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# Cutting-edge bioorthogonal chemistry: Innovations, practical applications, and emerging trends

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ABSTRACT

Bioorthogonal chemistry has emerged as a pivotal field in molecular science, offering transformative tools for applications in drug discovery, imaging, and molecular biology. This review provides a comprehensive analysis of recent advancements in bioorthogonal chemistry, emphasizing key innovations, practical applications, and future research directions. We explore state-of-the-art bioorthogonal reactions, including Staudinger ligation, strain-promoted azide-alkyne cycloaddition (SPAAC), and tetrazine ligation, detailing their mechanisms, advantages, and limitations. The review highlights significant innovations such as novel fluorogenic probes, improved catalysts, and enhanced reaction conditions that have expanded the utility and efficiency of these reactions. Practical applications are examined, showing how these advances have revolutionized fields like live-cell imaging, targeted drug delivery, and molecular labeling. Looking to the future, we discuss emerging trends and potential research avenues, including the integration of bioorthogonal chemistry with other advanced technologies and the development of new reaction methodologies. This review provides a detailed overview of the current state of bioorthogonal chemistry and outlines its future potential, serving as a valuable resource for researchers and practitioners in the field.

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## 1. Introduction

Imagine trying to fix a broken machine without turning it off or disrupting its normal operation [1]. That is the challenge that scientists face when they want to study and manipulate tiny machines inside our bodies, such as proteins, DNA, and other molecules, without causing any harm [2]. Bioorthogonal chemistry has emerged as a transformative discipline that bridges the gap between chemistry and biology, offering unprecedented opportunities to interrogate and manipulate biological systems with exquisite precision and control. Bioorthogonal chemistry, a term coined by Carolyn Bertozzi, represents a revolutionary approach in chemical biology that enables the study and manipulation of biological systems with unprecedented precision [3]. The foundation of bioorthogonal chemistry lies in the development of chemical reactions that proceed rapidly and selectively under physiological conditions, without interfering with native biochemical processes [4]. Since its inception, bioorthogonal chemistry has revolutionized our ability to manipulate and visualize biomolecules in living organisms, enabling breakthroughs in areas such as chemical biology, diagnostics, and drug discovery [5,6]. This concept has also challenged our ability to study biomolecules in their native environments, allowing researchers to selectively label, track, and manipulate biological molecules with minimal interference [7].

The bioorthogonal reaction must meet the following requirements [8,9]: (i) The reaction must occur at the temperature and pH of physiological environments. (ii) The reaction must provide products selectively and in high yields and must not be affected by water or endogenous nucleophiles, electrophiles, reductants, or oxidants found in complex biological environments. (iii) The reaction must be fast, even at low concentrations, and must form stable reaction products. (iv) The reaction should involve functional groups that are not naturally present in biological systems.

The key principles of bioorthogonality are selectivity [10], biocompatibility [11], and orthogonality [12], which have guided the development of novel chemical reactions that are capable of selectively modifying biomolecules in complex biological environments [13]. Selectivity refers to the ability of a bioorthogonal reaction to target specific functional groups or biomolecules within a complex biological environment, minimizing off-target effects and cross-reactivity [14]. Biocompatibility ensures that reaction components are nontoxic and compatible with living systems, allowing their use in a wide range of biological applications [15].


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**Table 1.** Comparison of different biorthogonal reactions.

| Aspect | Click chemistry (Huisgen cycloaddition) | Staudinger ligation | Diels-Alder reaction | Strain-promoted azide-alkyne cycloaddition | Tetrazine ligation |
|---|---|---|---|---|---|
| Reaction type | Cycloaddition | Ligation | Cycloaddition | Cycloaddition | Ligation |
| Key functional groups | Azide and alkyne | Azide and phosphine | Diene and dienophile | Azide and strained alkyne | Tetrazine and alkene |
| Reaction conditions | Mild, aqueous, room temperature | Mild, aqueous, room temperature | Mild to moderate, varies | Mild, aqueous, room temperature | Mild to moderate, varies |
| Reaction speed | Generally fast | Moderate | Moderate to slow | Fast | Moderate to fast |
| Biocompatibility | Generally high | Generally high | Moderate | Generally high | Moderate |
| Interference with native processes | Minimal | Minimal | Moderate | Minimal | Minimal |
| Applications | Imaging, protein labeling, drug delivery | Protein labeling, imaging | Biomolecule labeling | Imaging, protein labeling | Biomolecule labeling |
| Examples of Use | Copper(I)-catalyzed azide-alkyne cycloaddition | Staudinger reaction with azides and phosphines | Diels-Alder with strained dienes | SPAAC with cyclooctynes | Tetrazine-based ligations |
| Advantages | Versatile, well-established, high yield | High selectivity, mild conditions | Well-established, versatile | High selectivity, copper-free | Fast, high yield |
| **Aspect** | **Copper-free click chemistry** | **Oxime ligation** | **Michael addition** | **Photo crosslinking** | **Radical chemistry** |
| Reaction type | Cycloaddition | Ligation | Addition | Crosslinking | Addition |
| Key functional groups | Azide and alkyne | Aldehyde and ketone | Michael acceptor and donor | Photoreactive groups | Various reactive groups |
| Reaction conditions | Mild, aqueous, room temperature | Mild, aqueous, room temperature | Mild to moderate, varies | Mild to moderate, light activation | Varies, often mild |
| Reaction speed | Fast | Moderate | Moderate to fast | Fast (with light) | Variable |
| Biocompatibility | High | High | Moderate | Moderate to high | Variable |
| Interference with Native Processes | Minimal | Minimal | Minimal | Minimal (light-dependent) | Variable |
| Applications | Imaging, protein labeling | Protein labeling | Biomolecule labeling | Protein crosslinking | Biomolecule labeling |
| Examples of use | SPAAC (Copper-free) | Oxime-forming reactions | Michael addition for labeling | Photoaffinity labeling | Radical-based tagging |
| Advantages | No copper required, high yield | Mild conditions | Versatile, mild conditions | Specific, light-activated | High specificity |

Orthogonality refers to the independence of bioorthogonal reactions from native biological pathways, allowing them to proceed selectively and efficiently in the presence of other biomolecules [16]. As a result, bioorthogonal chemistry has become an indispensable tool for studying the dynamic interactions and functions of biomolecules in health and disease [17]. The driving force behind the development of bioorthogonal chemistry lies in the need for chemical tools that can seamlessly with the complexity of living systems. Traditional chemical reactions often suffer from limitations such as low selectivity, poor biocompatibility, and interference with cellular processes [18]. The journey of bioorthogonal chemistry began with the introduction of Staudinger ligation, a reaction that laid the groundwork for subsequent advances in the field [19]. This reaction, initially developed to modify cell surface glycans, demonstrated the potential of chemical tools to probe and manipulate biological systems in a noninvasive manner [20]. However, the real breakthrough came with the advent of the copper-free click reaction, pioneered by the Bertozzi group [21]. This innovation eliminated the cytotoxicity associated with copper-catalyzed reactions, allowing bioorthogonal reactions to be applied to living organisms, including mammals. Bioorthogonal chemistry seeks to overcome these challenges by providing reactions that are orthogonal to biological functionality, allowing researchers to introduce chemical probes and modifications into biological systems without disrupting their natural behavior [22]. The development of bioorthogonal chemistry has been driven by the quest to address fundamental questions in biology and medicine [23]. Bioorthogonal chemistry is like a special set of tools that chemists and biologists use to tinker with biological molecules in living organisms without causing any problems [24]. These tools are designed to be very precise, so they interact only with the molecules they are supposed to, leaving everything else alone. It is kind of like using a tiny wrench to tighten a bolt without touching anything else nearby [25]. Bioorthogonal chemistry has facilitated groundbreaking discoveries in areas such as proteomics [26], metabolomics [27], and cell signaling. Moreover, bioorthogonal chemistry has enabled the development of innovative diagnostic techniques and therapeutic strategies for diseases ranging from cancer to infectious disorders. The impact of bioorthogonal chemistry has been profound, influencing a wide range of applications, from live cell imaging to drug delivery and beyond [28]. Table 1 shows the different bioorthogonal reactions with a comparison of different properties that define the different aspects of click chemistry.

In this research paper, we will explore recent advances in bioorthogonal chemistry, including novel reaction methodologies, applications in biomedical research, and future directions for the field. In this review, we will dive into the key reactions that define this field, explore their applications, and discuss emerging trends that will shape the future of bioorthogonal chemistry. We will explore some of the cool tricks scientists have developed, such as reactions that can happen inside living cells and methods for imaging molecules in real-time. By examining the latest developments and challenges in bioorthogonal chemistry, we aim to provide insights into its potential to revolutionize our understanding of biology and improve human health.

## 2. Key bioorthogonal reactions

In recent years, significant progress has been made in the design and optimization of bioorthogonal reactions, leading to the development of new reaction methodologies within the living system. These advances have significantly expanded the scope and versatility of bioorthogonal chemistry, opening up new possibilities for studying complex biological processes in real time. One of the most notable advances is the discovery of strain-promoted azide-alkyne cycloaddition reactions (SPAAC) [29], which do not require toxic metal catalysts and exhibit rapid kinetics under physiological conditions. SPAAC reactions have found widespread applications in bioconjugation, molecular imaging, and drug delivery, because of their high selectivity and biocompatibility.

**Table 2.** Recent advances in bioorthogonal chemistry.

| Advancement | Reaction/Method | Key developments | Applications | References |
|---|---|---|---|---|
| Copper-free click chemistry improvements | Strain-promoted azide-alkyne cycloaddition (SPAAC) | Enhanced efficiency and selectivity, new cyclooctyne derivatives | Protein labeling, live-cell imaging | [1,2,28] |
| Tetrazine-based ligation | Tetrazine-ligations | Development of faster and more selective tetrazine reactions | Rapid biomolecule labeling, drug delivery systems | [3,4] |
| Photo-crosslinking advances | Photo-crosslinking reactions | Novel photoreactive probes and better spatial control | Protein crosslinking, dynamic imaging | [5,6] |
| Bioorthogonal probes for metabolomics | Oxime ligation | New probes for metabolite labeling | Metabolite profiling, cellular tracking | [7,8] |
| Biocompatible click chemistry | Copper-free click chemistry | Development of more biocompatible catalysts and ligands | Cellular imaging, biomolecular interactions | [9,10,31] |
| Dual bioorthogonal labeling | Dual bioorthogonal reactions | Techniques for simultaneous multi-tagging | Multi-color imaging, complex biological studies | [11,12] |
| Enhancements in Staudinger ligation | Staudinger ligation | New phosphine-based reagents for improved labeling | Targeted labeling imaging in complex systems | [13,14] |
| Development of new reactivity profiles | Novel bioorthogonal reactions | Introduction of new functional groups and reactivities | Targeted drug delivery, advanced biomolecule manipulation | [15,16] |
| Applications in drug discovery | Various bioorthogonal techniques | Integration of bioorthogonal chemistry in drug screening | Drug discovery, high-throughput screening | [17,18] |
| Innovations in in-vivo bioorthogonal reactions | In-vivo bioorthogonal reactions | Enhanced methods for in vivo tracking and manipulation | Live animal imaging, real-time cellular studies | [19,20] |

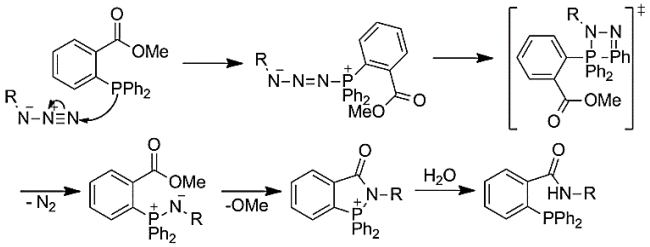


**Figure 1.** Staudinger ligation reaction.

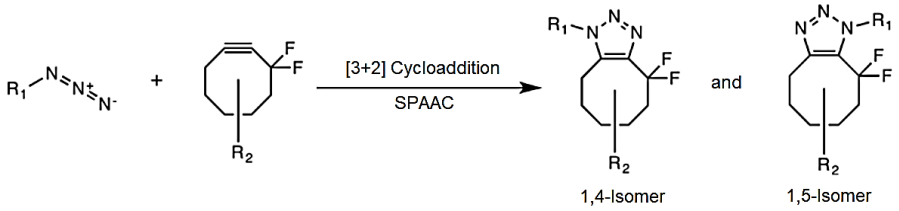


**Figure 2.** The strain promoted the azide-alkyne cycloaddition reaction.

Another promising development is the emergence of bioorthogonal catalysis, in which small-molecule catalysts facilitate bioorthogonal reactions in living systems. This approach allows site-specific labeling of biomolecules with various chemical functionalities, expanding the scope of bioorthogonal chemistry beyond traditional click chemistry reactions [30]. One notable recent development is the discovery of bioorthogonal reactions that can occur inside living cells. Traditionally, bioorthogonal reactions were limited to extracellular environments due to the harsh conditions found within cells, such as high concentrations of reactive molecules and complex biochemical pathways. However, researchers have overcome these challenges by designing reactions that are compatible with the intracellular environment. Table 2 shows the comparison of different biorthogonal reactions with reaction methods with recent advancements and its application in the real world.

Bioorthogonal chemistry is defined by a suite of chemical reactions that have been meticulously engineered to operate within the complex environment of living systems. Among the most prominent reactions are Staudinger ligation [31], strain-promoted azide-alkyne cycloaddition (SPAAC) [32], and tetrazine ligation [33]. Each of these reactions has unique advantages and has found different applications in chemical biology [34].

### *2.1. Staudinger ligation*

The Staudinger ligation, developed in the early 2000s, was one of the first reactions to be termed "bioorthogonal." This reaction involves the reduction of an azide by phosphine to form an amide bond, a process that proceeds under physiological conditions without producing toxic by-products (Figure 1) [35]. It was originally applied to label glycoproteins on the surface of living cells, providing a non-invasive method to study glycosylation patterns. Despite its slower kinetics compared to subsequent bioorthogonal reactions, Staudinger ligation laid the foundation for the development of more robust bioorthogonal tools [36,37].

### *2.2. Strain-promoted azide-alkyne cycloaddition (SPAAC)*

Based on the principles of the classical azide-alkyne "click" reaction, the SPAAC reaction was introduced as a copper-free alternative that could be safely used in living systems (Figure 2) [38]. When a strained alkyne is used, the reaction is driven by ring strain rather than by a metal catalyst, making it biocompatible and suitable for in vivo applications. SPAAC has been widely used to label biomolecules in live cells, and its application extends to the development of drug delivery systems in which bioorthogonal chemistry allows the targeted release of therapeutics [37,39].

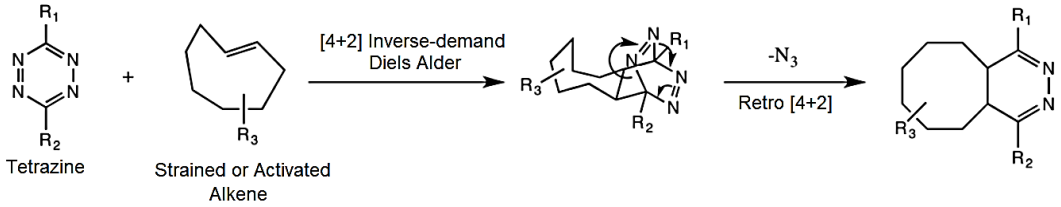


**Figure 3.** Tetrazine ligation reaction.

### *2.3. Tetrazine ligation*

Tetrazine ligation represents one of the fastest bioorthogonal reactions, characterized by the rapid Inverse Electron Demand Diels-Alder reaction between a tetrazine and a strained alkene (such as trans-cyclooctene) (Figure 3). This reaction has gained popularity because of its exceptional speed and the minimal concentration of reactants required, making it ideal for live cell imaging and real-time tracking of biomolecular processes. Tetrazine ligation has been instrumental in studying dynamic biological processes with high temporal resolution, further cementing the role of bioorthogonal chemistry in modern biology [40,41].

These key reactions not only expanded the toolkit available to chemical biologists, but have also inspired the development of new bioorthogonal reactions that continue to push the boundaries of what can be achieved in living systems [42]. The development of bioorthogonal reactions with enhanced selectivity and efficiency. Traditional bioorthogonal reactions, such as copper-catalyzed azide-alkyne cycloaddition (CuAAC), often suffer from limitations such as slow reaction kinetics, low selectivity, and cytotoxicity [43]. In response, researchers have engineered new bioorthogonal reactions that exhibit improved reaction rates, high selectivity, and minimal cytotoxicity, making them ideal for in vivo applications [44]. For example, bioorthogonal catalysis, in which small-molecule catalysts promote bioorthogonal reactions in living systems, has emerged as a powerful strategy to achieve the rapid and selective labeling of biomolecules *in vivo* [45]. Furthermore, bioorthogonal reactions based on bioorthogonal functional groups, such as strained alkenes, tetrazines and cyclooctynes, have been developed to overcome the limitations of traditional click chemistry reactions and expand the range of biomolecules that can be targeted [46]. Furthermore, recent advances in imaging technology have revolutionized the field of bioorthogonal chemistry by enabling real-time visualization of bioorthogonal reactions in living systems [47]. High-resolution imaging techniques, such as fluorescence microscopy, super-resolution microscopy, and positron emission tomography (PET), allow researchers to monitor bioorthogonal reactions with unprecedented spatial and temporal resolution, providing valuable information on cellular dynamics and physiological processes [48]. These imaging techniques have facilitated the development of new bioorthogonal probes and imaging agents for studying various biological phenomena, including protein dynamics, DNA replication, and cell signaling pathways [49,50].

## 3. Applications of bioorthogonal chemistry in biomedical research

The versatility of bioorthogonal chemistry has allowed its application in a wide range of biomedical research areas [51,52], including protein labeling and tracking [53], metabolic engineering [54], glycan imaging [55], and nucleic acid labeling [56]. For example, bioorthogonal chemistry-based protein labeling techniques, such as metabolic labeling with non-canonical amino acids and site-specific modification with bioorthogonal probes, have revolutionized the study of protein dynamics, interactions, and function in live cells and organisms [57]. Similarly, metabolic engineering strategies that utilize bioorthogonal chemistry have been employed to introduce bioorthogonal handles into cellular metabolites and pathways, enabling selective manipulation and visualization of metabolic processes in vivo. In the field of glycan biology, bioorthogonal chemistry has facilitated the imaging and profiling of complex carbohydrate structures on cell surfaces and tissues, shedding light on their roles in development, immunity, and disease [58]. Furthermore, nucleic acid labeling techniques have been instrumental in the study of DNA / RNA dynamics, replication, and repair mechanisms, providing new insights into genome biology and gene regulation [59]. Bioorthogonal chemistry has revolutionized biomedical research by providing powerful tools for studying and manipulating biological molecules with unprecedented precision and specificity [60]. These versatile techniques have found wide-ranging applications in various areas of biomedical research, allowing researchers to probe complex biological processes, elucidate disease mechanisms, and develop innovative diagnostic and therapeutic strategies [61]. One of the key applications of bioorthogonal chemistry is protein labeling and tracking. For example, researchers have used bioorthogonal chemistry to selectively label and track specific proteins in living cells and organisms [62]. By incorporating bioorthogonal functional groups, such as azides or alkynes, into target proteins and subsequently reacting them with complementary bioorthogonal probes, scientists can visualize protein localization, dynamics, and interactions in real time. This technique has been instrumental in studying protein trafficking pathways, signaling cascades, and protein-protein interactions involved in various cellular processes [63]. Metabolic engineering and imaging represent another important application of bioorthogonal chemistry. For example, metabolic labeling techniques have been used to selectively incorporate non-canonical amino acids (ncAAs) or sugar analogs containing bioorthogonal handles into cellular biomolecules [64]. By introducing these modified biomolecules into living cells, researchers can monitor metabolic pathways, study post-translational modifications, and visualize biomolecule dynamics in real time using fluorescence microscopy or other imaging modalities [65]. This approach has facilitated the discovery of new metabolic pathways, biomarkers, and therapeutic targets for metabolic disorders and cancer [66]. In the field of glycobiology, bioorthogonal chemistry has allowed researchers to study glycan structures and function with high precision [67]. For example, chemoenzymatic labeling strategies have been developed to selectively label glycans with bioorthogonal probes, such as azides or alkynes, for imaging and profiling studies. By selective labeling of glycans on cell surfaces or tissues, scientists can visualize glycan distribution patterns, characterize glycan-binding proteins, and elucidate glycan-mediated signaling pathways involved in immune responses, inflammation, and cancer metastasis [68,69]. Furthermore, bioorthogonal chemistry has found applications in nucleic acid labeling and genome engineering [70]. For example, researchers have utilized bioorthogonal functional groups to selectively modify DNA or RNA molecules with fluorophores [71], biotin tags, or other chemical moieties for

visualization or detection purposes [72]. This approach has been applied to study DNA replication, transcription, and repair mechanisms, as well as to develop new gene editing tools and gene delivery vectors for therapeutic applications, such as CRISPR/Cas9-based genome editing and RNA interference (RNAi) technologies [73,74].

### *3.1. Applications in live-cell imaging*

Bioorthogonal chemistry has revolutionized live cell imaging by enabling the precise labeling and tracking of biomolecules within their native cellular environments. This approach allows researchers to observe biological processes in real time, providing information on the dynamics of cellular function and molecular interactions that were previously inaccessible [75]. One of the most significant applications of bioorthogonal chemistry in live cell imaging is the labeling of glycans on the cell surface. Glycans play crucial roles in cell signaling, adhesion, and immune recognition, but their study has been challenging due to their structural diversity and complex biosynthetic pathways [76]. Bioorthogonal reactions, such as Staudinger ligation and SPAAC, have been used to selectively label glycan molecules with fluorescent tags [77]. This has enabled researchers to visualize the distribution and dynamics of glycan expression in different cell types and in response to various stimuli [78]. For example, a groundbreaking study used the copper-free click reaction to label and track sialic acid residues on the surface of live cells [79]. The ability to monitor glycan dynamics in real-time provided new insights into the role of glycosylation in cellular communication and immune response [80]. Another key application of bioorthogonal chemistry in live cell imaging is the real-time tracking of protein synthesis and localization [81]. Tetrazine ligation, with its rapid kinetics, has been particularly useful in this context [82]. By incorporating bioorthogonal handles into newly synthesized proteins, researchers can label these proteins immediately after synthesis, allowing visualization of their movement and interactions within the cell [83]. This approach has provided valuable information on the timing and spatial distribution of protein expression, shedding light on the mechanisms of gene regulation and protein trafficking [84]. In general, the integration of bioorthogonal chemistry into live cell imaging has opened up new avenues for studying complex biological systems that offer unprecedented spatial and temporal resolution.

### *3.2. Drug delivery and therapeutics*

The application of bioorthogonal chemistry in drug delivery and therapeutics represents one of the most promising avenues for the development of targeted and controlled therapies [85]. Using the unique properties of bioorthogonal reactions, researchers can design drug delivery systems that precisely activate therapeutic agents at the desired site of action, thus minimizing off-target effects and reducing systemic toxicity [86]. One of the pioneering approaches in this area involves the use of bioorthogonal reactions to activate prodrugs compounds that are biologically inactive until converted to an active form by a specific chemical reaction [87]. For example, the SPAAC reaction has been used to develop prodrugs that remain inert until they encounter a bioorthogonal partner at the target site. This approach has been successfully demonstrated in the treatment of cancer, where the prodrug is activated within the tumor microenvironment, avoiding healthy tissues from the cytotoxic effects of chemotherapy [88]. A notable example of this strategy is the use of bioorthogonal chemistry to activate a prodrug for the treatment of glioblastoma, a highly aggressive brain tumor [89]. Researchers designed a prodrug that could cross the blood-brain barrier and remain inactive until it reached the tumor site, where it was activated by a bioorthogonal reaction. This targeted activation resulted in a significant reduction in tumor size with minimal damage to surrounding healthy brain tissue [90]. In addition to prodrug activation, bioorthogonal chemistry has been used in the development of drug delivery systems that release therapeutic agents in response to specific biological triggers [91]. For instance, nanoparticles functionalized with bioorthogonal groups can be designed to release their payload upon encountering a complementary bioorthogonal reactant within the body. This approach has been explored for the targeted delivery of anticancer drugs, where nanoparticles accumulate at the tumor site and release the drug in response to the unique biochemical environment of the tumor [92]. Furthermore, bioorthogonal reactions have been used to create "click-to-release" systems, in which the binding of a drug to its target triggers a bioorthogonal reaction that releases the drug from its carrier [93]. This method allows precise control over the time and location of drug release, further enhancing the specificity and efficacy of treatment. The application of bioorthogonal chemistry in drug delivery and therapeutics not only enhances the effectiveness of existing treatments, but also paving the way for the development of new therapeutic modalities that can address previously intractable diseases. Table 3 describes the different applications of biorthogonal chemistry in the field of drug delivery and imaging.

## 4. Emerging trends in bioorthogonal chemistry

Bioorthogonal chemistry has emerged as a versatile platform for integrating with other cutting-edge technologies, enhancing their capabilities, and enabling new applications in biomedical research and healthcare [94]. By combining bioorthogonal chemistry with emerging technologies such as nanotechnology, CRISPR/Cas systems, and bioinformatics, researchers can develop synergistic approaches for studying and manipulating biological systems with unprecedented precision and specificity [95]. An area where bioorthogonal chemistry has made significant contributions is in the field of nanotechnology [96,97]. Nanomaterials, such as nanoparticles and nanoscale scaffolds, offer unique properties that make them ideal platforms for the delivery of bioorthogonal probes and therapeutic agents to specific cellular targets [98]. For example, researchers have developed bioorthogonal functionalized nanoparticles that can selectively bind to cancer cells and deliver cytotoxic drugs or imaging agents for targeted cancer therapy and diagnostics [99,100]. Furthermore, bioorthogonal chemistry has been used to modify the surfaces of nanomaterials with biomolecules, such as proteins or nucleic acids, enabling controlled assembly and functionalization of nanoscale structures for applications in drug delivery, tissue engineering, and regenerative medicine [101]. Another area of integration is with CRISPR/Cas systems, a revolutionary gene editing technology that allows precise genome modification [102]. By combining bioorthogonal chemistry with CRISPR/Cas systems, researchers can develop novel tools for labeling, imaging, and controlling gene expression with high spatiotemporal resolution [103]. For instance, researchers have engineered CRISPR/Cas systems with bioorthogonal tags that can be selectively labeled with fluorescent probes or affinity ligands using bioorthogonal chemistry, allowing for real-time visualization and manipulation of specific genomic loci or gene expression patterns in living cells and organisms. This approach has the potential to advance our understanding of gene regulation, cellular differentiation, and disease pathology, as well as to facilitate the development of gene therapy strategies to treat genetic disorders and cancer [104,105]. Therefore, bioorthogonal chemistry has been integrated with bioinformatics tools and computational modeling techniques to analyze complex biological data and predict molecular interactions with high accuracy [106,107].

**Table 3.** Application of biorthogonal chemistry in drug delivery and imaging.

| Application | Bioorthogonal reaction/method | Key features | Advantages | References |
|---|---|---|---|---|
| Targeted drug delivery in cancer therapy | Strain-promoted azide-alkyne Cycloaddition (SPAAC) | Use of clickable drug carriers with cancer-specific targeting | Improved precision in drug delivery to cancer cells | [1,2,32] |
| In vivo imaging of cellular processes | Fluorescence-activated click chemistry | Integration of fluorescent tags for real-time imaging | Non-invasive tracking of cellular activities | [3,4,77] |
| Development of diagnostic probes | Tetrazine-based ligation | Use of tetrazine-modified probes for imaging | High specificity and reduced background signal | [5,6,33] |
| Monitoring drug release in vivo | Bioorthogonal click chemistry | Real-time monitoring of drug release from carriers | Detailed insights into drug release dynamics | [7,8,31] |
| Imaging of tumor microenvironment | Photo-crosslinking Reactions | Light-sensitive crosslinkers for tumor imaging | Enhanced imaging contrast in complex tumor settings | [9,10,71] |
| Tracking biomolecular interactions | Staudinger ligation | Use of Staudinger reactions to track biomolecular interactions | Precise interaction mapping in biological systems | [11,12,37] |
| Multimodal imaging approaches | Dual bioorthogonal reactions | Combination of multiple imaging modalities | Comprehensive imaging with enhanced resolution | [13,14,92] |
| Development of smart drug delivery systems | Bioorthogonal probes with ph-sensitive ligands | Use of pH-sensitive ligands for targeted release | Controlled drug release in specific environments | [15,16] |
| Real-time visualization of cellular processes | Oxime ligation | Real-time visualization using oxime-labeled probes | Dynamic tracking of cellular processes | [17,18,83] |
| Imaging of protein-protein interactions | Bioorthogonal chemical reporter assays | Use of chemical reporters to study protein interactions | High-resolution imaging of protein interactions | [19,20,93] |
| Drug delivery via nanoparticles | Click chemistry with nanoparticles | Functionalization of nanoparticles for targeted delivery | Enhanced delivery efficiency and targeting precision | [21,22] |
| Real-time monitoring of enzyme activity | Bioorthogonal enzyme substrates | Use of bioorthogonal substrates for enzyme activity monitoring | Real-time insights into enzyme kinetics | [23,24,70] |

By combining experimental data with computational models, researchers can gain a deeper understanding of the mechanisms underlying biological processes and identify new targets for therapeutic intervention [108]. For example, bioorthogonal chemistry-based proteomics approaches, such as proximity labeling and cross-linking mass spectrometry, have been coupled with bioinformatics algorithms to map protein-protein interactions, identify protein complexes, and characterize signaling networks in cells and tissues [109]. This integrated approach enables researchers to decipher the molecular mechanisms driving disease progression and identify potential biomarkers for early diagnosis and targeted therapy [110]. As the field of bioorthogonal chemistry continues to expand, the search is driven by the quest for more efficient, selective, and versatile reactions that can be applied in increasingly complex biological settings. As the toolbox of bioorthogonal reactions grows, so does their potential impact across various domains of science and medicine. The other most promising trend in bioorthogonal chemistry is the development of fluorogenic reactions, bioorthogonal reactions that produce a fluorescent signal upon completion [111]. These reactions are particularly valuable for live cell imaging, where the ability to visualize reaction progress in real time can provide detailed insights into dynamic biological processes [112]. For example, the development of tetrazine-based fluorogenic reactions has enabled the tracking of biomolecules with minimal background noise, allowing high-resolution imaging of cellular events [113]. Another emerging area is the integration of bioorthogonal chemistry with synthetic biology [114]. By designing synthetic pathways that incorporate bioorthogonal reactions, researchers are creating new tools for the precise control of biological functions. These innovations are paving the way for the development of engineered cells that can respond to specific stimuli in a controlled manner, with potential applications in therapeutic gene editing, metabolic engineering, and the construction of synthetic tissues [115]. The application of bioorthogonal chemistry in vivo has garnered significant attention. The ability to conduct bioorthogonal reactions within living organisms opens new possibilities for targeted therapies, diagnostics, and real-time monitoring of disease progression [116]. Researchers are exploring the use of bioorthogonal reactions to create highly specific probes for imaging diseases such as cancer, where probes can be activated in the presence of tumor-specific markers, allowing early detection and monitoring of treatment efficacy [117]. Furthermore, the discovery and development of new bioorthogonal ligation partners are broadening the scope of this field. Innovations such as sulfur-fluoride exchange (SuFEx) chemistry [118] and strain-promoted inverse electron demand Diels-Alder (SPIEDAC) reactions [119] are expanding the range of chemical transformations that can be performed in biological environments. These advances not only enhancing the versatility of bioorthogonal chemistry but also drive the exploration of new applications in materials science, catalysis, and beyond. As bioorthogonal chemistry continues to evolve, its impact on science and medicine is expected to grow, offering novel solutions to long-standing challenges and opening new frontiers in research and innovation [120,121].

## 5. Challenges and future directions

Although bioorthogonal chemistry has made significant strides in the advancement of biomedical research and healthcare, several challenges remain that must be addressed to fully realize its potential [121]. As noted above, bioorthogonal chemistry methods must be compatible with biological components and must occur sufficiently rapidly to capture analytes of interest at low concentrations. However, reaction partners that undergo sufficiently fast reactions may not be selective and may not be sufficiently stable under physiological conditions [122]. Bioorthogonal methods that do not require catalysts would make the methods easier to use and reduce toxicity to organisms. The development of novel bioorthogonal functionalities and methods would make bioorthogonal chemistry more broadly useful. Additionally, exploring future directions in bioorthogonal chemistry offers exciting opportunities for further innovation and application.

### *5.1. Challenges*

#### *5.1.1. Biocompatibility*

Despite progress in the development of bioorthogonal reactions that are compatible with living systems, ensuring optimal biocompatibility remains a challenge. Some bioorthogonal reactions may still exhibit cytotoxicity or interfere with cellular processes, limiting their utility for in vivo applications. Addressing this challenge requires the development of new bioorthogonal reactions with improved biocompatibility and minimal off-target effects [123].

#### *5.1.2. Selectivity*

Achieving high selectivity is essential for bioorthogonal chemistry to accurately target specific biomolecules or cellular compartments within complex biological environments. However, achieving selectivity in the presence of competing biomolecules or endogenous functional groups can be challenging. Future research efforts should focus on the design of bioorthogonal reactions with enhanced selectivity and specificity for their biological targets [124].

#### *5.1.3. In vivo stability*

Bioorthogonal reactions must maintain their stability and efficiency in vivo to enable longitudinal studies and therapeutic applications. However, factors such as enzymatic degradation, metabolic turnover, and immune responses can affect the stability and performance of bioorthogonal probes and imaging agents in living organisms. Overcoming these challenges requires the development of bioorthogonal reactions and probes that are resistant to biological degradation and compatible with physiological conditions [125].

#### *5.1.4. Delivery and localization*

Efficient delivery and precise localization of bioorthogonal probes and therapeutic agents to target tissues or cells are critical to achieve optimal imaging and therapeutic outcomes. However, achieving selective delivery and controlled release of bioorthogonal agents in vivo remains a significant challenge. Strategies are needed to improve target efficiency, improve cellular uptake, and minimize off-target effects to maximize the clinical utility of bioorthogonal chemistry-based approaches [126].

### *5.2. Future directions*

#### *5.2.1. Development of novel reactions*

Continued efforts to develop novel bioorthogonal reactions with improved selectivity, biocompatibility, and in vivo stability are essential to expand the toolkit available to researchers. Innovations in reaction design, catalysis, and reaction kinetics hold promise for addressing current limitations and enabling new applications in biomedical research and therapy.

#### *5.2.2. Multimodal imaging and therapeutics*

Integrating bioorthogonal chemistry with multimodal imaging techniques, such as PET/MRI and PET/CT, offers opportunities for synergistic imaging and therapeutics. By combining different imaging modalities with complementary bioorthogonal probes, researchers can achieve enhanced sensitivity, spatial resolution, and functional information for diagnosing diseases and monitoring therapeutic responses in real time.

#### *5.2.3. Targeted drug delivery*

Bioorthogonal-chemistry-based approaches hold promise for the development of targeted drug delivery systems that can selectively deliver therapeutic agents to diseased tissues or cells while minimizing systemic toxicity. By conjugating bioorthogonal probes to drug molecules or nanocarriers, researchers can achieve site-specific drug delivery and controlled release, improving therapeutic efficacy, and reducing off-target effects.

#### *5.2.4. In vivo imaging and sensing*

Advancements in in vivo imaging and sensing techniques, such as bioluminescence imaging and photoacoustic imaging, offer new opportunities for noninvasive monitoring of biological processes in living organisms. By integrating bioorthogonal chemistry with these imaging modalities, researchers can develop novel probes and sensors to visualize molecular events and physiological processes in real time, enabling early detection of diseases and personalized treatment strategies.

#### *5.2.5. Bioorthogonal chemistry for remote control of biological systems*

Develop bioorthogonal reactions that can be controlled remotely using external stimuli such as light, ultrasound, or magnetic fields. This could lead to spatiotemporally controlled biological processes in tissues or organs, enabling noninvasive manipulation of cellular functions, targeted drug activation, or gene expression in specific regions of the body.

#### *5.2.6. Integration with artificial intelligence for predictive chemistry*

Using artificial intelligence (AI) and machine learning algorithms to predict and design new bioorthogonal reactions with desired properties, such as reaction speed, selectivity, and stability, in biological environments. Artificial intelligence can also be employed to optimize the use of bioorthogonal chemistry in complex biological systems, such as predicting off-target effects and enhancing reaction kinetics.

#### *5.2.7. Design of bioorthogonal reactions for single-molecule tracking*

The development of ultrasensitive bioorthogonal probes is crucial for enabling single-molecule tracking within living cells. These probes would empower researchers to monitor individual biomolecules with precision overtime and space, offering unparalleled insights into dynamic biological processes such as protein folding, enzymes kinetics and real-time signaling pathways.

#### *5.2.8. Bioorthogonal chemistry in synthetic organelles and artificial cells*

Bioorthogonal reactions specifically tailored for use in synthetic organelles or artificial cells could mimic cellular environments for studying complex biochemical reactions. These systems can be used to explore cellular behaviors, construct artificial life forms, or develop novel biobased factories for the production of pharmaceuticals or biofuels.

#### *5.2.9. Bioorthogonal reactions for dynamic biomaterial interfaces*

Bioorthogonal reactions can be created to engineer dynamic biomaterial interfaces that can change properties in response to biological signals. Such interfaces could be used in smart implants, wound healing materials, or drug delivery systems that adapt to body needs, enhancing their therapeutic potential and patient safety.

### *5.3. Exploration of new reaction modalities for soft matter and hydrogels*

Investigate new types of bioorthogonal reactions that work effectively in soft matter systems such as hydrogels, liquid crystals, or biomimetic materials. These reactions could enable the creation of intelligent and functionalized materials for applications in tissue engineering, biosensing, and drug-release systems.

#### *5.3.1. Development of bioorthogonal reactions for controlled protein degradation*

Novel bioorthogonal reactions to selectively tag proteins for degradation via cellular pathways such as the ubiquitin proteasome system or autophagy. This could lead to new methods for studying protein function, regulating protein levels, and developing targeted therapies for diseases such as cancer and neurodegenerative disorders.

#### *5.3.2. Incorporation into microfluidic and lab-on-a-chip systems*

Integrate bioorthogonal reactions into microfluidic devices and lab-on-a-chip systems to enable high-throughput screening, single-cell analysis, and real-time monitoring of biochemical reactions in miniaturized formats. This could revolutionize personalized medicine, diagnostics, and biochemical analysis.

#### *5.3.3. Use in rewritable biological circuits and logic gates*

Future work could explore the use of bioorthogonal reactions to create rewritable biological circuits or logic gates within cells, contributing to the development of synthetic biology tools for more complex biological computation, memory storage, or biosensing applications.

#### *5.3.4. Exploring bioorthogonal reactions for quantum dots and nanoparticles*

Bioorthogonal chemistry can be applied to modify and functionalize quantum dots and other nanoparticles for use in advanced imaging, sensing, and therapeutic delivery. Future research could focus on improving the stability, specificity, and biocompatibility of these nanomaterials through bioorthogonal strategies. In summary, addressing the challenges and exploring future directions in bioorthogonal chemistry holds great promise for advancing biomedical research and healthcare. By overcoming technical limitations, developing innovative approaches, and integrating bioorthogonal chemistry with emerging technologies, researchers can unlock new opportunities to study complex biological processes, diagnose diseases, and deliver targeted therapies with unprecedented precision and specificity.

## 6. Conclusions

Bioorthogonal chemistry has fundamentally transformed the landscape of chemical biology, providing researchers with powerful tools to study and manipulate biological systems with unparalleled specificity and control. From its origins with Staudinger ligation to the development of rapid and biocompatible reactions such as SPAAC and tetrazine ligation, bioorthogonal chemistry has enabled a wide array of applications, including live cell imaging, drug delivery, and therapeutic interventions. The unique ability of bioorthogonal reactions to operate within living organisms without interfering with native biochemical processes has opened up new avenues for research and clinical applications. These reactions not only facilitated the detailed study of cellular processes, but also have also driven the development of targeted therapies that can be precisely activated at the site of the disease, minimizing side effects, and improving treatment efficacy. As the field continues to evolve, emerging trends such as fluorogenic reactions, synthetic biology integration, and in vivo applications are poised to further extend the reach of bioorthogonal chemistry. The discovery of new ligation partners and reaction mechanisms is expanding the chemical toolkit available to researchers, enabling the exploration of new frontiers in science and medicine. In conclusion, bioorthogonal chemistry stands as a testament to the power of interdisciplinary collaboration, bridging the gap between chemistry and biology to create innovative solutions to complex biological challenges. As we look to the future, the continued development of this field promises to unlock new insights into the fundamental processes of life and contribute to the advancement of personalized medicine and other transformative technologies.

## Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
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