Bioorthogonal labeling tools to study pathogenic intracellular bacteria

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Chapter 1

General Introduction

Abstract

Over the years, the field of bioorthogonal chemistry has developed many biocompatible ligation reactions or 'click reactions', that are highly suitable for detection of biomolecules of interest by fluorescence-based analysis techniques. Click reactions are very interesting for the visualization of intracellular bacteria, due to the various challenges that are encountered when studying these organisms *in situ*. This chapter describes the basics of applying bioorthogonal chemistry to the study of intracellular bacteria.

1.1 The biochemistry of a cell

At the smallest level of organization, all life is built from cells. Cells are tiny compartments that exist to seclude the internal processes, that are essential to life, from the outside, that is considered lifeless. Life is fundamentally driven by chemistry, primarily used to metabolize fuel into energy (catabolism) and build new biomolecules (anabolism).[1] A cell consists of four major types of biological macromolecules; proteins, carbohydrates, lipids and nucleic acids. These macromolecules themselves are made from smaller biological building blocks, such as amino acids, sugars, fatty acids and nitrogenous bases.[2] The biochemistry of a cell is highly complex, and it has been estimated that around a billion reactions occur per cell per second. There are six major classes of biochemical reactions; oxidation/reduction, hydrolysis/condensation, ligation/cleavage, functional group transfer, isomerization and the formation/removal of carbon-carbon double bonds.[3] Due to their high activation energy and the relatively low temperature in a living cell, most biochemical reactions must be sped up by biological catalysts, called enzymes. Enzymes are usually proteins but in some cases RNA can function as an enzyme (ribozyme).[4] Compartmentalization of biochemical reactions inside cells and subcellular compartments is thought to have been crucial for life to evolve to the complexity of multicellular organisms such as humans.[5]

1.2 Bioorthogonal chemistry & click chemistry

Bioorthogonal chemistry refers to chemical reactions that can be selectively performed within the complex environment of a living cell, without interacting or interfering with the biological system.[1] The term bioorthogonal was coined by Bertozzi in 2003 and refers to orthogonality between the artificially introduced reaction and all biochemical reactions that occur within a living system (i.e. the cell).[2,3] True bioorthogonality requires high selectivity, fast kinetics, biocompatibility of the reaction, biological and chemical inertness of the introduced functional group and the resulting product, as well as accessible engineering of the biomolecule.[1,3,4] The first bioorthogonal reaction is arguably the Staudinger ligation, in which an organic azide is irreversibly ligated to a triphenylphosphine moiety to form an amide group (**Figure 1A**).[5–7] Historically, various attempts were made to obtain bioorthogonality already in 1990 by the group of McCarthy[8], with a condensation reaction between hydrazine and aldehyde moieties and in 1997 by the group of Bertozzi[9], with a condensation reaction between hydrazide and levulinoyl moieties. However, since the functional groups used in these reactions are not unique in nature and the products of these reactions are instable, they cannot be considered truly bioorthogonal.

Although fundamentally different in nature, the terms bioorthogonal chemistry and click chemistry are often used interchangeably. The term click chemistry was coined by Sharpless in 1998 and refers to ligation reactions in which two functional groups can be joined selectively, quickly and with high yield in aqueous solution, producing a physiologically stable product without toxic byproducts.[10] Although some click reactions can be performed in live cells, biocompatibility is not a requirement. Click reactions are ideally suited for the production of biologically active molecules in organic synthesis, as well as for biochemical ligation reactions, in which a reporter moiety is connected to a 'click handle'-containing biomolecule of interest, to facilitate the analysis of said biomolecule.[11] In practice, the reporter is usually a fluorophore for fluorescent measurements or a biotin for enrichment of the tagged biomolecule (e.g., for mass spectrometry analysis).[12] Bioorthogonal reactions can be considered the ideal click reactions for ligations in living cells, due to their biocompatibility and bio-inertness of their click handles.

The first reported click reaction was the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC; **Figure 1B**), also known as the copper-catalyzed Huisgen cycloaddition (ccHc), discovered simultaneously but independently by the group of Sharpless[13] and the group of Meldal[14]. Although this reaction can be performed successfully in fixed cells (and live cells under special conditions[15,16]), this reaction cannot be considered as a true bioorthogonal reaction, due to cytotoxicity of the Cu(I)-catalyst.[17] To avoid this toxicity, Bertozzi and coworkers then went on to develop a copper-free click reaction, using the intrinsic ring-strain of cyclooctynes to overcome the activation energy of the azide-alkyne cycloaddition.[18,19] This reaction is known as the Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC; **Figure 1C**) or strain-promoted Huisgen cycloaddition (spHc). In order to overcome the suboptimal reaction kinetics of these cycloadditions, another copper-free click reaction was then developed, combining the ring-strain of a cyclooctyne with the stereoelectronic tunability of a nitrone moiety. This reaction was termed the Strain-Promoted Alkyne-Nitrone Cycloaddition (SPANC; **Figure 1D**).[20]

Although many other bioorthogonal reactions have been developed to date[21,22], the most notable recent addition is the inverse electron demand Diels–Alder (IEDDA) reaction, due to its excellent reaction kinetics, chemical orthogonality and biocompatibility.[23] The IEDDA reaction involves a cycloaddition between a 1,2,4,5-tetrazine and a (strained) alkene group, and is often referred to as the tetrazine ligation reaction (**Figure 1E**).[24,25] Both reaction partners of this reaction can be extensively tuned to optimize the reaction in terms of speed, selectivity and stability of the product. Several different strained alkenes have been developed,

including norbornene[26], cyclopropene[27], spirohexene[28] and trans-cyclooctene (TCO)[25,29].

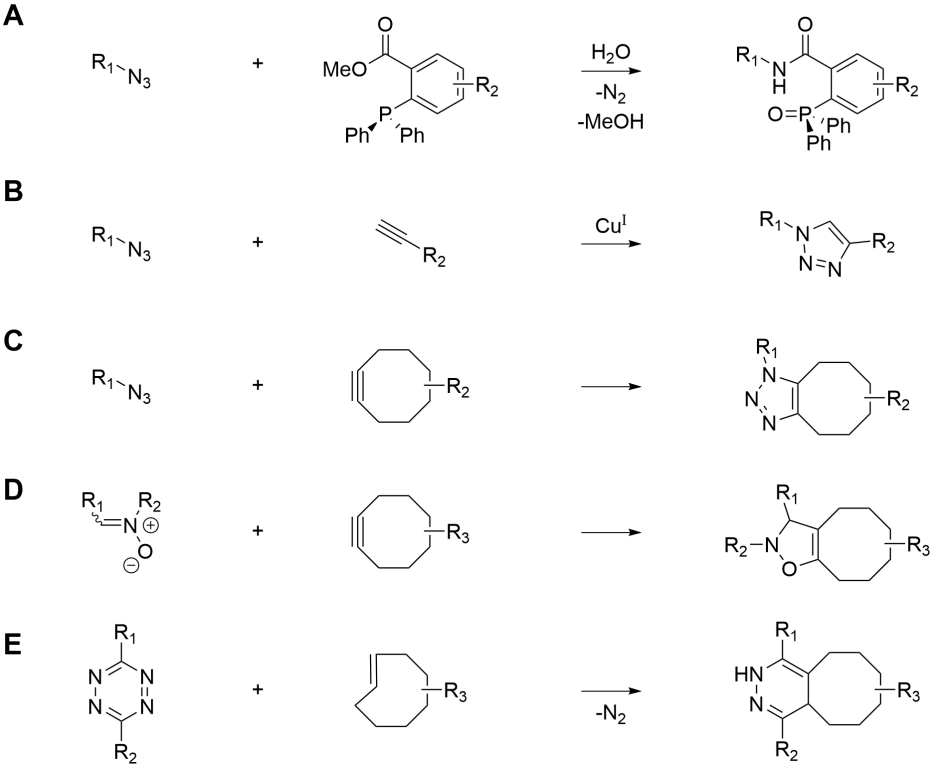

Figure 1. Overview of the most common bioorthogonal (click) reactions. (**A**) Staudinger ligation (a.k.a. Staudinger-Bertozzi ligation). (**B**) Cu(I)-catalyzed Azide-Alkyne Cycloaddition (CuAAC, a.k.a. copper-click reaction). (**C**) Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC, a.k.a. copper-free click reaction). (**D**) Strain-Promoted Alkyne-Nitrone Cycloaddition (SPANC). (**E**) Inverse Electron-Demand Diels-Alder reaction (IEDDA), here showing the reaction between a tetrazine and a TCO (tetrazine ligation).

1.3 Bioorthogonal metabolic labeling

Metabolic labeling (also known as metabolic engineering) involves the incorporation of a detectable chemical label into a biomolecule of interest, using the native metabolism of the organism.[30] This is achieved by creating a synthetic analogue of a building block (e.g., an amino acid) that can be incorporated into larger biomolecules by the organism. The synthetic analogue needs to be sufficiently similar to the original, in order to be accepted by the cellular machinery, while containing a detectable label. Metabolic labeling can be achieved either by one-step labeling, in which the initial label can be detected directly (e.g. fluorophore or radioactive isotope), or two-step (direct) labeling, in which the initial label can be selectively ligated to a reporter molecule for detection (e.g. click handle), also known as bioorthogonal metabolic labeling.[30] The main advantage of bioorthogonal metabolic labeling is its broad compatibility with common analysis methods such as fluorescence microscopy, flow cytometry and proteomics[31] without interfering with the cellular processes[32], which is often observed for one-step labeling approaches[33].

Bioorthogonal metabolic labeling was originally developed by the group of Bertozzi, in order to selectively label and modify cell surface glycans.[9,34,35] In this study, a levulinoyl-analogue of *N*-acetylmannosamine (ManLev) was incorporated by the cells into the cell surface associated sialic acids. A hydrazide-functionalized biotin moiety could then be selectively ligated to the levulinoyl-modified sialic acids and subsequently visualized using a fluorophore-modified avidin moiety (which selectively binds to biotin).[9] Switching to an azide-analogue of *N*-acetylmannosamine (ManNAz), in combination with the more biocompatible Staudinger ligation, eventually made it possible to study cell surface glycans in living animals.[36] Bertozzi then continued to modify this strategy, using SPAAC to visualize the azide-modified sialic acid, which proved possible in living animals as well.[37] Although CuAAC is insufficiently biocompatible to be performed in live cells, it proved possible to metabolically incorporate an alkyne-analogue of *N*-acetylmannosamine (ManNAl) into living animals and perform the click reaction *ex vivo* after harvesting the organs.[38]

At present, bioorthogonal metabolic labeling has been successfully achieved for virtually all biomolecules[33], including carbohydrates[39] as described above, but also proteins[40], lipids[41–43], nucleic acids[44–46] and bacterial cell wall components[47–49]. Although the method originated with carbohydrates, bioorthogonal labeling of proteins has gained a lot of attention over the years.[40] Some bioorthogonal

analogues of amino acids can be directly incorporated by an organism, when they are recognized by the native translational machinery (i.e. aminoacyl-tRNA synthetase).[50] This technique was termed Bioorthogonal Non-Canonical Amino acid Tagging (BONCAT) and provides both an azide and an alkyne analogue of L-methionine, that are readily incorporated into newly synthesized proteins.[50,51] BONCAT has been successfully applied to many different organisms including bacteria[52,53], mammalian cells[51,54], whole animals[55–58], plants[59], parasites[60] and viruses[61,62].

1.4 Bioorthogonal metabolic labeling of intracellular bacteria

One research field in which bioorthogonal metabolic labeling can be applied is the study of intracellular pathogenic bacteria and their interaction with the host cell. Both the extracellular and intracellular life cycle of pathogenic bacteria can be studied using bioorthogonal labeling to visualize the newly synthesized bacterial components, such as the membranes, cell wall or cytoplasmic content under various conditions. For example, the effect of antibiotics on peptidoglycan synthesis can be studied using D-alanine analogues[63] or the effect of nutrient limitation on mycomembrane remodeling using trehalose analogues[64]. Another possibility is to study the metabolic activity of viable bacteria using BONCAT.[65] Finally, various bioorthogonal analogues can be used to label bacteria of interest and study their intracellular fate, after uptake by phagocytic immune cells such as macrophages or dendritic cells. This approach has previously been used to visualize degradation of non-pathogenic *Escherichia coli* (*E. coli*)[66] and survival and proliferation of *Salmonella enterica* serovar Typhimurium (*Stm*)[67]. Additionally, this approach could be used to study more dangerous pathogenic intracellular bacteria such as *Mycobacterium tuberculosis* (*Mtb*) and the effect of antibiotics on the intracellular survival of those bacteria.

The broad compatibility of bioorthogonal metabolic labeling allows for many fluorescence-based analysis methods to be used. For example, when using BONCAT, the incorporation of bioorthogonal amino acids into the bacterial proteins can be assessed using SDS-PAGE, followed by in-gel fluorescence analysis. Additionally, the average labeling efficiency per bacterium can be assessed using flow cytometry. Finally, several microscopy techniques can be used to visualize the fluorescently labeled bacteria, including confocal fluorescence microscopy, Stochastic Optical Reconstruction Microscopy (STORM) and Correlative Light-Electron Microscopy (CLEM). Since STORM provides the best spatial resolution in terms of fluorescence and CLEM can provide the ultrastructural context of the host

cell, a combination of the two (STORM-CLEM) would provide the most detailed information on the intracellular bacterium, *in situ*.

1.5 Aim of this thesis

The aim of this thesis is to expand on the previously developed bioorthogonal CLEM technique for the study of intracellular bacteria. In **chapter 2**, the principle of bioorthogonal metabolic labeling for the study of intracellular bacteria is discussed in more detail and recent developments in the field are reviewed. In **chapter 3**, the stability of several commonly used click handles is tested within the chemically harsh intracellular environment of phagocytic immune cells, to confirm that they can be used to study the degradation or survival of intracellular bacteria. In **chapter 4**, bioorthogonal metabolic labeling of *Stm* is optimized for the development of STORM-CLEM, to study intracellular pathogenic bacteria in more detail. In **chapter 5**, dual bioorthogonal metabolic labeling of *Mtb* is presented and combined with a fluorescent protein. The resulting triple-label *Mtb* are studied using CLEM, and the effect of various first line antibiotics on the intracellular distribution and shape of *Mtb* is quantified. Additionally, the bioorthogonal label retention of intracellular *Mtb* is measured by flow cytometry, after retrieval of the bacteria by selective host cell lysis. In **chapter 6**, a summary of the previous chapters is provided, along with several future prospects which can be pursued, following the findings of this thesis.

1.6 References

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