Bioorthogonal chemistry in living animals

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Bioorthogonal chemistry refers to those chemical reactions that can be performed in living systems without interfering with native biological processes. Since its first introduction by Bertozzi et al. in 2003 [1], it has attracted an explosion of interests due to its great impact on studies of the complex biological processes as well as on therapeutic applications. The majority of previous efforts have been focused on developing and/or discovering new reactions, as well as expanding their applications, particularly within living cells. To this end, versatile bioorthogonal reactions have been created, which emerged as a powerful toolbox for live-cell investigations [2]. Nevertheless, beyond these cellular applications, expanding bioorthogonal reactions to living animals is another fascinating frontier. Although still in its infancy, bioorthogonal chemistry in living animals has great potential for dissecting the complex biological processes at the whole animal level, with significant clinical implications (e.g. clinic diagnosis, prodrug therapy and drug delivery). Indeed, efforts have already been made to carry out bioorthogonal chemistry in several model animals ranging from Caenorhabditis elegans to mice [3]. Due to the page limitation, this perspective article will mainly focus on the original reports of the reactions that have been tested in animals, followed by discussions of their future directions.

A few bioorthogonal chemistry studies have been conducted in animals so far, which can be divided into two classes according to the reaction type: namely the ligation reaction and the cleavage reaction.

**Bioorthogonal Ligation Chemistry in Living Animals**

Biological studies often require the specific labeling of a target for visualization and/or modification purposes. This is usually achieved by a two-step approach: a reaction handle is first introduced into the target specifically and then the reporter group is conjugated with the pre-installed handle on the target via a bioorthogonal ligation reaction [2]. However, performing ligation reactions in living animals is more challenging than in living cells. For example, lower concentrations in animals require faster reaction kinetics, while issues on the bioorthogonality of reaction handles as well as the stability and permeability of reagents need to be more carefully addressed. Bertozzi and coworkers first used the Staudinger–Bertozzi ligation reaction in living mice in 2004 (Fig. 1a) [1]. However, the insufficient ligation rate made the authors seek alternative reactions with faster kinetics. Through a series of optimizations, they eventually obtained the strain-promoted alkyn-azide cycloaddition (SPAAC) reaction with satisfying reaction rate and reagent stability (Fig. 1b), which was then used to analyse the glycan expression and trafficking during zebrafish development [1].

Among all the bioorthogonal reactions developed so far, the inverse electron demand Diels–Alder reaction (iEDDA) possesses the fastest reaction kinetics, with an incredible $k_2$ up to $10^6$ M$^{-1}$ s$^{-1}$ [2]. The great reaction rate and efficiency rapidly promoted the applications of iEDDA reaction in living animals. In 2010, the Robillard group first conducted the iEDDA ligation reaction in living mice for clinical diagnosis of cancer by introducing a SPECT (single-photo emission computed tomography) imaging agent indium-111 in vivo [4]. Soon after, these elegant works inspired many other studies on applying such ligation chemistry in living animals (Fig. 1c and d).

**Emerging Bioorthogonal Cleavage Chemistry in Living Animals**

Recently, the iEDDA ligation reaction has been redirected as a ‘click and release’ strategy, in which the electron rearrangement process of an iEDDA cycloaddition product from trans-cyclooctene (TCO) and 1,2,4,5-tetrazine (TZ) results in elimination of the TCO group in case that a cleavable linker (e.g. carbamate group) is placed at the allylic position on TCO [5]. Our group first applied this reaction on proteins in vitro and in living cells. With our continued interests in function manipulation of proteins, we coupled this iEDDA cleavage reaction with the genetic code expansion technique as a general protein-activation strategy in living cells (Fig. 1e) [6].

Due to its fast reaction rate and excellent bioorthogonality, we soon exploited this iEDDA-based cleavage chemistry in living animals. We were particularly interested in employing this iEDDA-triggered cleavage chemistry for kinase activation.
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(a) $\text{N}^+\text{N}^-\text{N}^+ + \text{MeO}^+\text{O}^-\text{Ph}_3\text{F}^- \xrightleftharpoons[-\text{N}_2, + \text{H}_2\text{O}]{\text{Staudinger-Bertozzi}} \text{N}^+\text{O}^-\text{Ph}_3\text{F}^-\text{N}^+$

(b) $\text{N}^+\text{N}^-\text{N}^+ + \text{[Cyclic]} \xrightarrow[\text{SPAAC ligation}]{-\text{N}_2} \text{N}^+\text{N}^-\text{N}^+$

(c) $\text{N}^+\text{N}^-\text{N}^+ + \text{R}^-\text{N}^-\text{N}^+ \xrightarrow[\text{iEDDA ligation}]{-\text{N}_2} \text{R}^-\text{N}^-\text{N}^+$

(d) $\text{N}^+\text{N}^-\text{N}^+ + \text{R}^-\text{N}^-\text{N}^+ \xrightarrow[\text{iEDDA ligation}]{-\text{N}_2} \text{R}^-\text{N}^-\text{N}^+$

Bioorthogonal decaying reactions in living animals

(e) $\text{N}^+\text{O}^-\text{O}^+\text{N}^-\text{N}^+ + \text{R}^+\text{N}^-\text{N}^+ \xrightarrow[-\text{N}_2, - \text{CO}_2\text{H}]{\text{iEDDA cleavage}} \text{N}^+\text{H}^+\text{N}^+$

Figure 1. Bioorthogonal reactions in living animals. (a–c) are revised from [6]; (f) is revised from [10].

in living cells and living mice. For example, by replacing Src kinase bearing a TCO-caged lysine residue at its key catalytic site, the resulting kinase variant Src-K295TCOK becomes inactive until iEDDA-triggered lysine decaging to regenerate the native lysine residue. Next, we implanted cells expressing Src-K295TCOK, in living mice as a xenograft model. Upon tail vein injection of the compound Di-methyl-TZ, Src-K295TCOK quickly underwent the decaging process in the mice to convert back to the native Src enzyme. Obvious activation of Src, as determined by the autophosphorylation level, was observed within one hour [7]. This study represents an important step in chemical regulation of kinases, one type of the most important enzymes in biological processes, in living animals. A prodrug activation strategy in living animals was also reported by the Robillard group recently, in which TCO-protected doxorubicin was conjugated with a cancer-targeted antibody. After the antibody targeted to cancer cells in living mice, the attached prodrug can be specifically cleaved upon injection of a TZ molecule to release the anticancer drug doxorubicin nearby to kill cancer cells [8]. Oneto et al. designed another strategy based on this iEDDA-triggered cleavage chemistry to treat cancer specifically. In this report, TZ-modified hydrogel was implanted into the cancer site in living mice, while the TCO-caged prodrug was administered into the whole mice body. As the TCO caging group could only be decaged by TZ, the prodrug around the TZ-modified hydrogel will be activated and kill the nearby cancer cells selectively (Fig. 1e) [9].

Because many biological processes require a very fast activation rate, we have systematically optimized the iEDDA cleavage reaction to improve the decaging rate. We discovered that the electron withdrawing substituents (EWG) on TZ could accelerate the cycloaddition step but suppress the following elimination step. In contrast, alkyl substituents could facilitate the elimination step. Based on these findings, we designed a series of unsymmetrical Tzs with an EWG at one side and an alkyl group at the other side (Fig. 1f). These newly designed Tzs could activate a caged enzyme in living cells with significantly improved decaging rates, which showed promising potential for further utilization in living animals [10].

OUTLOOK AND CHALLENGES

Although a few bioorthogonal reactions, including ligation and cleavage ones, have been successfully demonstrated in living animals, the bioorthogonal toolbox for living animals remains to be further established. New types of reactions are particularly in high demand, not only for presenting more choices, but also for addressing and compensating the deficiencies of the current available reactions. For instance, the relatively slow kinetics of the Staudinger–Bertozzi ligation and the SPAAC reaction as well as the complicated synthesis procedures of the iEDDA reagents hindered the application of these reactions. Introduction of bioorthogonal handles into specific targets in animals is another critical issue. Metabolic incorporation is conventionally utilized, but the site-specificity of this approach is usually not satisfying. Therefore, affinity conjugation (e.g. antibody) and xenograft implant are occasionally used to improve the specificity. Recently, the extension of the genetic code expansion technique into living animals offers a powerful method to specifically install the bioorthogonal handles. However, the incorporation efficiency and availability of the chemicals are the bottleneck for broad applications of this strategy.
In addition, since the development and applications of bioorthogonal reactions in living animals are still emerging, more applications of such chemistry in diverse animals merit further expansion. Bioorthogonal ligation reactions may facilitate the labeling of specific cells or tissues with functional molecules that permits biological studies within intact animals. For example, labeling nerve cells with potential-response fluorescent molecules would offer a visual detection tool for monitoring nerve-signaling transduction. Meanwhile, function regulation of proteins by using bioorthogonal cleavage reactions may enable a comprehensive understanding of the functional role of the target protein at the whole-animal level.

Finally, together with the rapid development of precise gene-editing methods (e.g. CRISPR-Cas9), improved antibody/immuno-cell therapies and specific oncogene inhibitors, bioorthogonal chemistry that is compatible with living animals will give novel insights and even solutions to many clinical questions, such as early-stage disease screening, site-specific drug delivery and controlled in situ drug release or synthesis.

Conflict of interest statement. None declared.

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