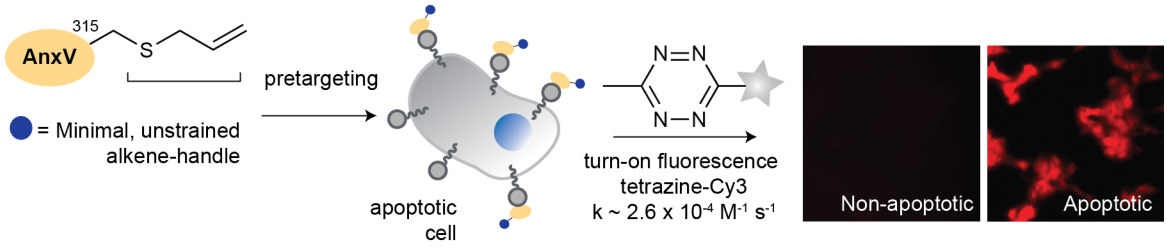

Figure 2. Pre-targeting of apoptotic cells with Anexin V-Sac followed by IEDDA labelling with fluorogenic Tz-Cy3. Specific labelling of apoptotic cells pre-targeted with Anexin V-Sac and subsequently labelled with Tz-Cy3. Cells were imaged 3 h after addition of the fluorogenic dye. Non-apoptotic cells were included as a control. Apoptotic cells are shown red. (Angew Chem Int Ed 2016,14683)

Future application: Current bioorthogonal reactions usually require the installation of bulky non-natural side-chains (norbornene or *trans*-cyclooctyne) and suffer from lack of selectivity (reaction of biological nucleophiles) and metabolic stability. We have developed an approach that uses small handles that do not disturb a protein's structure and activity and that may be activated in a temporal manner to undergo a rapid, fluorogenic reaction in living systems. We are interested in probing and characterizing the uptake and intracellular processing pathways of key proteins involved in disease progression, namely interleukins in solid cancers and leukaemias, in order to guide the development of targeted strategies for intracellular drug-delivery. This reaction and approach can be generalized to follow protein/receptor complex internalisation and degradation pathways.

Researcher: Research in the GBernardes Lab tackles a range of biological problems of fundamental importance to understand and fight human disease primarily through the use of chemistry principles. Since the start of the CIG project, and supported by additional generous funding from the Royal Society and the EU including an ERC Starting Grant (€1.5 million over five years), I have established a research group of 11 postdoctoral researchers and 15 PhD students who apply an interdisciplinary approach to Chemical Biology, from synthetic to computational chemistry and from molecular biology to immunology. I have published 64 independent papers

(89 in total) that were cited >4200 times leading to an h-index of 33, and have filed 7 patents. My research has been recognised by a number of prizes including the Chem Soc Rev Emerging Investigator Lectureship and the RSC Harrison–Meldola Memorial Prize both in 2016.

Since June 2018, I became a tenured University Lecturer in Chemical Biology at the Department of Chemistry, University of Cambridge. At the same time, I run a lab at the Instituto de Medicina Molecular in Lisbon where my CIG project was based. While our work in Cambridge focuses more on the synthetic aspects of our research programme, focus is placed on biology at iMM Lisbon. The award of the CIG project, just after finishing my postdoc, was key so that I could start establishing my independent research programme and also to obtain the preliminary data that allowed me then to secure additional funding, for example the ERC StG.