

## REVIEW

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## A powerful bioorthogonal toolbox boosting the development of immune theranostics

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Bioorthogonal chemistry encompasses a series of rapid and selective reactions that proceed under physiological conditions without interfering with essential biological functions. Over the past few decades, benefiting from its simplicity and efficiency, bioorthogonal chemistry has provided non-chemists with a powerful toolbox to advance chemical biology research and develop innovative biomedical strategies, leading to significant breakthroughs in disease treatment. With a growing understanding of pathological biology and immunology, immune theranostics has emerged as a next-generation strategy for precise disease diagnosis and treatment, as exemplified by the clinical success of CAR-T cell therapy. Indeed, prevalent advances have demonstrated that bioorthogonal chemistry significantly facilitates the construction of immune theranostic platforms. Recognizing its growing importance, this review provides an overview of the ideal bioorthogonal reactions suitable for biomedical applications. Additionally, we systematically summarize promising bioorthogonal-enabled applications for the *ex vivo* construction of immunotherapeutic agents (e.g. antibody–drug conjugates, vaccines, engineered cells, etc.). Furthermore, we also highlight recent developments in *in vivo* immunomodulation and precision diagnostics *via* bioorthogonal chemistry. Finally, we discuss the existing challenges and limitations associated with bioorthogonally driven immune theranostics, aiming to inspire future research toward enhancing its practical performance and clinical translation.

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## 1. Introduction

Bioorthogonal chemistry refers to a large scope of rapid and selective reactions that could happen in a mild physiological environment without perturbation of endogenous functional molecules or disturbance of the biological system.<sup>1</sup> It is recognized as a powerful and sophisticated toolbox to facilitate and accurately manipulate intricate biological events, greatly boosting the development of chemical biology in the last few decades.<sup>2</sup> Recently, the 2022 Nobel Prize in Chemistry has been awarded for bioorthogonal click chemistry, ushering in a new wave of interest from the scientific community. In particular, bioorthogonal reactions, regarding the ligation processes between two specific components, have paved the way for investigating biomolecules under complicated living conditions. Moreover, these reactions enable the direct analysis of biocomponents (e.g., glycans, lipids, nucleic acids, proteins, etc.) through the biological processes in cells,<sup>3</sup> resulting in numerous significant advancements in chemical biology, such as cellular surface engineering and targeted site-specific

labeling.<sup>4</sup> Along with the development of molecular mechanisms, bioorthogonal chemistry could also rapidly release the payload from pre-caging precursors, termed the click-to-release reaction.<sup>5</sup> These cleavage reactions contribute to a new era of manipulating bioactive molecules in a controllable manner, inspiring novel prodrug strategies in biomedical applications. Benefiting from the maturation of powerful bioorthogonal chemistry, it provides numerous opportunities for researchers in non-chemistry fields, especially for biologists, immunologists, and clinician scientists, to implement their chemical biology design and construct biomedical strategies for significant breakthroughs of various disease treatments.

Notably, with a deep understanding of immune networks, immunotherapy has been heralded as new-generation therapeutics,<sup>5</sup> witnessed by the successful clinical practice,<sup>6</sup> where bioorthogonal chemistry also significantly contributes to immunotherapeutic development. In particular, immune checkpoint blockers (ICBs) and chimeric antigen receptor (CAR) T-cell therapy provide new options for patients, which reshape the current oncology approaches.<sup>7</sup> Meanwhile, the established immunotherapeutics also play critical roles in combating bacterial or viral infections *via* the development of vaccines,<sup>8</sup> especially in the recent global prevalence of COVID-19, illustrating the universal applicability towards diverse diseases.<sup>9</sup> In principle, compared with conventional therapies, especially for combating tumors, immunotherapy could eradicate malignant

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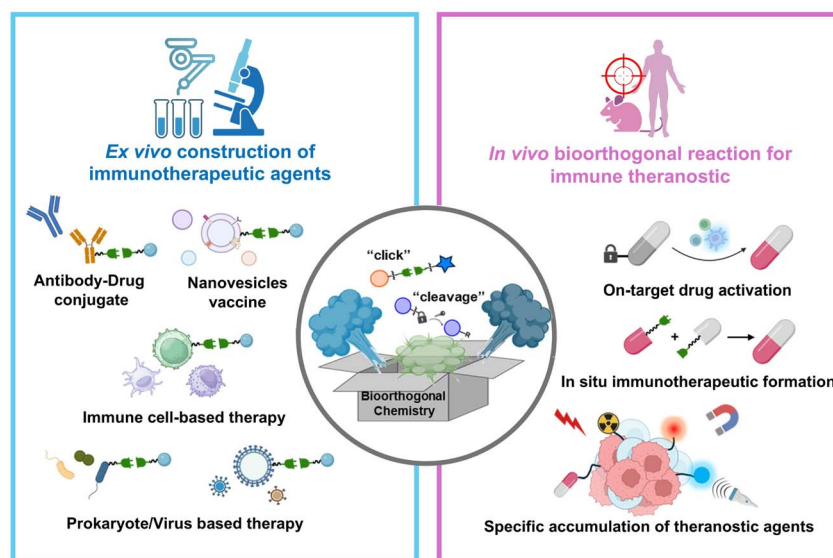


Fig. 1 Powerful bioorthogonal chemistry toolbox for *ex vivo* construction of immunotherapeutic agents and *in vivo* reactions for smart immune theranostics.

tumors in the late stages, elevating survival with reduced side effects. The antitumor immunity could promote global immune activation for metastasis suppression and build up long-term immune memory against tumor relapse.<sup>10</sup> However, due to the systemic administration of immunotherapeutic agents in the current bedside practice, it may elicit off-target side effects. Besides, for CAR T-cell therapy, the treatment of solid tumors has been hampered by poor infiltration of T-cells into deep tissues.<sup>11</sup> Hence, recent advances in immunotherapy have applied bioorthogonal chemistry to overcome these limitations by targeting the activation of immune-modulating agents and improving the accumulation of CAR T cells in malignant tumors.<sup>12</sup> Similarly, bioorthogonal chemistry also boosted the *ex vivo* construction of vaccines to maximize their therapeutic efficacy while minimizing their potential off-target toxicity.

Intriguingly, bioorthogonal chemistry has already become the essential toolbox for the detailed exploration of intricate immune systems and the establishment of novel therapeutic approaches. In parallel, the development of clickable biolabeling also offers the promise to monitor and visualize the immune-related biomarkers as reliable criteria, evaluating the efficacy of immunomodulation. In particular, the combination of theranostics into immunotherapy, termed immune theranostics, could improve the diagnosis and understanding of disease status, thereby establishing personalized immune-targeting strategies and amplifying the efficacy of individual treatments. Along with all these striving developments of bioorthogonal chemistry and a deeper understanding of complex immune systems, recently, emerging advances are boosting the revolution of immune theranostic strategies, potentiating their biomedical applications from bench to bedside. Therefore, it is the prime moment to reconsider and explore how bioorthogonal chemistry can enhance the effectiveness of immunotherapies and address their existing challenges.

In this review, we have presented a comprehensive summary of the current scope of broadly applicable bioorthogonal reactions, exemplifying how reaction design provides mechanistic insight into the opportunities for immune theranostics. More importantly, we have highlighted emerging avenues of recent immune theranostic strategies with the assistance of bioorthogonal click chemistry, including *ex vivo* construction of immunotherapeutic agents and *in vivo* bioorthogonal engineering for precise diagnosis and targeted immunotherapy (Fig. 1). In particular, we will introduce the recent advances and formulate guidelines for systematically addressing how to utilize bioorthogonal chemistry to construct sophisticated and reliable immunotherapies, including the construction of immunotherapeutic agents (e.g., nanovaccines, biomimetic agents, and antibody–drug conjugates), targeted immunotherapy activation, site-specific delivery of immune drugs and combinational immune theranostics. Finally, we also discuss the current limitations and obstacles in bioorthogonal chemistry-mediated immune theranostics, which may provide meaningful insights for novel improvements toward future practice.

## 2. Bioorthogonal reaction scope for the development of immune theranostics

As the pioneer in the field, in 2003, Carolyn Bertozzi and her collaborators proposed the term “bioorthogonal chemistry” to define the fundamental principles of the biocompatible Staudinger ligation reaction, originally developed in the 1990s.<sup>1,2b,13</sup> It refers to a modular, highly selective, mild, and high-yield coupling reaction involving complementary functional groups that do not exist in biological systems, are inert to biological components, and are prone to mutual reactions under



biological conditions. Since then, many bioorthogonal chemistry reactions have been applied to biological systems for various functionalizations, especially ligation reactions which enable the covalent assembly of two clickable fragments into an intact molecule, facilitating precise construction of functional agents in complex biological environments (Fig. 2A). In particular, the pioneering Staudinger ligation involves the reaction of azide with phosphines (e.g. triphenylphosphine) or phosphite esters to form an imine phosphonane intermediate. These intermediates, upon hydrolysis, can yield the corresponding amines and phosphine oxides (such as triphenylphosphine oxide), thereby providing a method for the preparation of amines through the reduction of azide. Although the Staudinger ligation reaction has become a choice for many biological ligation reactions and certain uses in *in vivo* labeling, some drawbacks still collectively affect its wide applications, such as the non-specific oxidation of phosphine reagents in biological systems and the relatively slow kinetics for dynamic tracking of biological processes (e.g.  $7.7 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$ ). High-concentration phosphine reagents can solve this problem to a certain extent, but high background signal interference also follows. Therefore, other bioorthogonal click reactions with faster reaction kinetics have been developed as alternatives.

This concept laid the foundation for the development of copper(i)-catalyzed azide-alkyne cycloaddition (CuAAC), in which a Cu(I) catalyst promotes the formation of stable 1,2,3-triazole rings between azide and alkynyl groups, enabling the precise conjugation of tumor-targeting ligands. In 2013, Professor Fokin proposed the classic mechanism of CuAAC – the multi-nuclear Cu(I) catalytic dehydrogenation mechanism based on the step-by-step and single-core copper mechanism proposed in 2002. First, Cu coordinates with alkynes to form Cu-alkyne- $\pi$ -complexes. Then, through the deprotonation of alkynes, it is transformed into a Cu- $\sigma$ ,  $\pi$ -alkyne intermediate, and this key intermediate must be formed after the deprotonation of terminal alkynes. Only metallic copper with strong  $\sigma$  ligands (such as N-heterocyclic carbenes) can prevent the deprotonation of alkynes and allow the CuAAC reaction of internal alkynes, which greatly limits the application of CuAAC. CuAAC, a well-established click reaction, is characterized by its rapid reaction kinetics (e.g.  $10\text{--}100 \text{ M}^{-1} \text{ s}^{-1}$ ), achieving high efficiency, gentle reaction conditions, and outstanding chemical selectivity in biological systems.<sup>14</sup> As a result of its outstanding characteristics, the bioorthogonal reaction has become the benchmark reaction for *in vivo* biomolecule labeling and tracking,<sup>15</sup> in which CuAAC has been commonly

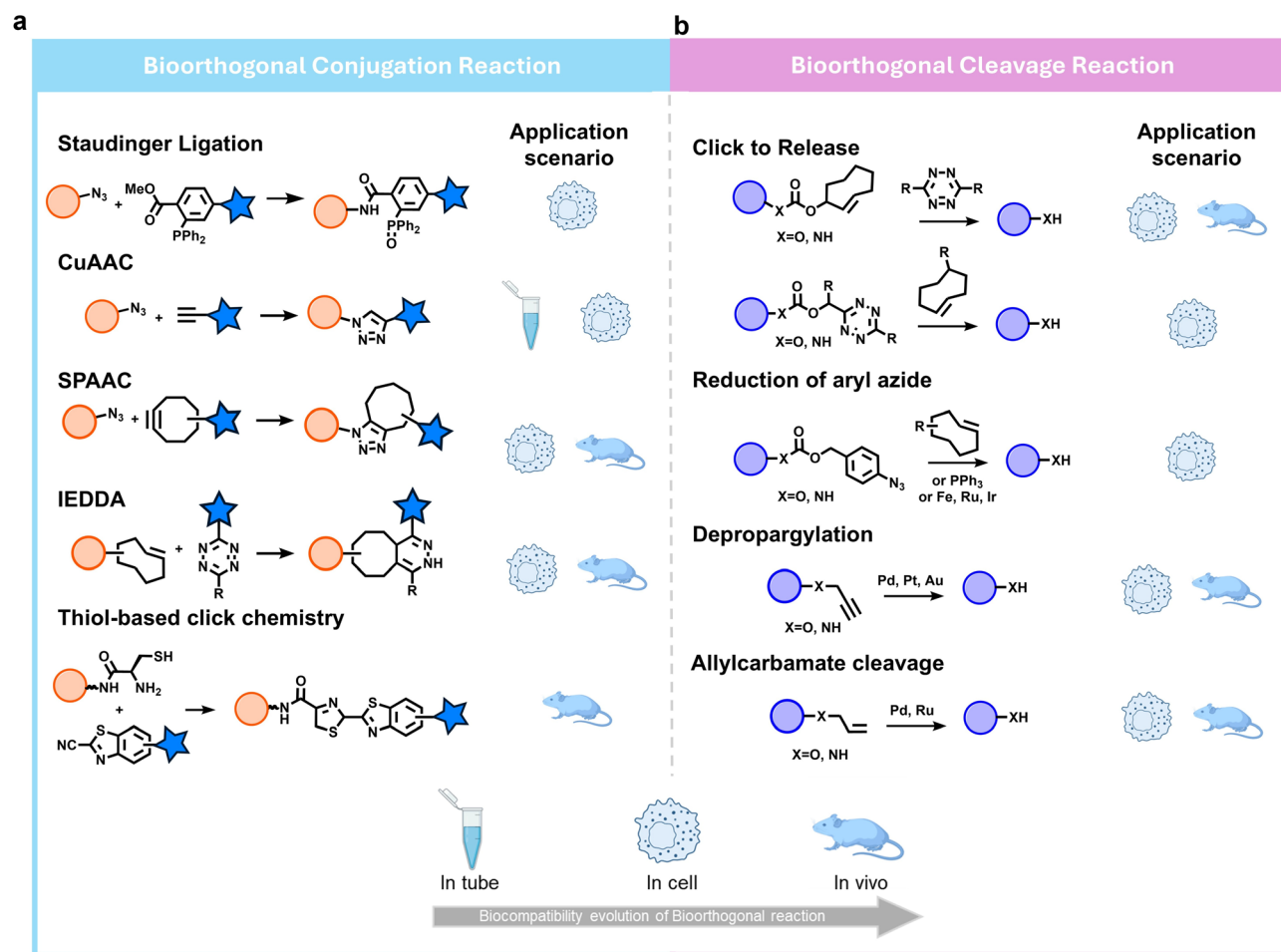


Fig. 2 Representatives of bioorthogonal conjugation reactions (A) and cleavage reactions (B) and their suitable application scenario for the development of immune theranostics.

applied in drug discovery to enable on-site drug synthesis for treating various diseases.<sup>16</sup> Although CuAAC has been significantly adopted in *in vitro* research, there are still some drawbacks that hinder its application *in vivo*. For example, the Cu(I) in CuAAC is mainly obtained by *in situ* reduction of Cu(II) with sodium ascorbate. However, Cu(I) is prone to promoting the generation of reactive oxygen species (ROS) in organisms, while sodium ascorbate mediates the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), eventually leading to biological damage. This greatly limits the further application of CuAAC *in vivo*. Noting the reliance on Cu(I) catalysts, many studies have attempted to improve the biocompatibility of the CuAAC reaction through ingenious methods, such as developing ligands with good water solubility to accelerate the CuAAC reaction while stabilizing Cu(I) to limit Cu(I)-mediated cytotoxicity.<sup>17</sup>

Concerns about the cytotoxicity of Cu(I) species led to the alternative development of copper-free strain-promoted alkyne-azide cycloaddition (SPAAC). This process naturally proceeds between a strained alkyne and an azide, allowing fast coupling without metal catalysis and retaining biocompatibility.<sup>18</sup> SPAAC can be carried out efficiently mainly for two reasons: (1) the chemical potential energy of the azide matrix and the alkyne matrix as reactants is very high. When the cyclization reaction generates a stable triazole, it can release more than 188 kJ mol<sup>-1</sup> heat, meeting the high energy conditions required by the reactants; (2) azide and alkyne are difficult to react with biological macromolecules under reaction conditions, and they are inert to most other reaction reagents and solvents. In addition, the cyclic alkyne group and azide group show weak polarity and have little effect on the chemical properties of the connection structure, meeting the selectivity requirements of biomedical applications both *in vitro* and *in vivo*. Therefore, SPAAC takes advantage of the high ring tension of cyclic alkynes themselves to undergo chemically regioselective click reactions without the participation of copper catalysts. Although SPAAC eliminates metal-related toxicity, it suffers from relatively slower reaction kinetics (e.g. 1–60 M<sup>-1</sup> S<sup>-1</sup>).<sup>19</sup> To improve the reaction kinetics, electron-withdrawing fluorine atoms can be incorporated into the cyclooctyne reagent. For example, difluorocyclooctyne (DIFO), whose reactivity can be improved to be comparable to that of CuAAC, can be applied to *in vivo* bioorthogonal imaging of azide-labeled sugars.<sup>20</sup> Moreover, compared with a simple clickable substrate of CuAAC, the cyclooctyne with a bulky structure suffered from a tedious synthesis route and limitation of large-scale production, which pose a cost concern for practical translation.<sup>21</sup>

A major breakthrough was achieved with the inverse-electron-demand Diels-Alder (IEDDA) reaction, which is a catalyst-free [4 + 2] cycloaddition between an electron-deficient 1,2,4,5-tetrazine (diene) and an electron-rich strained alkene such as *trans*-cyclooctene (TCO), enabling site-specific bioorthogonal modification without external catalysts.<sup>22</sup> Mechanistically, the tetrazine first undergoes a concerted cycloaddition with the dienophile to give a dihydropyridazine adduct that immediately expels N<sub>2</sub> via a retro-Diels-Alder step, furnishing a stable product while maintaining compatibility with biological media. IEDDA, typically happening between tetrazine-TCO pairs,

stands out for outstanding kinetics (e.g. 1–10<sup>6</sup> M<sup>-1</sup> S<sup>-1</sup>) under physiological conditions.<sup>23</sup> So far, an increasingly broad substrate scope has been noted, as new dienophiles and tetrazines are developed for improved balance between their stability and reactivity, which enables efficient biolabeling at sub-micromolar concentrations and pre-targeted *in vivo* bioimaging. Nonetheless, practical challenges remain behind this powerful bioorthogonal reaction, which hinder its widespread application in biological research compared with the strain-promoted alkyne-azide cycloaddition. A major limitation lies in the ongoing development of incorporating dienophile groups into biomolecules and living systems. Due to their relatively bulky molecular structures and steric hindrance, dienophiles remain less accessible and versatile compared to the well-established azide-based modifications. Benefiting from remarkable efforts and advances in recent years, beyond conventional chemical conjugation, emerging approaches such as metabolic labeling<sup>24</sup> and genetic code expansion<sup>25</sup> have enabled the introduction of clickable moieties into diverse cellular or microbial biomacromolecules. In addition, tetrazines as reactive partners in IEDDA chemistry exhibit an inherent reactivity-stability trade-off, wherein higher reaction rates are often accompanied by reduced stability in biological environments due to susceptibility to hydrolysis and reduction. To address this limitation, novel IEDDA substrates are urgently demanded to enhance chemical stability without significantly compromising reaction kinetics.

Additionally, nature-inspired bioorthogonal reactions with improved biocompatibility have attracted increasing attention, among which thiol-based click reactions represent a versatile and promising alternative strategy. These reactions involve thiol-ene or thiol-yne chemistry, leveraging the high nucleophilicity of thiols to form stable thioether bonds under mild conditions. Inspired by the synthetic pathway of D-luciferin structure, a thiol-based condensation between D-cysteine (D-Cys) and 2-cyanobenzothiazole (CBT) with moderate kinetics was developed (e.g. 9.1 M<sup>-1</sup> S<sup>-1</sup>), where the fragments could react under different controllable physiological conditions.<sup>26</sup> However, thiol-rich proteins in organisms also bring challenges to the application of these reactions. Therefore, many specifically activated cysteine derivatives have been well developed and applied for better drug retention and probe signal amplification.

Distinctively, bioorthogonal chemistry is not limited to fragment conjugation, while it also holds broader potential to restore endogenous biological functions via specific and controllable bond cleavage mechanisms (Fig. 2B).<sup>27</sup> Mechanistically, these reactions involve functional group-masked reactants that undergo bond cleavage under physiological conditions, converting from an inactive to an active state.<sup>28</sup> This strategy facilitates the on-site production of active therapeutic agents, broadening the scope of targeted treatment applications. In 2013, Robillard and colleagues demonstrated that the inverse-electron-demand Diels-Alder (IEDDA) reaction serves as an efficient bioorthogonal cleavage mechanism to activate prodrugs.<sup>29</sup> Exhibiting exceptional efficiency, this “click-to-release” system has been extensively applied to prodrug and





protein activation in living organisms.<sup>30</sup> Additionally, Bernardes *et al.* developed a vinyl ether/tetrazine (Tz-vinyl ether) system, enabling the controlled release of alcohol-containing compounds shielded using vinyl ethers.<sup>30c</sup> Furthermore, in 2006, Meggers and colleagues introduced a Ru-catalyzed deprotection reaction for cleaving Alloc-protected amines in cellular environments.<sup>31</sup>

Moreover, transition metals, including iridium (Ir), iron (Fe), platinum (Pt), palladium (Pd), and gold (Au), have also been widely explored for their catalytic properties in eliminating protecting groups.<sup>32</sup> In 2014, Chen *et al.* employed various palladium catalysts, including Pd(dba)<sub>2</sub> and allyl<sub>2</sub>Pd<sub>2</sub>Cl<sub>2</sub>, to successfully achieve site-specific depropargylation at pre-masked lysine residues within proteins of interest, thereby restoring protein function in living cells.<sup>33</sup> Building upon the excellent orthogonality of metal-catalyzed reactions, Mascarñas *et al.* simultaneously demonstrated Ru complex-catalyzed deallylation and Au complex-mediated carbon-carbon bond cyclization within HeLa cells, achieving the generation of multiple fluorescent signals *via* transition metal catalysis. This process enables precise intracellular activation of proteins and therapeutic agents, providing a highly controlled approach for drug release and biochemical regulation within living cells.<sup>34</sup> In addition, bioactive inorganic metal complexes can also be synergistically exploited to achieve bioorthogonal cleavage. Bernardes *et al.* developed a metal-mediated bond-cleavage strategy utilizing platinum complexes (*e.g.*, K<sub>2</sub>PtCl<sub>4</sub> or cisplatin) for prodrug activation. Under aqueous conditions, rapid decaging of pentynoyl tertiary amides and *N*-propargyl groups occurs, enabling intracellular activation of protected analogues of cytotoxic agents, including 5-fluorouracil and monomethyl auristatin E.<sup>35</sup> This process enables precise intracellular activation of proteins and therapeutic agents, providing a highly controlled approach for drug release and biochemical regulation within live cells.

Overall, continuous efforts have been devoted to expanding the scope of bioorthogonal ligation and cleavage reactions, thereby enriching the arsenal of these powerful chemical tools. So far, increasing mechanistic insights into these reactions have allowed their strengths and weaknesses to be more clearly defined, thereby refining their applicability to specific biological settings, especially in immune theranostic strategies (Table 1). In particular, the catalyst-required CuAAC has demonstrated strong potential for *ex vivo* applications, benefiting from its small-sized clickable substrates and high reaction kinetics, enabling the efficient construction and screening of immunotherapeutic agents, such as vaccines, antibody-drug conjugates, and small functional molecules. However, the inherent toxicity of externally introduced copper catalysts poses a major barrier to the *in vivo* application of CuAAC in immune theranostics. On the other hand, catalyst-free bioorthogonal reactions, especially SPAAC and IEDDA, have been more widely employed for on-target immune modulation in living systems due to their intrinsic biocompatibility and operational simplicity. Meanwhile, a variety of well-established strategies (*e.g.*, chemical conjugation, metabolic labeling, genetic code expansion, *etc.*) have been developed to enable the installation of these

bioorthogonal handles onto cellular structures (*e.g.*, cytoplasmic membrane, subcellular organelle, *etc.*) for efficient bioorthogonal engineering. Given the existing limitations in immunotherapy, including concerns over immunogenicity, off-target effects in normal tissues, and suboptimal immune activation, *in vivo*-applicable bioorthogonal click reactions provide promising approaches to improve targeting precision and significantly boost the overall therapeutic outcome. However, the SPAAC and IEDDA reactions are still challenged by issues such as the bulky structure of clickable substrates, limited stability in physiological environments, and difficulties in large-scale synthesis. As a result, the development of next-generation bioorthogonal chemistry continues to attract significant attention. Nevertheless, existing reaction platforms have already carved out substantial roles in biomedical developments and have demonstrated great promise for translational applications in immune theranostics.

### 3. *Ex vivo* construction of immunotherapeutic agents *via* bioorthogonal chemistry

Typically, as a powerful and facile chemistry tool, the bioorthogonal click reaction could rapidly construct the bioactive conjugates and functionalize the biological components. This chemical toolbox plays significant roles in the design of antibody-drug conjugates, biomimetic nanoplateforms, and cell-based immunotherapy, which may be discussed in detail in the following sections.

#### 3.1 Facile construction of antibody-drug conjugates

Antibody-drug conjugates (ADCs) have established themselves as a transformative therapeutic platform, achieving significant clinical success in targeted disease treatment.<sup>36</sup> Their key advantage lies in the ability to precisely deliver cytotoxic payloads to diseased sites, thereby significantly enhancing the clinical applicability by minimizing adverse systemic effects. Moreover, for immune-regulatory design, ADCs can load immunostimulatory payloads or leverage immune-activating antibodies to engage specific receptors (*e.g.*, PD-L1,<sup>37</sup> CD22, CD74, *etc.*) on tumor or immune cells, ultimately enhancing systemic antitumor immunity. However, most ADCs are typically synthesized *via* nonspecific modifications of inherent active cysteine or lysine residues on antibodies, which inevitably result in heterogeneous products with varying drug-to-antibody ratios (DAR) and mixed pharmacological properties.<sup>38</sup> Such heterogeneity markedly compromises the potency, pharmacokinetic stability, antigen-binding affinity, and overall tolerability of ADCs. Accordingly, the construction of site-specific and homogeneous multimeric conjugation products has emerged as a critical research direction, attracting substantial research attention for improving the therapeutic consistency and performance of ADCs.

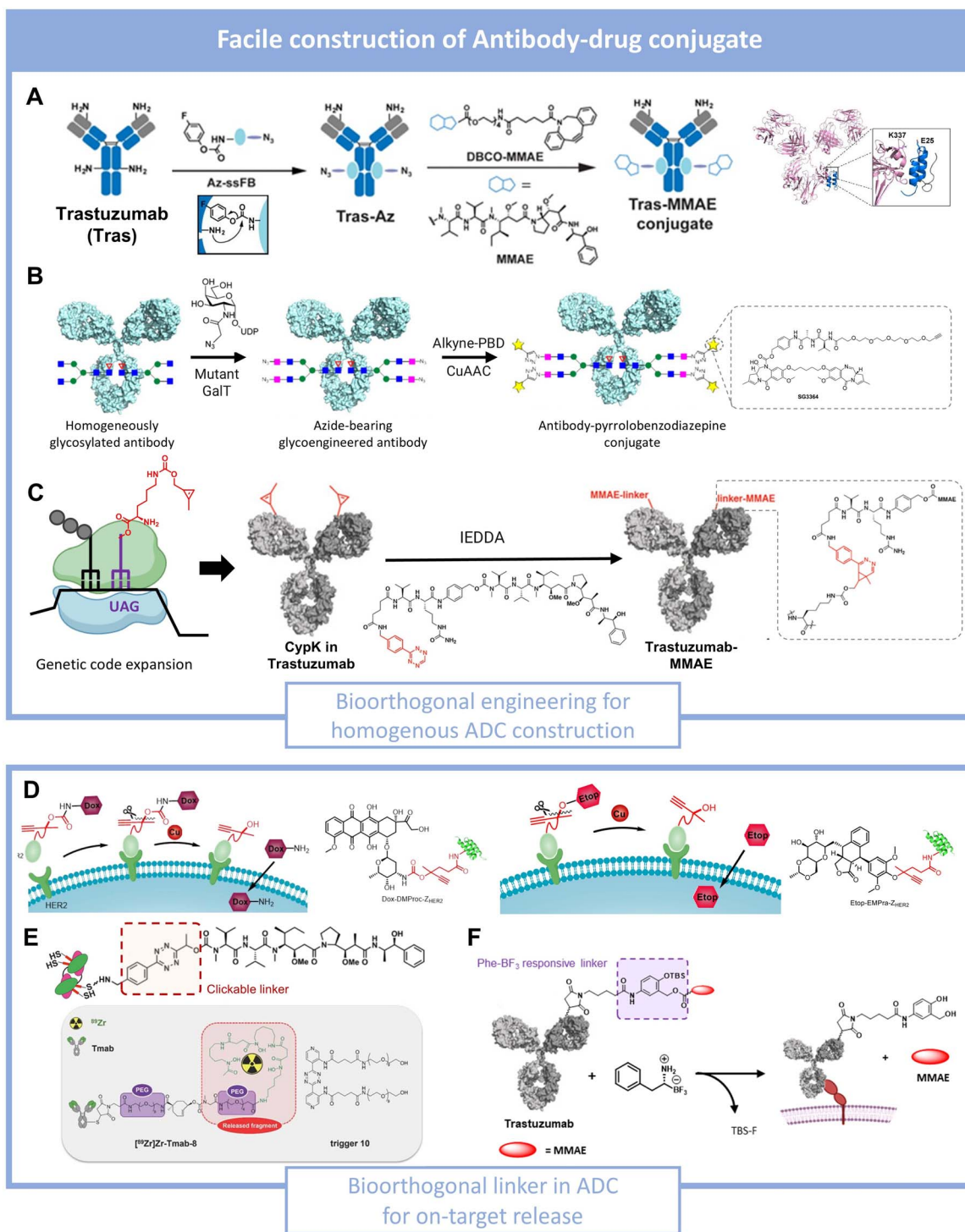
In recent years, the incorporation of bioorthogonal chemistry has introduced powerful strategies for advancing ADC design and optimization.<sup>39</sup> Bioorthogonal chemistry has



Table 1 Comparison of diverse bioorthogonal reactions highlighting their kinetics, advantages and limitations, and suitable application scenarios

Reaction type	Reaction rate ( $M^{-1} s^{-1}$ )	Advantages	Limitations	Applicable scenarios in immune theranostics
Staudinger ligation	$\sim 10^{-3}$	<ul style="list-style-type: none"> <li>● Catalyst free</li> </ul>	<ul style="list-style-type: none"> <li>● Slow reaction rate</li> <li>● Instability under oxidative stress</li> </ul>	<ul style="list-style-type: none"> <li>● Glycan metabolic engineering</li> </ul>
CuAAC	$\sim 10-100$	<ul style="list-style-type: none"> <li>● Compatible in physiological environments</li> <li>● Fast reaction rate</li> <li>● Mild reaction conditions</li> <li>● Broad substrate scope</li> <li>● Stable clickable substrates with minimal perturbation of biofunction</li> </ul>	<ul style="list-style-type: none"> <li>● High concentration required</li> <li>● Requires an external copper catalyst</li> <li>● Cytotoxicity and immunotoxicity</li> <li>● Poor suitability for <i>in vivo</i> applications</li> </ul>	<ul style="list-style-type: none"> <li>● Protein labeling and target screening</li> <li>● <i>Ex vivo</i> construction of antibody–drug conjugates</li> <li>● <i>In situ</i> therapeutic formation by catalysts</li> </ul>
SPAAC	$\sim 1-60$	<ul style="list-style-type: none"> <li>● Catalyst free</li> <li>● High selectivity</li> <li>● Compatible in physiological environments</li> <li>● Established approach for biomolecule modification</li> </ul>	<ul style="list-style-type: none"> <li>● Moderate reaction rate</li> <li>● Bulky chemical structure</li> <li>● Tedious synthesis and high cost</li> <li>● Instability of clickable substrates</li> </ul>	<ul style="list-style-type: none"> <li>● <i>Ex vivo</i> construction of: (a) antibody–drug conjugates (b) Host cell-derived biomimetic nanoplatfoms (c) Adoptive cell therapy (d) Prokaryote/virus-based immune activators</li> <li>● <i>In vivo</i> localized accumulation of immune theranostics</li> <li>● <i>In situ</i> therapeutic formation and enhanced accumulation</li> </ul>
IEDDA	$\sim 10^2-10^6$	<ul style="list-style-type: none"> <li>● Fastest reaction rate</li> <li>● Catalyst free</li> <li>● Compatible in physiological environments</li> <li>● Broad substrate scope</li> </ul>	<ul style="list-style-type: none"> <li>● Bulky chemical structure</li> <li>● Tedious synthesis and high cost</li> <li>● Instability of clickable substrates</li> <li>● Limited approach to modify biological molecules</li> </ul>	<ul style="list-style-type: none"> <li>● <i>In vivo</i> localized accumulation of immune theranostics</li> <li>● <i>In situ</i> formation and enhanced accumulation of immunotherapeutics</li> </ul>
Cysteine ligation	$\sim 1-10$	<ul style="list-style-type: none"> <li>● Catalyst free</li> <li>● Superior compatibility in physiological environments</li> </ul>	<ul style="list-style-type: none"> <li>● Moderate reaction rate</li> <li>● Potential interference by thiol groups in biological systems</li> </ul>	<ul style="list-style-type: none"> <li>● <i>In vivo</i> localized accumulation of immune theranostics</li> <li>● <i>In situ</i> formation and enhanced accumulation of immunotherapeutics</li> </ul>
Click for release	Small molecule-activated cleavage ( <i>e.g.</i> , IEDDA, aryl azide reduction, <i>etc.</i> )	<ul style="list-style-type: none"> <li>● Catalyst free</li> <li>● Compatible in physiological environments</li> <li>● Mild reaction conditions</li> <li>● Broad flexibility for diverse payload</li> <li>● Easily modified on biomolecules</li> <li>● Large reaction scope</li> </ul>	<ul style="list-style-type: none"> <li>● Variable reaction rate and partial with poor kinetics</li> <li>● Compromised selectivity in physiological environments</li> <li>● Instability of clickable substrates</li> <li>● External catalyst required</li> <li>● Instability and toxicity of catalysts in physiological environments</li> <li>● Immunogenicity of metal catalysts</li> <li>● Synthetic cost and poor scalability</li> </ul>	<ul style="list-style-type: none"> <li>● Immunotherapeutic activation <i>via</i> bioorthogonal cleavage reactions</li> <li>● Controllable cargo release from antibody–drug conjugates</li> </ul>
Transition metal-catalyzed cleavage ( <i>e.g.</i> Ru, Pt, Pd, Au, Cu, <i>etc.</i> )	$< \sim 10^3$	<ul style="list-style-type: none"> <li>● Fast reaction rate</li> <li>● Flexibility in material, structure, and function of catalysts</li> </ul>		





**Fig. 3** Facile construction of antibody–drug conjugates. (A) Schematic illustration of the pClick prepared homogeneous ADCs, modifying Tras with Az-ssFB/FAM and conjugating DBCO–MMAE by copper-free click chemistry. Reprinted with permission from ref. 40. Copyright 2021 Ivy Spring International. (B) Glycoengineered anti-EphA2 antibody site-specifically conjugated with pyrrolobenzodiazepine via CuAAC to yield a homogeneous, potent ADC. Reprinted with permission from ref. 41. Copyright 2016 American Chemical Society. (C) Schematic presentation of genetic code expansion introducing CypK into trastuzumab, enabling tetrazine ligation with MMAE to yield a stable, HER2-selective antibody–drug conjugate. Reprinted with permission from ref. 43. Copyright 2018 John Wiley and Sons. (D) Schematic presentation of copper-triggered bioorthogonal cleavage enables traceless ADC payload release on tumor cells, reversible cell surface modifications, and site-specific reversible mutagenesis for ligand–receptor modulation. Reprinted with permission from ref. 46. Copyright 2019 American Chemical Society. (E) IEDDA-based clickable linker enabling biocompatible click-to-release of ADC payloads. Reprinted with permission from ref. 44. Copyright 2020 American Chemical Society. (F) Schematic illustration of conjugation of peptide drugs (MMAE) with the trastuzumab antibody via a Phe-BF<sub>3</sub> response linker to form ADCs. Reprinted with permission from ref. 48. Copyright 2020 Springer Nature.



especially played a key role in antibody functionalization. Notably, the small size of bioorthogonal functional groups allows their facile incorporation into specific protein domains or amino acid residues through chemical labeling, enzymatic ligation, or genetic code expansion. This enables precise control over the number and position of conjugated drugs, thereby facilitating the rapid construction and screening of homogeneous multimeric ADCs to identify the most efficacious formulation. For precise bioorthogonal decoration *via* chemical labeling, recently, Xiao *et al.* developed a proximal ligand-induced chemical conjugation strategy (pClick) to produce homogeneous ADCs from the native antibody without additional genetic engineering or post-synthesis treatment (Fig. 3A).<sup>40</sup> They applied an azide-containing antibody binding peptide, named Az-ssFBn, which incorporates the crosslinker 4-fluorophenyl carbamate lysine (FPheK). Upon binding to the antibody, the modified FB protein formed a site-specific covalent crosslink with nearby lysine residues, yielding a high-purity, homogeneous conjugate bearing the same amount of azide functionalities. Such a method successfully constructed the ADC by linking the MMAE peptide drug to the trastuzumab antibody *via* the SPAAC reaction, achieving a high conjugation yield (>90%) and a defined drug-to-antibody ratio (DAR) of 2. The pClick also constructs the homogeneous DUPA-OKT3 bispecific, which targets both CD3<sup>+</sup> T cells and PSMA<sup>+</sup> tumor cells, promoting T cell activation and antitumoral immunomodulation. Distinctively, Thompson *et al.* established a one-step chemoenzymatic strategy for the generation of homogeneous ADCs with a defined DAR (from 2 to 4), employing a glyco-engineering-based approach in combination with bioorthogonal chemistry (Fig. 3B).<sup>41</sup> The authors introduced four azide-functionalized glycans onto the antibody using the mutant galactosyltransferase and UDP-GalNAz. Then, the alkynyl pyrrolobenzodiazepine dimer was selected as a payload to rapidly conjugate to the modified antibody by the CuAAC click reaction. The homogeneous ADCs with an uncleavable bioorthogonal linker showed highly potent and specific cytotoxic activity against the murine xenograft PC3 prostate cancer model.

Despite the ability of proximity-guided chemical and enzymatic strategies to achieve site-specific and homogeneous bioorthogonal antibody conjugation, the scope of modifiable sites on antibodies remains inherently restricted, posing challenges for broader design flexibility. Notably, genetic code expansion provides a versatile platform for introducing noncanonical amino acids (ncAAs) bearing orthogonal reactive handles (*e.g.*, ketone, azide, alkyne, alkene, *etc.*) at predetermined sites within antibodies. This enables the precise synthesis of homogeneous ADCs with enhanced drug-loading efficiency and improved antitumor potency.<sup>42</sup> For instance, Oller-Salvia, B. *et al.* introduced a high-yielding mammalian expression platform enabling the site-specific incorporation of a cyclopropene-modified lysine analogue (CypK) into the HER2-targeting trastuzumab *via* genetic code expansion, which demonstrates the homogeneity with 2 clickable cyclopropenes on each antibody (Fig. 3C). The cytotoxic MMAE was further modified with tetrazine groups, which enable the rapid click conjugation

between trastuzumab(CypK)2 and MMAE *via* the IEDDA reaction.<sup>43</sup> All these advancements highlight how bioorthogonal chemistry has enabled diverse and efficient approaches for the streamlined construction of homogeneous multimeric ADCs.

However, current site-specific modification strategies for ADCs still face several limitations that warrant further investigation. These include prolonged reaction times, which often require several hours to days, and the undesired formation of hydrolytically labile byproducts, which may compromise conjugate stability and efficiency. To address these challenges, very recently, the Scheeren and Verdoes groups introduced a novel technique termed “ubitagging,” which enables the rapid and high-purity construction of structurally homogeneous ADCs.<sup>44</sup> Specifically, diverse bioactive cargos ranging from proteins to small molecules can be rapidly attached to antibodies or nanobodies through multiple ubiquitin-based fusion tags, achieving efficient conjugation within 30 minutes. Moreover, the authors efficiently constructed a bispecific T-cell engager and nanobody–antigen conjugates targeting dendritic cells, both of which elicited robust T-cell responses, thereby boosting the applicable potential of ADC platforms in immunotherapy. In the future, the integration of bioorthogonal chemistry for antibody engineering is expected to accelerate the synthesis and scalable production of ADCs while ensuring site-specific and structurally homogeneous conjugation. Moreover, expanding accessible modification sites on antibodies will be crucial for increasing the drug-loading capacity and enhancing the overall therapeutic potency, thereby facilitating the clinical translation of ADCs, particularly in immune modulation and related therapeutic areas. In traditional ADCs, cytotoxic drugs are delivered to targets *via* linkers attached to antibodies.

Another major contribution of bioorthogonal chemistry to ADC development lies in the design of linkers between functionalized antibodies and bioactive payloads. In ADC design, antibodies serve as precise targeting vehicles that navigate cytotoxic payloads specifically to diseased sites. Subsequently, a series of established linkers will respond to specific pathological stimuli (*e.g.*, enzymes, pH, ROS, *etc.*) within the disease microenvironment, thereby facilitating the controlled release of the therapeutic payload from the antibody.<sup>45a</sup> So far, some commonly used responsive linkers in ADCs are designed based on short peptides (*e.g.*, Val–Cit, Gly–Gly–Phe–Gly, *etc.*), glucuronide, or disulfide bonds that are selectively cleaved by tumor-overexpressed biomarkers. However, these conventional linkers often prematurely release drugs in off-target sites, leading to toxic side effects and making it challenging to control the sufficient amount of drug released at the desired location.<sup>45b</sup> Importantly, the bioorthogonal chemistry toolbox enables the design of linkers that are inert to physiological stimuli but can be selectively cleaved *via* click-to-release reactions. This allows the payload to remain stably conjugated during circulation and be precisely released only upon exposure to an external bioorthogonal trigger after antibody-mediated accumulation within the disease site, thereby significantly enhancing the stability and specificity of ADC linkers. In particular, transition metal-catalyzed click-to-release reactions offer a reliable strategy for the controlled cleavage of bioorthogonal ADC



linkers upon orthogonal activation. For instance, Chen *et al.* systematically evaluated a library of alkyne-functionalized linkers alongside a range of transition metal catalysts (including Cu, Ni, Ru, Pd, Co, and Fe) to identify the optimal cleavable linker–catalyst pair capable of achieving efficient and selective bioorthogonal activation. The authors successfully identified two linkers, dsProc and dsPra, which can selectively cage the active amine or hydroxyl groups of drug molecules, respectively, and chemically link them to the HER2-targeting antibody for the successful construction of antibody-conjugated prodrugs, Dox-DMProc- $Z_{HER2}$  and Etop-EMPra- $Z_{HER2}$ . Under the stimulation by selected copper(I)-BTAA catalysts, the pretargeted ADC underwent specific linker cleavage, enabling the “traceless” release of the active payload within the tumor microenvironment. Such a bioorthogonal linker minimizes off-target drug release from ADCs in normal tissues, ultimately contributing to enhanced treatment accuracy, reduced adverse effects, and greater clinical applicability (Fig. 3D).<sup>46</sup>

However, the potential toxicity associated with exogenous metal catalysts remains a significant concern, which limits their broad application *in vivo*. As a result, increasing attention has been directed toward the use of small-molecule triggers, which shows better biocompatibility, for inducing click-to-release cleavage of ADC linkers. Notably, the Robillard group pioneered the first-in-class development of IEDDA-based click linkers, introducing a chemically unique design in which discrete *trans*-cyclooctene or tetrazine moieties serve as modular linkers to conjugate therapeutic payloads to antibodies with high specificity and efficiency (Fig. 3E).<sup>47</sup> Driven by a complementary IEDDA trigger after the pre-accumulation of ADCs, the payload is rapidly and selectively released from the antibody scaffold, exemplifying a highly efficient small molecule-mediated click-to-release strategy. Another landmark contribution was made by the Liu group, who developed an innovative bioorthogonal reaction based on Phe-BF<sub>3</sub>-mediated desilylation. As proof of concept, this system couples peptide drugs (MMAE) with trastuzumab antibodies *via* a *tert*-butyldimethyl silyl (TBS)-based linker to form ADCs. Under the Phe-BF<sub>3</sub> triggering, the peptide payloads were efficiently released from ADCs and accumulated within the tumor area. Moreover, this innovative bioorthogonal chemistry could selectively cleave gasdermin proteins from nanoparticles to induce tumor cell pyroptosis. Pyroptosis further released inflammatory cytokines, such as IL-1 $\beta$ , which activated CD4<sup>+</sup> and CD8<sup>+</sup> T-cell-mediated antitumor immune responses (Fig. 3F).<sup>48</sup>

Collectively, these advances address critical challenges in traditional ADCs, especially in achieving precise site-specific conjugation and improving product homogeneity. Meanwhile, bioorthogonal chemistry has also inspired a new generation of linker design strategies, including the rapid formation of non-cleavable conjugation linkers (*e.g.*, triazole moiety *via* CuAAC) and cleavable linkers in response to external bioorthogonal molecular triggers. However, current bioorthogonal-enabled ADC platforms still face several concerns that limit their clinical translation. Notably, these non-cleavable linkers may compromise the intrinsic bystander effect of ADCs, thereby sacrificing the therapeutic efficacy to some extent in exchange for

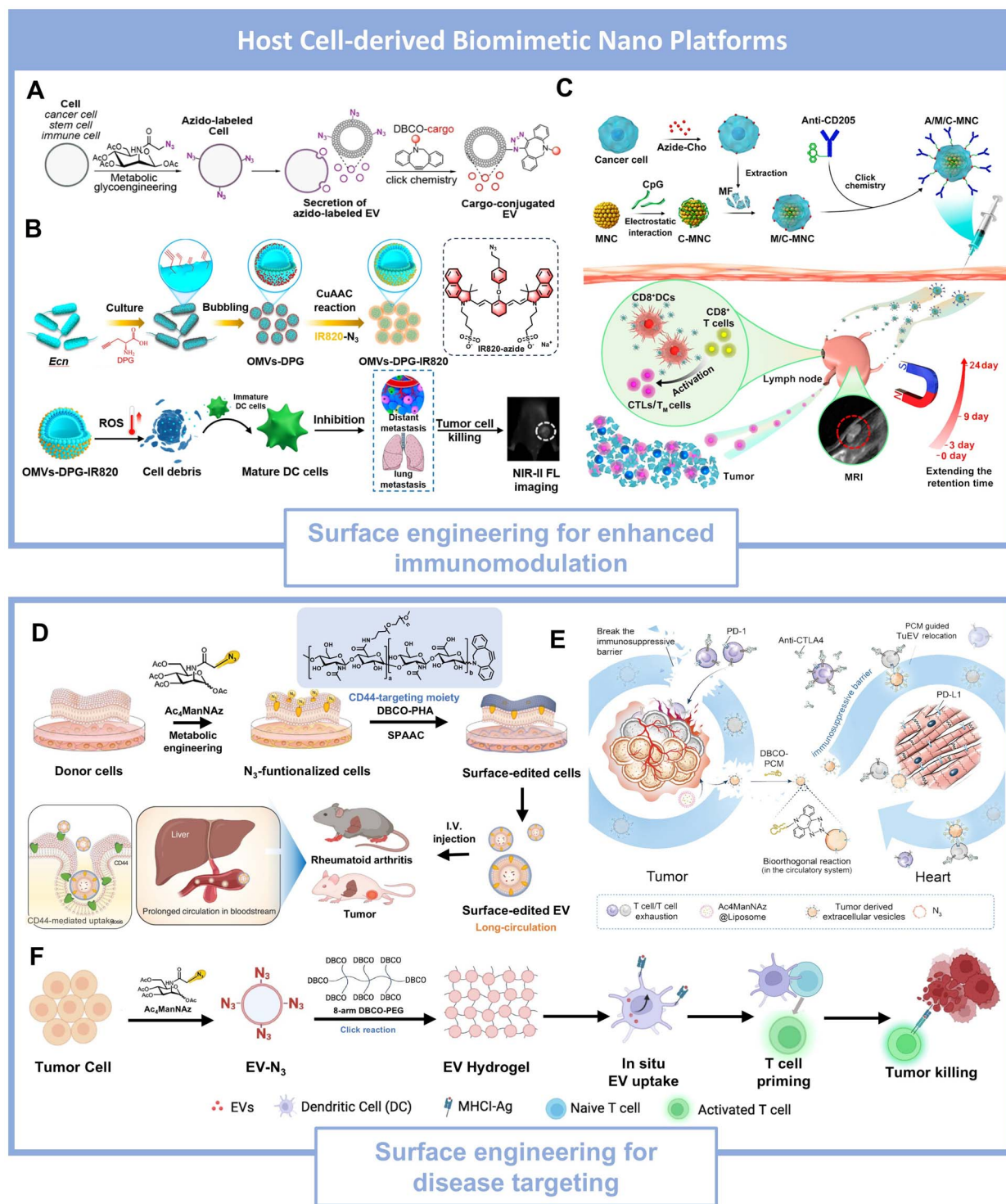
enhanced safety. In contrast, bioorthogonal cleavable linkers (*e.g.*, TCO, tetrazine, *etc.*) require careful optimization to balance reaction kinetics with intrinsic stability, to ensure their feasibility for *in vivo* applications. More importantly, the requirement of exogenous triggers to initiate drug release necessitates multiple administrations in clinical settings. The timing between the two doses must be carefully optimized to prevent undesired linker cleavage during systemic circulation, which may require the integration of real-time monitoring for ADC distribution. Additionally, the design of ADCs needs to consider their potential for combination with immunotherapy, like selecting immunomodulatory antibodies or employing bi-specific antibodies,<sup>49</sup> to maximize antitumor immune responses, thereby enhancing the clinical applicability and therapeutic efficacy of ADC-based strategies.

### 3.2 Host cell-derived biomimetic nanoplatfoms

Biomimetic nanoplatfoms have emerged as promising immunomodulatory therapies due to their precise targeting capabilities and prolonged circulation time within biological systems.<sup>50</sup> Among these, cell membrane-coated nanocomposites and cell-derived extracellular vesicles (EVs) have become core components of biomimetic nanoplatfoms for immunotherapy.<sup>51</sup> These systems mimic natural biological processes, offering enhanced biocompatibility and immune evasion while efficiently delivering therapeutic payloads to specific sites. Bioorthogonal surface functionalization has been introduced as a key strategy to further enhance the therapeutic efficacy of these platforms. This technique enables site-specific ligands, antibodies, or immunomodulator conjugation onto these biomimetic platforms, thereby improving selective interactions with immune cells or the tumor microenvironment.<sup>52</sup> Additionally, bioorthogonal cargo coatings allow for efficient encapsulation and controlled release of immunostimulants, such as cytokines, antigens, or immune checkpoint inhibitors, ultimately enhancing the therapeutic potential of these biomimetic systems.<sup>53</sup>

Importantly, extracellular vesicles (EVs) are critical for immune system modulation. In certain cases, they function as antigen-presenting vesicles (APC-derived vesicles), capable of carrying and presenting antigens to activate dendritic cells (DCs) or T cells, thereby enhancing adaptive immune responses.<sup>54</sup> Notably, bioorthogonal engineering on the EV surface allows the incorporation of specific antigens or immunoadjuvants, thereby improving the specificity and efficacy of immune responses. For instance, Wang *et al.* utilized bioorthogonal chemistry to modify tumor-derived extracellular vesicles by introducing azido groups onto their surface, enabling precise conjugation of the TLR9 agonist CpG *via* strain-promoted azide–alkyne cycloaddition (SPAAC), thereby enhancing their immunostimulatory capacity (Fig. 4A).<sup>55</sup> This modification improved antigen presentation (evidenced by increased MHCI-SIINFEKL complex expression) and effectively activated CD8<sup>+</sup> T cell proliferation and IFN- $\gamma$  secretion. Meanwhile, tumor cell-derived extracellular vesicles have also demonstrated favorable homologous targeting capabilities,





**Fig. 4** Host cell-derived biomimetic nanoplateforms. (A) Schematic illustration of the metabolically labeled cells producing azido-tagged EVs via glycoengineering of unnatural sugars. Reprinted with permission from ref. 55. Copyright 2023 Springer Nature. (B) Scheme of the construction of OMV-DPG-IR820 and its application in phototherapy with antitumor immune activation guided by NIR-II fluorescence imaging. Reprinted with permission from ref. 58. Copyright 2024 American Chemical Society. (C) Schematic presentation of the fabrication of A/M/C-MNC by integrating magnetic nanotechnology with bioorthogonal chemistry, coating azide-labeled cancer cell membranes onto magnetic nanoclusters (MNCs). Reprinted with permission from ref. 60. Copyright 2019 American Chemical Society. (D) Schematic presentation of the preparation of PHA-EVs and their *in vivo* fates after systemic administration. Reprinted with permission from ref. 61. Copyright 2021 John Wiley and Sons. (E) Schematic illustration of the BioMeDer strategy modulating tumor/heart immunosuppressive barriers and redirecting PCM-modified TuEVs via bioorthogonal chemistry. Reprinted with permission from ref. 62. Copyright 2024 John Wiley and Sons. (F) Schematic illustration of injectable EV hydrogels prepared via click chemistry enabling DC activation and CD8<sup>+</sup> T cell priming for tumor immunotherapy. Reprinted with permission from ref. 63. Copyright 2025 Springer Nature.



enabling precise activation of immune responses in the carcinoma regions.

Distinctively, extracellular vesicles derived from other sources, such as bacteria-derived outer membrane vesicles (OMVs), naturally carry pathogen-associated molecular patterns (PAMPs) on their surface, making them potent immune stimulators capable of activating both innate and adaptive immune responses.<sup>56</sup> Due to their high density of PAMPs, OMVs can serve as intrinsic adjuvants, effectively enhancing immune cell activation and amplifying inflammatory signaling, thereby promoting antigen presentation and immune response. More importantly, bacteria possess a high degree of genetic programmability, and the availability of modification by metabolic engineering tools enables the efficient incorporation of bioorthogonal “handles” into outer membrane proteins or cell wall components on secreted OMVs. Such bioorthogonal modifications not only preserve the intrinsic immunogenicity conferred by PAMPs but also endow OMVs with additional functionalities, including precise targeting, efficient antigen presentation, and synergistic adjuvant activation. In particular, Zhao *et al.* developed bidirectional immune regulation OMVs (PEG/Se@OMV-CD47 nb) to activate macrophages and enhance antitumor immunotherapy, where the orthogonal modification offers the spatiotemporal selectivity for precise immune therapy. The OMVs modified with azide groups were collected from Ac<sub>4</sub>GalNAz-treated *E. coli*, while CD47 nanobodies were genetically fused onto the OMV surface. Subsequently, a polyethylene glycol layer containing diselenide bonds was coated onto OMVs *via* the SPAAC reaction to form PEG/Se@OMV-CD47nb, enabling the compromised immunogenicity of OMV-CD47nb and the radiation-triggered release of active antigen-presenting OMVs. Such bio-orthogonally-engineered OMVs amplify the antitumoral immune effect by promoting phagocytosis of macrophages and their native abundant PAMPs.<sup>57</sup> Distinctively, Li *et al.* employed an unnatural amino acid (DPG) metabolic labeling strategy to modify OMVs, enabling the *in vitro* conjugation of the photosensitizer IR820 onto the vesicle surface *via* the CuAAC reaction. The IR820-OMVs conjugated generated ROS in response to light irradiation, inducing the burst of tumor-associated antigens. Together with the intrinsic PAMPs of OMVs, these released antigens significantly promoted dendritic cell maturation and enhanced antitumor immune responses (Fig. 4B). Concurrently, the surface-conjugated IR820 enabled NIR-II fluorescence imaging of the tumor, allowing real-time monitoring of therapeutic efficacy within this immune theranostic platform. The study demonstrated that these engineered OMVs not only enhanced the recruitment and activation of immune cells but also improved their targeting precision and immunomodulatory capabilities through precise modifications, resulting in a significant increase in tumor-associated T-cell infiltration and enhanced tumor treatment efficacy.<sup>58</sup>

In addition to utilizing naturally occurring extracellular vesicles, artificially engineered biomimetic nanoparticles, such as membrane-camouflaged nanovesicles, can also effectively activate the immune system while maintaining targeting capability and excellent biocompatibility, as their composition closely resembles that of native cells, minimizing immune

rejection.<sup>59</sup> By conjugating immunotherapeutic agents, such as antibodies, adjuvants, or antigens, onto their surface, they could be applied to activate the immune response for combating disease progression. Recently, Xie *et al.* combined magnetic nanotechnology with bioorthogonal chemistry to develop a vaccine by coating azide-labeled cancer cell membranes onto magnetic nanoclusters (MNCs) (Fig. 4C). This approach manually extended the lymph node retention time and promoted efficient antigen cross-presentation by dendritic cells.<sup>60</sup>

However, heterologous bio-derived vesicle-based therapy has certain limitations, such as limited accumulation of therapeutic cargo and non-specific distribution in normal tissues, resulting in undesired self-immune disorder. To solve such problems, bioorthogonal chemistry offers an effective solution by facilely conjugating active targeting molecules (*e.g.*, peptide, glycan, antibody, *etc.*) onto the surface of EVs. The targeting ability could drive these immune-activating vesicles to accumulate within diseased sites, thereby improving the precision of immunotherapy and reducing the off-target toxicity in the normal tissue. In particular, Park *et al.* used Ac<sub>4</sub>ManNAz metabolic labeling to incorporate azide groups into donor cell membranes and covalently conjugated polyethylene glycol-functionalized hyaluronic acid (DBCO-PHA) onto the cell surface *via* copper-free click chemistry (Fig. 4D). The resulting PHA-EVs demonstrated enhanced CD44 receptor-mediated targeting capabilities and effectively polarized macrophages toward an anti-inflammatory M2 phenotype, thereby alleviating inflammation.<sup>61</sup> Similarly, Li *et al.* metabolically labeled tumor-derived EVs (TuEV-N<sub>3</sub>) with azide groups and utilized bioorthogonal reactions to conjugate myocardial-targeting peptides, producing dual-functional EVs (TuEV-PCM) capable of modulating both myocardial and tumor microenvironments (Fig. 4E). This strategy successfully alleviated immune checkpoint inhibitor-induced cardiotoxicity by increasing PD-L1 expression on myocardial cells and enhancing CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltration, significantly boosting antitumor immunity.<sup>62</sup>

Meanwhile, witnessed by the development of biomedical engineering, novel drug delivery platforms (*e.g.*, hydrogel, microneedles, *etc.*) offer alternative strategies to introduce *in situ* delivery of immune-active EVs in the lesion site, thereby maximizing therapeutic benefit. Importantly, bioorthogonal chemistry allows facile construction of these engineering delivery platforms without perturbing the native biological function of EVs. For instance, hydrogels can act as depot vaccines that prolong local residence and long-term release of antigen-presented EVs in the disease area. Wang *et al.* developed an injectable, viscoelastic, and tunable EV-based hydrogel using a clicking assembly. During host cell culture, the authors applied Ac<sub>4</sub>ManNAz to metabolically label nascent EVs with azide groups on their surface. These azido-EVs were then crosslinked through highly efficient strain-promoted azide-alkyne cycloaddition with DBCO-functionalized 8-arm PEG, thereby using EVs themselves as structural “building blocks” of the hydrogel. Compared with conventional EV suspensions, the EV hydrogel exhibited prolonged *in situ* stability (exceeding four weeks) and elicited stronger CD8<sup>+</sup> T-cell responses with





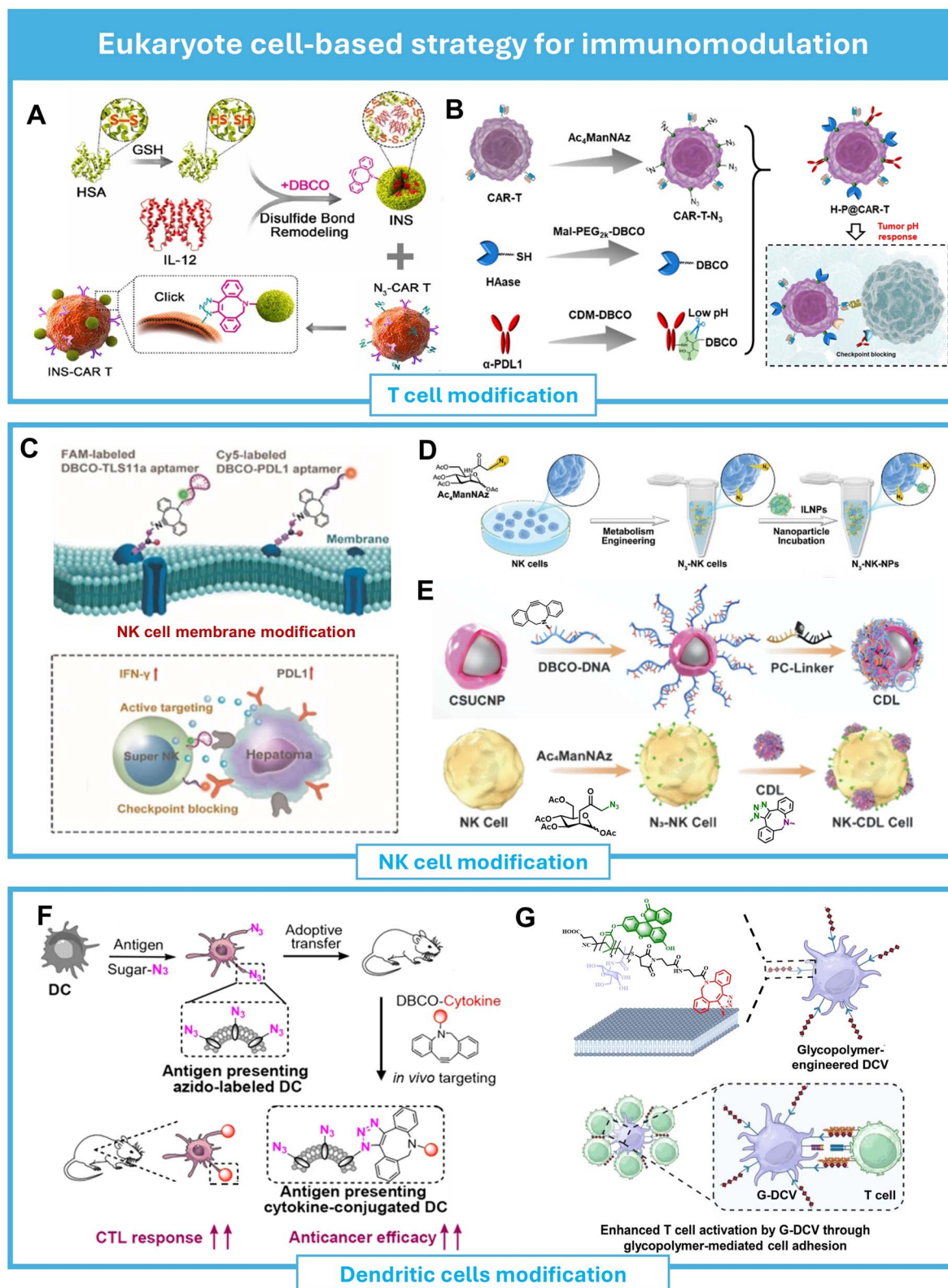


Fig. 5 Eukaryote cell-based strategy for immunomodulation. (A) Schematic illustration of the construction of functionalized INS-CAR T bi-hybrids by covalently conjugating redox-responsive nanoparticles loaded with the stimulant I-12 (INS) onto CAR T cells. Reprinted with permission from ref. 66. Copyright 2022 Elsevier. (B) Schematic presentation of the tumor ECM-degrading enzyme (HAase) and checkpoint blockade antibody ( $\alpha$ -PDL1) engineered on CAR-T cells by metabolic glycan biosynthesis and click chemistry. Reprinted with permission from ref. 67. Copyright 2022 American Chemical Society. (C) Schematic illustration of the preparation of aptamer-equipped NK cells through metabolic glycan biosynthesis and click reaction. Reprinted with permission from ref. 70. Copyright 2020 John Wiley and Sons. (D) Schematic of bio-orthogonal azide ( $-\text{N}_3$ ) groups and ILNPs introduced on NK cell surfaces via metabolic engineering and antigen-antibody interactions.





sustained immune activation, resulting in markedly improved antitumor efficacy (Fig. 4F). Notably, combination with anti-PD-1 and CpG adjuvant produced even greater therapeutic benefit, underscoring its potential for precise tumor vaccination and immunotherapy only targeting the disease area.<sup>63</sup>

As a highly specific chemical modification technology, bioorthogonal chemistry, when integrated with metabolic labeling techniques, has shown immense application potential in biomimetic nanoplatfoms, EV functionalization, and immune cell vaccine development. These studies not only address the challenges of targeting specificity and stability in traditional immunotherapy but also offer flexible design strategies for the precise treatment of complex diseases with multiple targets. Future efforts to optimize the efficiency and biosafety of bioorthogonal reactions are expected to drive the clinical translation of biomimetic nanoplatfoms for cancer, inflammatory diseases, and beyond.

### 3.3 Eukaryote cell-based strategy for immunomodulation

Chimeric Antigen Receptor T (CAR-T) cells are genetically engineered T lymphocytes that express synthetic receptors to recognize and kill specific tumor cells, representing a powerful form of personalized immunotherapy. With the clinical translation of CAR-T cell therapy, cell-based immune modulation treatments have revealed significant potential,<sup>64</sup> particularly in the realm of anti-tumor therapies. By precisely engineering host cells, sufficient therapeutic efficacy can be achieved, offering patients effective systemic treatments. Bioorthogonal chemistry has emerged as an essential tool in this process, widely applied to the modification and engineering of cells, especially through metabolic labeling and genetic engineering techniques. These technologies notably enhance the functional diversity of cells, enabling the use of cargo-loaded cells such as T cells, natural killer (NK) cells, and dendritic cells (DCs) in immunotherapy.<sup>65</sup>

While CAR T-cell therapy has achieved remarkable success in hematological cancers, challenges remain in treating solid tumors, particularly due to immune suppression in the tumor microenvironment and limited cell penetration. Bioorthogonal chemistry has been integrated into CAR T-cell modification to improve the therapeutic efficacy of CAR T cells in tumor environments. Notably, Cai *et al.* loaded IL-12 nanoactivators (INS) onto CAR T cells through a click chemistry reaction, constructing INS-CAR T cells. The data show that INS-CAR T cells significantly enhance the anti-tumor effect in mice. Through the immune feedback mechanism, the local release of IL-12 promotes the proliferation, activation, and tumor inhibition of CD8<sup>+</sup> CAR T cells (Fig. 5A) while significantly reducing systemic toxicity.<sup>66</sup> Distinctively, Zhao *et al.* utilized bioorthogonal click chemistry as the core strategy to construct a chemically dual-functionalized CAR-T system (H-P@CAR-T) (Fig. 5B). Through DBCO-azide coupling, hyaluronidase (HAase) and an acid-

responsive anti-PD-L1 antibody ( $\alpha$ -PDL1) were precisely and efficiently covalently anchored onto the CAR-T cell surface. HAase endowed the CAR-T cells with enhanced tumor tissue penetration, while  $\alpha$ -PDL1, linked *via* an acid-sensitive mal-imide linker, was responsively released within the tumor microenvironment to chemically block the PD-1/PD-L1 immunosuppressive pathway, thereby restoring and amplifying T-cell antitumor activity.<sup>67</sup> Building upon this bioorthogonal approach,  $\gamma\delta$  T cells, a unique subset of unconventional T lymphocytes, can also be engineered to enable tumor-targeted adoptive cell therapy.  $\gamma\delta$  T cells could recognize antigens in an MHC-independent manner, while they combine the innate-like rapid response with adaptive immune memory, exhibit strong cytotoxic activity against a wide spectrum of tumors. Very recently, Lin *et al.* employed click chemistry to conjugate PD-L1-specific nanobodies onto the surface of  $\gamma\delta$  T cells. The engineered  $\alpha$ PD-L1- $\gamma\delta$  T cells directly induced cancer cell pyroptosis through PD-L1 binding while also recruiting and activating CD8<sup>+</sup> T cells *via* the CCR5/CCL5 axis. This approach “heated” cold tumors, reshaping the tumor microenvironment and realizing the antitumor immunotherapy with  $\gamma\delta$  T cells.<sup>68</sup>

As crucial immune cells, natural killer (NK) cells possess powerful tumor-killing capabilities, boosting the development of CAR-NK as a new modality for cancer immunotherapy.<sup>69</sup> CAR-NK cells are engineered NK cells equipped with synthetic receptors that enable targeted tumor recognition and killing, offering potent anti-cancer activity with a lower risk of graft-versus-host disease compared to CAR-T cells. Through bioorthogonal chemistry, researchers have enhanced the targeting ability of NK cells to tumor cells and inhibited immune evasion. In particular, Yang *et al.* enhanced the targeting of NK cells to liver cancer cells (HepG2) by modifying specific aptamers on the NK cell surface, significantly improving their anti-tumor activity. Compared to NK cells modified with a single aptamer, dual-aptamer-modified NK cells demonstrated stronger anti-tumor effects (Fig. 5C).<sup>70</sup> Similarly, to boost the interaction between tumor cells and NK cells, Cai *et al.* installed azide on NK-cell membranes using metabolic glycoengineering, subsequently anchored stimuli-responsive IL-21-releasing nanoparticles (ILNPs) on glycoengineered NK cells to construct a bioorthogonally targeted live-cell nanocarrier (N<sub>3</sub>-NK-NPs) (Fig. 5D). *Via* the intratumoral injection of glycan derivatives, the tumor was pre-labeled with a complementary BCN-based clickable moiety. Together, these steps enable precise and efficient NK-tumor binding *via* click chemistry. The released IL-21 acted as an adjuvant to promote the proliferation and differentiation of NK cells and enhance the maturation and durable persistence of NK cells. This chemistry-immunology synergistic strategy remodels the immunosuppressive tumor microenvironment and achieves potent antitumor efficacy with favorable safety.<sup>71</sup> Similarly, to enhance the crosstalk of NK-tumor cells,

Reprinted with permission from ref. 71. Copyright 2022 John Wiley and Sons. (E) Schematic illustration of the integration of CDL nanoparticles with NK-92 cells by metabolic engineering and click chemistry.<sup>72</sup> Copyright 2024 American Chemical Society. (F) Schematic of azido-sugar metabolism in DCs, leading to membrane incorporation as glycoproteins and glycolipids. Reprinted with permission from ref. 73. Copyright 2023 Springer Nature. (G) Schematic illustration of the modification of DC vaccines with glycopolymers by copper-free click chemistry and metabolic glycoengineering. Reprinted with permission from ref. 74. Copyright 2023 John Wiley and Sons.



Liang *et al.* developed a near-infrared (NIR) light-activated DNA nanotentacle system (CDL) based on CSUCNPs-DBCO-DNA@-Linker (Fig. 5E), which provides the additional spatiotemporal controllability. Upon NIR irradiation, the nanoparticles exposed DBCO groups that underwent click chemistry with  $N_3$ -modified NK-92 and tumor cells, driving immune-tumor cell assembly. This strategy significantly enhanced NK-92 cytotoxicity and effectively inhibited tumor migration and lung metastasis.<sup>72</sup>

Moreover, dendritic cells (DCs) are professional antigen-presenting cells that bridge innate and adaptive immunity by capturing, processing, and presenting antigens to T cells, thereby orchestrating immune responses. Importantly, bioorthogonal chemistry also offers new solutions to modify DC cells, which demonstrates the universality to cover a broad range of immune cells. For instance, Wang *et al.* metabolically labeled DCs with azide groups and utilized bioorthogonal click chemistry to conjugate immunomodulatory molecules, such as IL-15 and IL-2, onto the DC surface. This strategy significantly enhanced the antigen presentation capability and T-cell activation potential of DCs (Fig. 5F), leading to delayed tumor growth and improved survival rates in mouse tumor models.<sup>73</sup> Similarly, Chen *et al.* engineered glycopolymer-modified DC vaccines (G-DCVs) *via* copper-free click chemistry in combination with metabolic glycoengineering. This approach enhanced DC-T-cell adhesion through carbohydrate-lectin interactions, resulting in augmented antigen-specific T-cell activation (Fig. 5G). Notably, G-DCVs exhibited superior tumor suppression in B16-OVA melanoma models, and when combined with immune checkpoint inhibitors, they further improved therapeutic outcomes, demonstrating a promising strategy for potentiating DC-based cancer immunotherapy.<sup>74</sup>

Beyond the wide range of immune cells, tumor cells themselves can also be engineered *via* bioorthogonal conjugation to construct therapeutic platforms that exploit their draining lymph node homing ability, thereby substantially expanding avenues for immunotherapy. Recently, Li *et al.* created an antigen-presenting cell (APC)-like tumor cell platform (DPNL) by labeling a clickable azide group with  $Ac_4ManNAz$  on the cellular membrane and subsequently coupling HMME-loaded DBCO liposomes *via* the SPAAC reaction. The cells were cryoshocked to terminate proliferative activity, yet retained tumor and draining lymph node homing. Under biomedical ultrasound, the sonosensitizers loaded in DPNL induce the immunogenic cell death of tumor cells, which subsequently activates NK-cell immunity through the NKG2D-NKG2DL axis and boosts T-cell responses in lymph nodes. In triple-negative breast cancer mouse models, combining DPNL with anti-PD-L1 prolongs survival and suppresses lung metastasis.<sup>75</sup>

Although bioorthogonal chemistry has made significant progress in the field of immunotherapy, there are still some challenges, such as how to further improve the targeting of therapy, optimize the sustainability of cellular function, and overcome issues related to immune evasion and immune tolerance. With the continuous advancement of technology, bioorthogonal chemistry will continue to play a key role in the field of immunotherapy, enhancing its targeting and immune response to tumors.

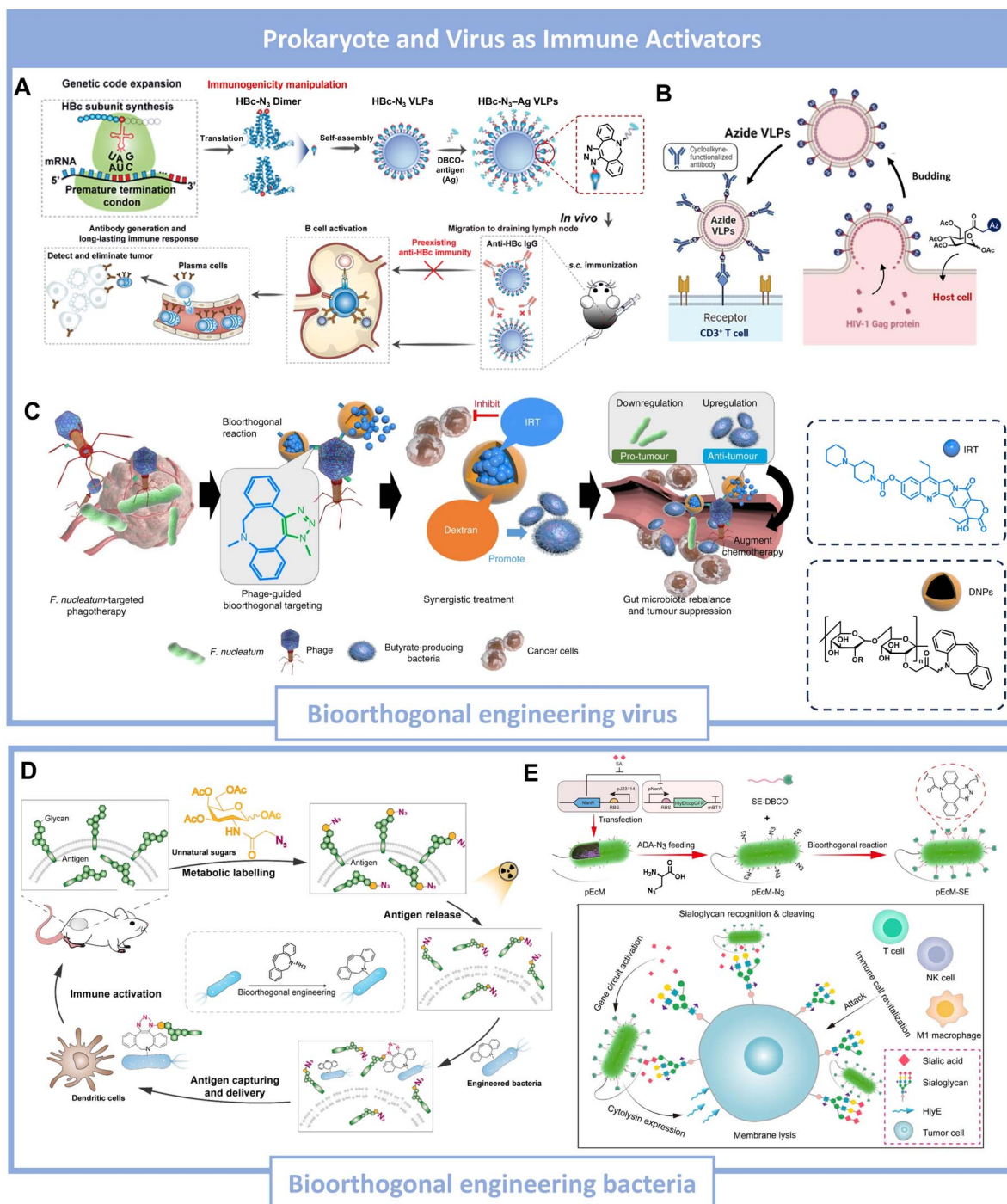
### 3.4 Clickable engineering of prokaryotes and viruses as immune activators

In recent years, the application of bioorthogonal chemistry in the functionalization of prokaryotes, viruses, and virus-like particles (VLPs) has emerged as a promising strategy for enhancing their immunoregulatory efficacy.<sup>59</sup> Beyond surface engineering of host cells, microorganisms such as bacteria, phages, and VLPs can also be engineered through bioorthogonal reactions to maximize their immunomodulatory potential. This strategy plays a particularly important role in the development of vaccines and therapeutic agents, offering treatment options for a range of diseases, including cancer, bacterial infections, and viral infections. By utilizing bioorthogonal chemistry, these microorganisms and nanoparticles can be “clicked” with specific ligands, antibodies, or other immunomodulatory molecules, thereby enhancing immune recognition and activation.

The presence of widespread preexisting immunity to virus-like particles (VLPs), combined with their poor tissue-specific targeting *in vivo*, presents a major obstacle to their effective use as vaccine vectors in immune therapy. Recently, studies by Cheng *et al.* have demonstrated the application of bioorthogonal chemistry in VLPs, significantly enhancing their immunogenicity as tumor vaccines. By utilizing the genetic code expansion, the hepatitis B core (HBc) VLPs were selectively incorporated with the clickable azide group in the desired site of protein. They precisely modified the exogenous tumor antigen, mucin-1 (MUC1), with complementary DBCO ligands for rapid construction of VLP vaccines. Such bioorthogonally engineered VLPs not only boost the immunogenicity of MUC1 but also prevent the side immunogenicity from VLPs themselves, successfully and persistently increasing the anti-MUC1 immune response (Fig. 6A), leading to efficient tumor clearance in the lung metastatic mouse model.<sup>76</sup> Similarly, Laomeephol *et al.* applied an alternative method to functionalize the surface of VLPs with azide groups, by harvesting the VLPs from the metabolic-labelled host cell, where the recombinant HIV-1 Gag protein self-assembles into VLPs incorporating the azide-modified membrane. Subsequently, they covalently attached the targeting antibody to VLPs *via* the SPAAC bioorthogonal reaction (Fig. 6B), to achieve the enhanced internalization in CD3<sup>+</sup> T cells by peptide-MHC complex recognition for specific T cell identification and antibody-receptor mediated endocytosis. Collectively, these bioorthogonal approaches for VLP modification could significantly enhance the targeting ability and uptake efficiency of VLPs against the immune system, boosting their development as reliable and efficient vaccine vectors.<sup>77</sup>

In addition, bioorthogonal engineering of viruses has also been extensively applied to bacteriophages (phages), which represent a unique class of viruses with exceptional modifiability, making them promising candidates as efficient delivery vectors for drugs and genetic materials. Benefiting from the inherent specificity of phages toward diverse bacterial strains, the phages could be facilely functionalized with bioorthogonal groups to load therapeutics and target delivery of bioactive cargos into the lesion site, offering precise and programmable





**Fig. 6** Clickable engineering of prokaryotes and viruses as immune activators. (A) Schematic illustration of the site-specific modification of HbC VLPs to manipulate the immunogenicity. Reprinted with permission 76. Copyright 2023 John Wiley and Sons. (B) Schematic of the antibody modified with cycloalkyne groups covalently conjugated to azide-functionalized VLPs by a bioorthogonal click reaction. Reprinted with permission from ref. 77. Copyright 2024 Elsevier. (C) Schematic presentation of the phage-guided biotic–abiotic hybrid nanosystem and its therapeutic effects. Reprinted with permission from ref. 78. Copyright 2019 Springer Nature. (D) Schematic of the engineered bacteria capturing metabolically labeled tumor antigens after radiotherapy and delivering them to dendritic cells to trigger adaptive immune responses. Reprinted with permission from ref. 79. Copyright 2025 American Chemical Society. (E) Schematic illustration of sialidase-chimeric bioengineered bacteria construction and their CAR-T cell-mimetic antitumor mechanism. Reprinted with permission from ref. 80. Copyright 2024 American Chemical Society.

platforms for disease intervention. For instance, Zheng *et al.* isolated a phage strain from human saliva that could specifically lyse the pro-tumoral *Fusobacterium nucleatum*.

Importantly, L-azidohomoalanine was added to the culture medium of phage-infected *F. nucleatum* to obtain azide-modified phages for further bioorthogonal conjugation of dextran-





based nanoparticles loaded with antitumoral irinotecan (IRT) (Fig. 6C). The phage-guided system could automatically target and lyse the pro-bacteria within the tumoral milieu while delivering the IRT for on-target tumor growth suppression. These results demonstrate the feasibility of applying bioorthogonal chemistry for the engineering of phage-based vectors and offer promising strategies to modulate the tumor microenvironment, particularly the intratumoral microbiota, thereby remodelling the tumor microenvironment and enhancing antitumor immunotherapy.<sup>78</sup>

Meanwhile, live bacteria inherently carry abundant surface antigens and exhibit active self-propulsion behaviour in the physiological environment, which was recognized as a new type of immunoregulator. Compared with conventional adoptive cell therapies, they more readily colonize hypoxic tumor regions and penetrate dense tumor stroma, providing a superior route for *in situ* immune activation within tumors. On this basis, bioorthogonal chemistry can facilitate engineer bacteria to load disease-associated antigens or functional molecules to amplify antigen-driven innate immunity and enable more effective and precise immunotherapy. Recently, Wu *et al.* developed a prokaryote-based immunotherapeutic strategy by bacteria-boosted antigen delivery. They chemically modified *Salmonella* with DBCO through the NHS ester labeling reaction (Fig. 6D). Simultaneously, tumor cells were metabolically labelled using Ac<sub>4</sub>ManAz, resulting in the covalent presentation of azide groups on tumor-associated antigens. Upon synergistic radiotherapy, the released azide-tagged antigens were recaptured by DBCO-modified *Salmonella*, which subsequently delivered them to dendritic cells for antigen presentation and immune activation. This bioorthogonal bacteria system enhanced cross-presentation of antigens in the tumor area and expanded systemic antitumoral CD8<sup>+</sup> T-cell responses.<sup>79</sup> Distinctively, Zhang *et al.* leveraged bioorthogonal chemistry to enhance the precision of bacteria-mediated immunotherapy by constructing a tumor-selective antigen expression platform (Fig. 6E), which imitates the antitumor modality by CAR-T cells. *Escherichia coli* MG1655 was metabolically labeled with the azido-functionalized amino acid ADA-N<sub>3</sub>, allowing the subsequent conjugation of sialidase (SA) to the bacterial surface *via* the SPAAC reaction. Furthermore, a sialic acid-responsive gene circuit was introduced into the bacteria to drive the expression of hemolysin E (HlyE) upon recognition of glycoantigens cleaved by orthogonally linked SA, thereby inducing targeted tumor cell lysis. These representative examples highlight the diverse strategies for integrating bioorthogonal chemistry with bacteria, characterized by their simple, efficient, and diverse labeling modalities. More importantly, such orthogonal modifications significantly enhance the potential and functional versatility of bacteria as immune activators in theranostic applications.<sup>80</sup>

These studies collectively highlight that the application of bioorthogonal chemistry in the functionalization of prokaryotes and viruses can significantly enhance the effectiveness of immunotherapy. By enabling precise modification of bacteria, phage, and virus-like nanoparticles, this approach opens new avenues for personalized immunotherapy. It represents a critical step in developing more effective and targeted therapeutic

strategies by enhancing immune activation while minimizing immune evasion. However, its widespread application still faces several challenges. First, these prokaryotes and viruses are often recognized as foreign entities by the host immune system, which may lead to their rapid clearance and immune interference *in vivo*, thereby reducing their effectiveness as on-target immunotherapeutics. Second, the biodistribution and retention of bacteria and bacteriophages *in vivo* are difficult to precisely control, which could not only cause off-target effects in non-diseased tissues but also trigger systemic inflammation or immune overactivation, suggesting the necessity of a detoxifying process for these agents. In addition, strategies based on metabolic labeling and orthogonal modifications usually rely on multi-step procedures, increasing the uncertainty and operational barriers in clinical translation. Finally, administration of such agents, which are inherently associated with infection risks, may inevitably cause concern and anxiety among patients. Therefore, it is crucial to implement stringent management policies to standardize and regulate the clinical use of these immunotherapeutic agents.

## 4. *In vivo* bioorthogonal reaction for on-target immunotherapy

Intriguingly, the site-specific bioorthogonal reaction *in vivo* could induce the targeting therapeutic activation, which revolutionizes the development of precise theranostics. The precise regulation of the immune microenvironment in the disease area could optimize the therapeutic performance and reduce the off-target side effects.

### 4.1 Immunotherapeutic activation *via* the bioorthogonal cleavage reaction

Due to the systemic administration of immunotherapeutics, the non-specific immunogenicity in normal tissues still elicits awareness during the development of immunotherapy.<sup>81</sup> Along with the development of the prodrug strategy, the researchers pay more attention to modifying the caging group on the bioactive compounds, which could be specifically released under the stimulation within the lesion site.<sup>82</sup> A number of overexpressed biomarkers and abnormal changes in the pathological environment, such as pH, enzymes, and oxidative pressure, are not only recognized as the fingerprint of disease progression but also applied as the endogenous trigger to selectively uncage the prodrug for the on-target therapeutic performance. However, these endogenous stimuli may raise concern about the selectivity and specificity of treatment, as the minimal biochemical differences between pathological and normal tissues may contribute to off-target effects, potentially compromising the therapeutic efficacy.<sup>83</sup> In contrast, exogenous activation strategies present a compelling alternative, as they can be engineered to achieve precise spatiotemporal control over prodrug activation, thereby enhancing therapeutic outcomes while minimizing adverse effects.<sup>84</sup>

Beyond endogenous triggers, bioorthogonal chemistry has emerged as a powerful tool in prodrug activation, leveraging





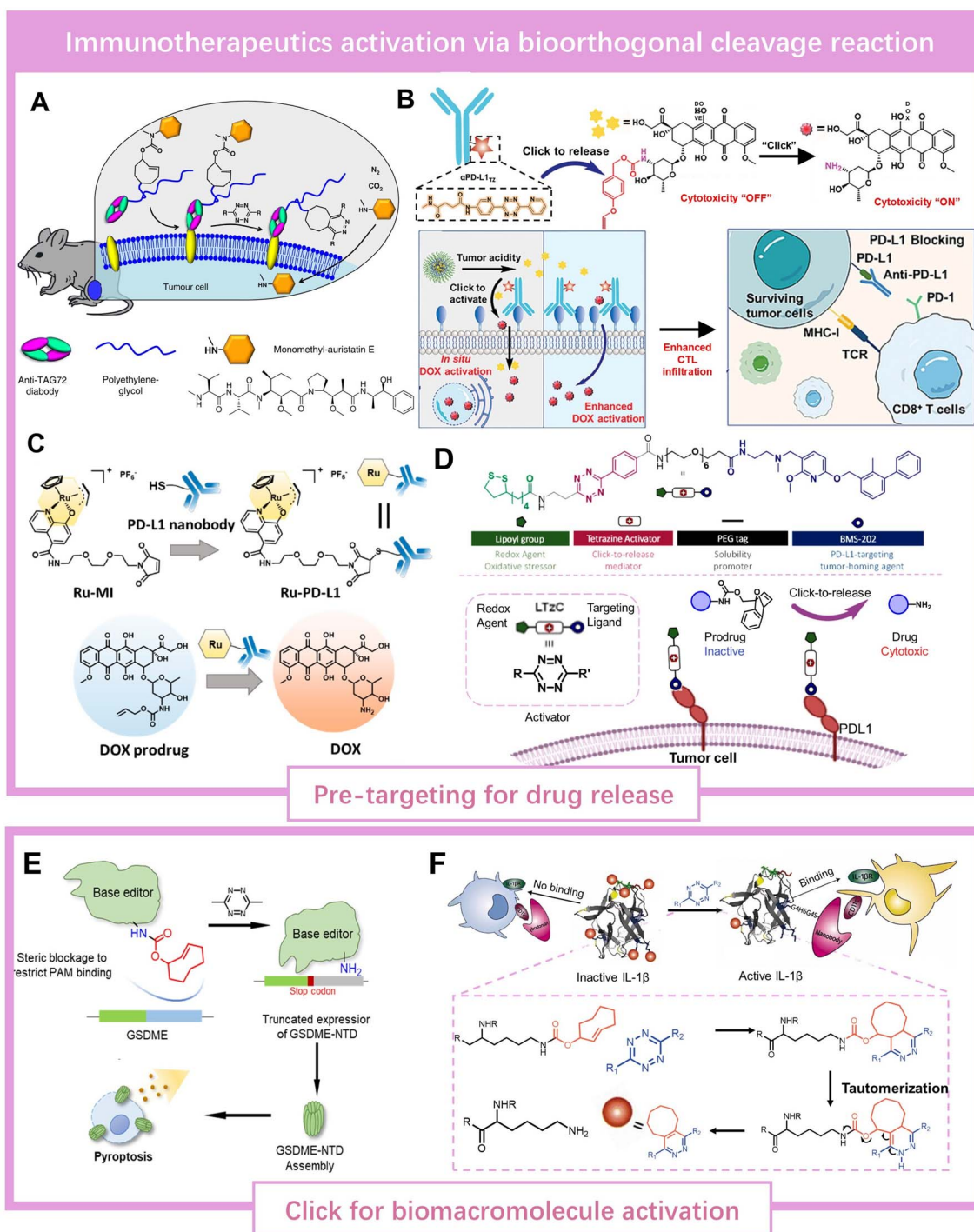


Fig. 7 (A) Schematic illustration of the bioorthogonally-activatable antibody–drug conjugate (tc-ADC) targeting malignant tumors and specifically releasing payloads from the ADC by tetrazine triggers. Reprinted with permission from ref. 85. Copyright 2018 Springer Nature. (B) Schematic presentation of tetrazine moieties modified on the anti-PD-L1 antibody ( $\alpha$ PD-L1T2) for prodrug activation to achieve combinational chemo-immunotherapy. Reprinted with permission from ref. 86. Copyright 2024 John Wiley and Sons. (C) Schematic illustration of fusing a ruthenium catalyst to an anti-PD-L1 nanobody, combining immune checkpoint blockade with Dox-induced chemotherapy. Reprinted with permission from ref. 87. Copyright 2023 American Chemical Society. (D) Schematic diagram of the conjugation of tetrazine with the small-molecule PD-L1 inhibitor (BMS-202) to targeted prodrug activation. Reprinted with permission from ref. 88. Copyright 2022 American Chemical Society. (E) Schematic presentation of the bioorthogonally activatable base editor (BaseBAC), enabling *in situ* induction of cell-type-specific pyroptosis. Reprinted with permission from ref. 89. Copyright 2022 American Chemical Society. (F) Schematic illustration of the bioorthogonally-activatable IL-1 $\beta$ -based immunocytokine via the IEDDA reaction. Reprinted with permission from ref. 90. Copyright 2025 The Royal Society of Chemistry.



highly selective chemical reactions to achieve targeted release of active immunotherapeutics. Recent advancements in this field have constructed powerful reaction scopes for bioorthogonal activation, such as inverse electron-demand Diels–Alder (IEDDA) reactions and metal element (*e.g.*, Pt, Pd, Ru, *etc.*) catalyzed cleavage. Moreover, to obtain the pre-targeting of the prodrug or bioorthogonal trigger in the lesion site, diverse affinity ligands (*e.g.*, antibody, aptamer, peptide, *etc.*) were applied to efficiently accumulate the bioactive cargo and locoregionally activate therapeutics. Recently, Rossin *et al.* introduced an innovative bioorthogonally-activatable antibody–drug conjugate (tc-ADC), which was designed for targeting non-internalizing receptors on tumor cells and specifically releasing payloads from antibodies by bioorthogonal tetrazine triggers (Fig. 7A). The tc-ADC comprises a diabody linked to therapeutic agents, monomethyl auristatin E (MMAE), *via* a *trans*-cyclooctene (TCO) moiety. Upon systemic administration, the ADC selectively accumulates in tumor tissues. The subsequent intravenous injection of a tetrazine-based activator induces the bioorthogonal IEDDA reaction with the TCO linker, resulting in the rapid release of MMAE directly within the tumor microenvironment. The treatment of tc-ADC and tetrazine trigger led to significant tumor regression in LS174T colon carcinoma and OVCAR-3 ovarian carcinoma xenografts mice models. These findings underscore the successful application of bioorthogonal chemistry, as an external stimulation, to achieve the *in vivo* activation of therapeutics from ADC.<sup>85</sup> Similar advancements in antibody pre-targeting and bioorthogonal uncaging were reported by Wang *et al.*, where tetrazine moieties were modified onto the anti-PD-L1 antibody ( $\alpha$ PD-L1TZ) for the pre-accumulation of bioorthogonal triggers (Fig. 7B). The synergistic chemioimmunotherapy system innovatively combines immune checkpoint inhibition with targeted chemotherapy. The vinyl ether-doxorubicin (DOX-VE) was encapsulated in the tumoral pH-responsive mPEG-*b*-PAEMA nanoparticles. The DOX-VE prodrug was released from the polymer carrier within the tumor acidic microenvironment, subsequently facilitating the tetrazine-vinyl ether reaction with pre-binding  $\alpha$ PD-L1TZ to release the activated chemotherapeutics, doxorubicin. The immunogenic cell death (ICD) in tumor cells through on-target chemotherapy is a form of regulated cell death that activates adaptive immunity by releasing death-associated molecular patterns (DAMPs) and tumor antigens, thereby stimulating effective antitumor immune responses. The ICD significantly enhances the therapeutic response to  $\alpha$ PD-L1 within an immune-suppressive tumor microenvironment. The combined effect of PD-L1 blockade and the targeted release of DAMPs potentiates antitumor efficacy in both *in vitro* and *in vivo* tumor models, ultimately promoting a durable immunological memory that mitigates the risk of tumor recurrence and metastasis.<sup>86</sup>

Furthermore, the bioorthogonal activation by metal catalysis could also *in situ* activate therapeutic functions, accompanied by immunomodulation for selective disease treatment. Zhao *et al.* developed a nanobody–catalyst conjugate (Ru-PD-L1) by fusing a ruthenium catalyst to an anti-PD-L1 nanobody, combining immune checkpoint blockade with Dox-induced chemotherapy (Fig. 7C). Ruthenium catalysts (Ru-MI) were

conjugated to the nanobody *via* the maleimide linker for thiol group labeling, selectively localized in the PD-L1 overexpressed tumor area after systemic administration. Subsequently, the Dox prodrug undergoes the allylcarbamate cleavage elicited by the Ru-mediated bioorthogonal reaction, releasing the active Dox for immunogenic cell death and DAMP burst.<sup>87</sup> Beyond the selection of antibodies for the construction of pre-targeting bioorthogonal activators, the small molecule inhibitors of disease overexpressed protein could also be functionalized as affinity ligands to achieve the selective labeling of bioorthogonal handles in disease areas. Recently, Unciti-Broceta *et al.* introduced an innovative strategy in which tetrazine (Tz) was conjugated to a small-molecule PD-L1 inhibitor (BMS-202) to enable targeted prodrug activation (Fig. 7D). This approach facilitated the application of hydrocarbon-bridged fluorogenic molecules for tumor imaging or drug molecules (*e.g.*, doxorubicin, mTOR-targeted inhibitor, *etc.*) for therapeutic intervention. By directing Tz to PD-L1, the platform not only enhanced tumor retention but also improved therapeutic efficacy through immunomodulatory effects, ensuring an optimal reaction rate and localized drug concentration.<sup>88</sup>

So far, most click-to-release approaches rely on antibodies or small-molecule ligands directed against overexpressed tumor receptors (*e.g.*, HER2 and PD-L1) to achieve precise pre-targeting and subsequent site-specific prodrug release. Nevertheless, membrane receptor expression exhibits significant heterogeneity across patients and even within individual tumors, which may lead to uneven drug distribution and variable therapeutic outcomes. Besides, the drug activation predominantly takes place extracellularly, where a part of the cleaved drug may not permeate across the membrane barrier, thereby diminishing the overall therapeutic utility.

Hence, the design of prodrug release systems is no longer confined to the extracellular milieu but is increasingly focused on precise subcellular organelle-specific activation. Organelles such as mitochondria, endosomes, and lysosomes are central to physiological regulation, particularly in immunity through inflammasome activation and antigen presentation. To address this requirement, Vrabel *et al.* designed tetrazines modified with delocalized cationic isoquinolinium groups for selective mitochondrial accumulation, leveraging the tetrazine-*trans*-cyclooctene (TCO) click-to-release reaction to achieve organelle-specific activation of the antitumoral drug, niclosamide.<sup>91</sup> This chemistry markedly enhanced the potency and reduced off-target effects, thereby demonstrating the feasibility of organelle-targeted click chemistry for precise immune regulation. Pushing this strategy forward, van Kasteren *et al.* developed a lysosome-targeted tetrazine (LysoTz) by appending a (2-aminoethyl)-morpholine handle to a 1,2,4,5-tetrazine scaffold, enabling organelle-specific IEDDA click-to-release. The probe accumulates in lysosomes *via* pH-dependent protonation, where it triggers TCO deprotection through pyridazine elimination.<sup>92</sup> Using LysoTz, the authors restored the activity of TCO-caged iNKT ligands within lysosomes and clarified their CD1d-dependent processing in antigen-presenting cells. When applied to long peptide antigens for CD8<sup>+</sup> T-cell activation, LysoTz did not yield lysosome-dependent uncaging or



presentation, indicating that these substrates do not transit the lysosome and are processed in early endosomal compartments. This study demonstrates that this strategy can monitor and explain antigen presentation pathways and key links at the organelle scale. The above examples together show that organelle-targeted “click-to-release” bioorthogonal chemistry has great potential in immune theranostics and are expected to reduce systemic toxicity while improving the efficacy.

Besides, apart from the recovery of therapeutic activity of small molecule drugs, bioorthogonal chemistry also offers promise to regulate the bioactive function of biomacromolecules (*e.g.*, protein, nucleic acids, *etc.*), realizing the *in situ* immunomodulation and therapy. For instance, Ngai *et al.* developed a bioorthogonally activatable base editor (BaseBAC) for *in situ* initiation of cell-type-specific pyroptosis, providing a new insight for immunologic regulation and the potential approach of antitumoral immunotherapy (Fig. 7E). The bioorthogonal blocking modification was initially introduced at the PAM-interacting residue of Cas9, thereby generating a chemically activatable Cas9 variant with restricted DNA substrate binding. This system was successfully applied to target genes such as GSDME, inducing selective editing that resulted in truncated expression of its N-terminal domain and triggering cell pyroptosis for activation of the immune system.<sup>89</sup> Moreover, in recent years, protein immunomodulators such as cytokines (*e.g.*, IL-2 and IFN- $\alpha$ ) have effectively activated the immune system and achieved clinical success in selected

indications. However, systemic administration of these immunogenic cytokines produces dose-dependent adverse effects due to widespread exposure and broad receptor distribution, which restrict the dose intensity and narrow the therapeutic window.<sup>93</sup> To address this challenge, van Kasteren *et al.* applied click-to-release chemistry to modify the immunocytokines and realize the site-specific activation of these immune regulators. IL-1 $\beta$  cytokines were caged at lysine residues with *trans*-cyclooctene (TCO) carbamates and then coupled to unmodified targeting nanobodies *via* sortase-mediated ligation to yield localizable immunocytokines. At the target site, tetrazine-triggered IEDDA reactions followed by pyridazine elimination uncaged cytokine activity *in situ* while preserving the binding of the targeting moiety.<sup>92</sup> Collectively, this bioorthogonal activation strategy targeting biomacromolecules establishes more of a broad scope of immunotherapeutic activation, which potentiates more precise regulation of conventional drugs, such as monoclonal antibodies, cytokines and antigenic vaccines (Fig. 7F).<sup>90</sup>

Intriguingly, the bioorthogonal activation systems are not only constructed based on the small molecule design or protein modification but also expand their scope in the nanomaterial for on-target immunotherapy. Witnessed by the development of nanotechnology and materials science, the bioorthogonal triggers could also be integrated into nanoconjugates (*e.g.*, metal-organic-framework, covalent-organic-framework, *etc.*), selectively activating the prodrug as the external stimuli.<sup>94</sup> In particular, Sun *et al.* developed a novel cancer immunotherapy

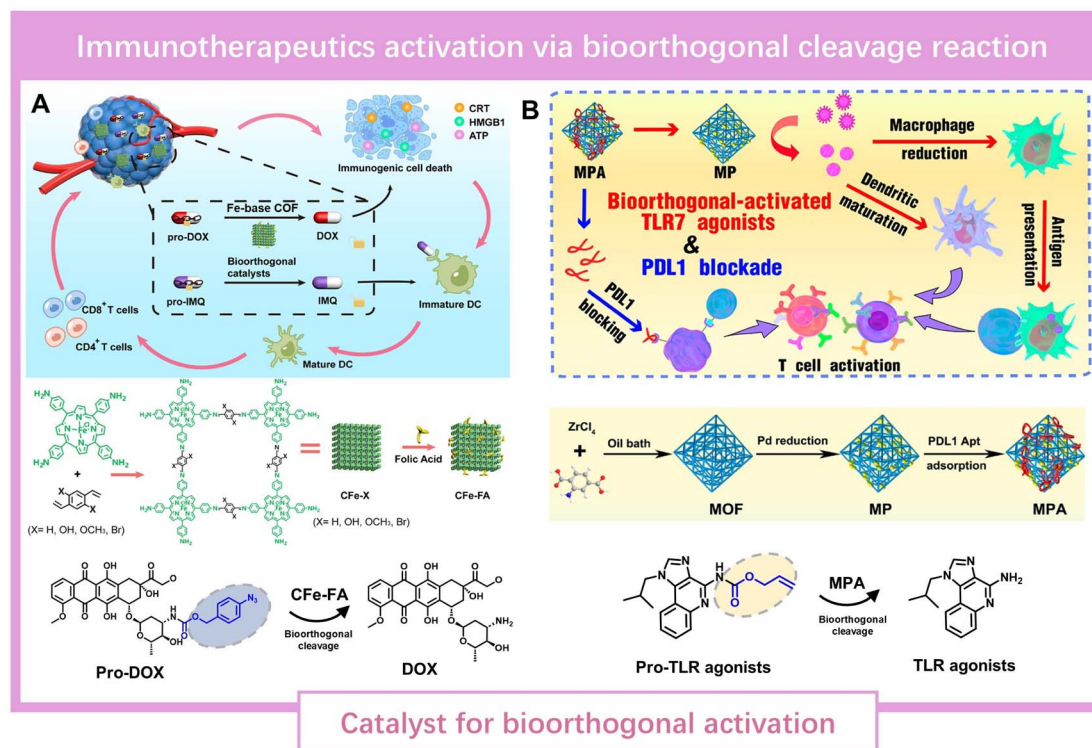


Fig. 8 (A) Schematic illustration of a novel cancer immunotherapy strategy employing a covalent organic framework (COF)-based catalytic system for bioorthogonal activation of prodrugs (Pro-DOX and Pro-IMQ). Reprinted with permission from ref. 95. Copyright 2023 American Chemical Society. (B) Schematic illustration of the Pd-doped MOF catalyst for targeted pro-TLR agonist activation in the tumor microenvironment via PD-L1 specific recognition. Reprinted with permission from ref. 96. Copyright 2023 American Chemical Society.



approach utilizing a covalent organic framework (COF)-based catalytic system (Fig. 8A). This platform employs a biocompatible folic acid-decorated COF-based Fe(II) catalyst, facilitating the *in situ* activation of chemotherapeutics (e.g., Doxorubicin) and toll-like receptor 7 agonists (e.g., imiquimod), which induces immunogenic cell death and releases tumor-associated antigens that stimulate a robust antitumor immune response.<sup>95</sup> Similarly, Wei *et al.* introduced a Pd-doped MOF catalyst for specific prodrug activation within the tumor area, while a PD-L1 aptamer was conjugated to the MOF, denoted as MPA (MOF@Pd-AptPDL1) (Fig. 8B). On one hand, the MOF@Pd (MP) complex catalyses the *in situ* uncaging of TLR7 agonists through allylcarbamate cleavage, which facilitates macrophage repolarization from the M2 to M1 phenotype while also promoting dendritic cell maturation, thereby enhancing the recruitment and activation of effector T cells. Simultaneously, the liberated PD-L1 aptamers disrupt the PD-1/PD-L1 signaling interaction between T cells and cancer cells, thereby reversing tumor-induced immunosuppression and stimulating systemic immune activation in living systems.<sup>96</sup> These approaches pave the way for the construction of nanoscale bioorthogonal triggers for on-target immunotherapeutic activation and selective immunomodulation for efficient treatment.

Although metal-based bioorthogonal catalytic platforms show promise in prodrug activation and immunomodulation, their stability remains a major limitation. In complex *in vivo* environments, metal catalysts are prone to interference from hydrolysis or redox decomposition, which compromises their catalytic efficiency. More importantly, albeit these representative studies claim that metal-based nanocomposites exhibit minimal toxicity at low doses while maintaining sufficient catalytic efficiency, their long-term safety and the potential toxicity of degradation byproducts in living systems still require comprehensive evaluation. Therefore, from the perspective of safety and translational feasibility, small molecules may be more suitable as functional modules for bioorthogonal prodrug activation.

## 4.2 *In situ* therapeutic formation and enhanced accumulation by bioorthogonal chemistry

Beyond the activation of the prodrug by bioorthogonal cleavage of the caging group, the on-target click toolbox also offers the promise for *in situ* therapeutic formation to concentrate the bioactive compound within the lesion site. Bioorthogonal reactions offer great promise for *in situ* ligation and covalent crosslinking of drug fragments, providing novel strategies for the localized generation of therapeutic molecules, particularly for drugs and immunomodulators with similar molecular structures of clicking-formed linkers.<sup>97</sup> Conventional Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry relies on sufficient copper availability to catalyse triazole formation. However, the copper concentration in the physiological environment remains a dynamic homeostasis, rendering it insufficient for direct catalysis of click formation.<sup>98</sup> To address this limitation, the introduction of exogenous metal catalysts or the precise manipulation of endogenous copper levels can

facilitate *in situ* CuAAC, thereby enabling localized drug synthesis. For instance, Zhu *et al.* developed a self-adaptive metal-organic framework (ZIF-90@P-A Nanoparticles) designed to enhance intracellular copper(I) levels, where the MOF structure incorporates reductants, such as sodium ascorbate, promoting their reduction to promote copper(I) species levels within the tumor site (Fig. 9A). This *in situ* generation of copper(I) enables efficient catalysis of azide-alkyne cycloaddition to form the antitumoral resveratrol analogues. ZIF-90@P-A was modified with adenosine triphosphate (ATP) aptamers to achieve tumor-targeting localization. Such antitumoral drug formation was mediated by CuAAC bioorthogonal reactions in carcinoma cells along with minimal off-target cytotoxicity, highlighting its potential for safe and efficient therapeutic applications.<sup>99</sup> Moreover, the bioorthogonal ligation strategy for *in situ* drug synthesis can be implemented using catalysis-free click chemistry (e.g. strain-promoted azide-alkyne cycloaddition (SPAAC), inverse electron-demand Diels-Alder (IEDDA), *etc.*), which do not require external stimulation or intervention.<sup>100</sup> In recent years, the advancement of target protein degradation has prompted increasing interest in the design of heterobifunctional molecules, introducing a number of emerging candidates as protein degraders, such as Proteolysis Targeting Chimera (PROTAC)<sup>101</sup> and Lysosome-Targeting Chimera (LYTAC), which are stepping into clinical trials for further investigation.<sup>102</sup> The heterobifunctional drug design paradigm, which employs a linker to conjugate two bioactive molecular warheads, presents significant opportunities for harnessing click chemistry to achieve rapid drug formation and screening. In particular, He *et al.* employed CuAAC chemistry to rapidly construct a PD-L1-targeting degrader (PBL1) by conjugating the small-molecule inhibitor BMS-202 to a dendritic DNA scaffold (Fig. 9B). This heterobifunctional degrader leverages scavenger receptor (SR)-mediated endocytosis to redirect PD-L1 from the cell surface to lysosomes for degradation. As a result, surface PD-L1 expression is significantly reduced, while off-target cytotoxicity is minimized compared to conventional small-molecule inhibitors. Both *in vitro* and *in vivo* evaluations demonstrated the therapeutic efficacy of PBL1, supporting its potential as a safer and more precise strategy for PD-L1 immune checkpoint modulation.<sup>103</sup> While heterobifunctional degraders can be synthesized efficiently using rapid CuAAC, their *in vivo* utility is still constrained by the large molecular size, limited tissue and cellular penetration, and off-target toxicity. Recently, leveraging copper-free bioorthogonal chemistry for the *in situ* assembly of clickable fragments into intact degraders has emerged as a novel strategy to improve their pharmacokinetic properties, reduce off-target toxicity, and enhance cellular penetration attributed to the smaller molecular size of these split precursors. In particular, as a proof-of-concept, Lebraud *et al.* designed in-cell click-formed proteolysis targeting chimeras (CLIPTACs), which split the conventional structure of heterobifunctional PROTAC into two clickable fragments (Fig. 9C). The affinity binding moiety targeting the protein-of-interest was modified with TCO, denoted as JQ1-TCO. Meanwhile, the warhead targeting CRBN E3 ligase was modified with tetrazine as the complementary fragment, which could undergo





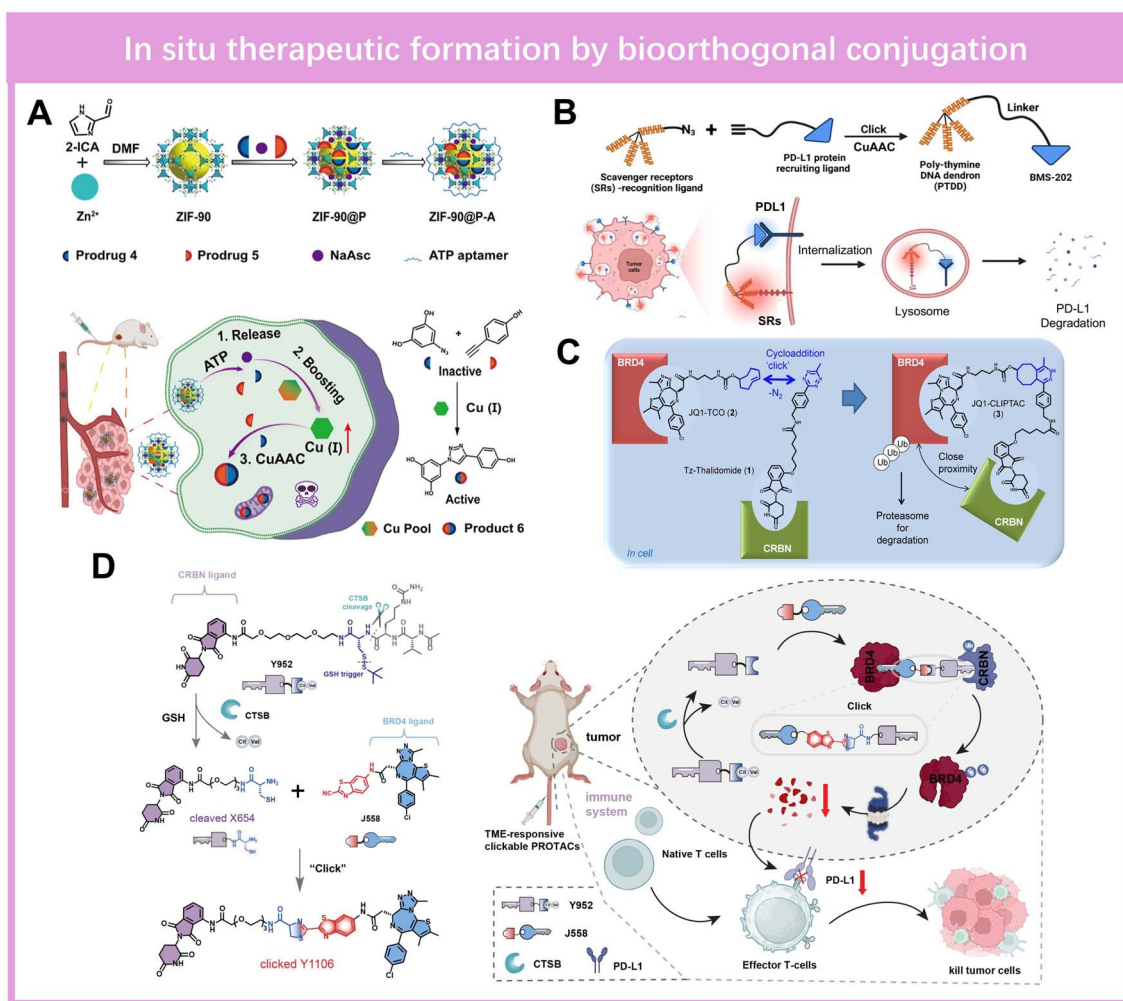
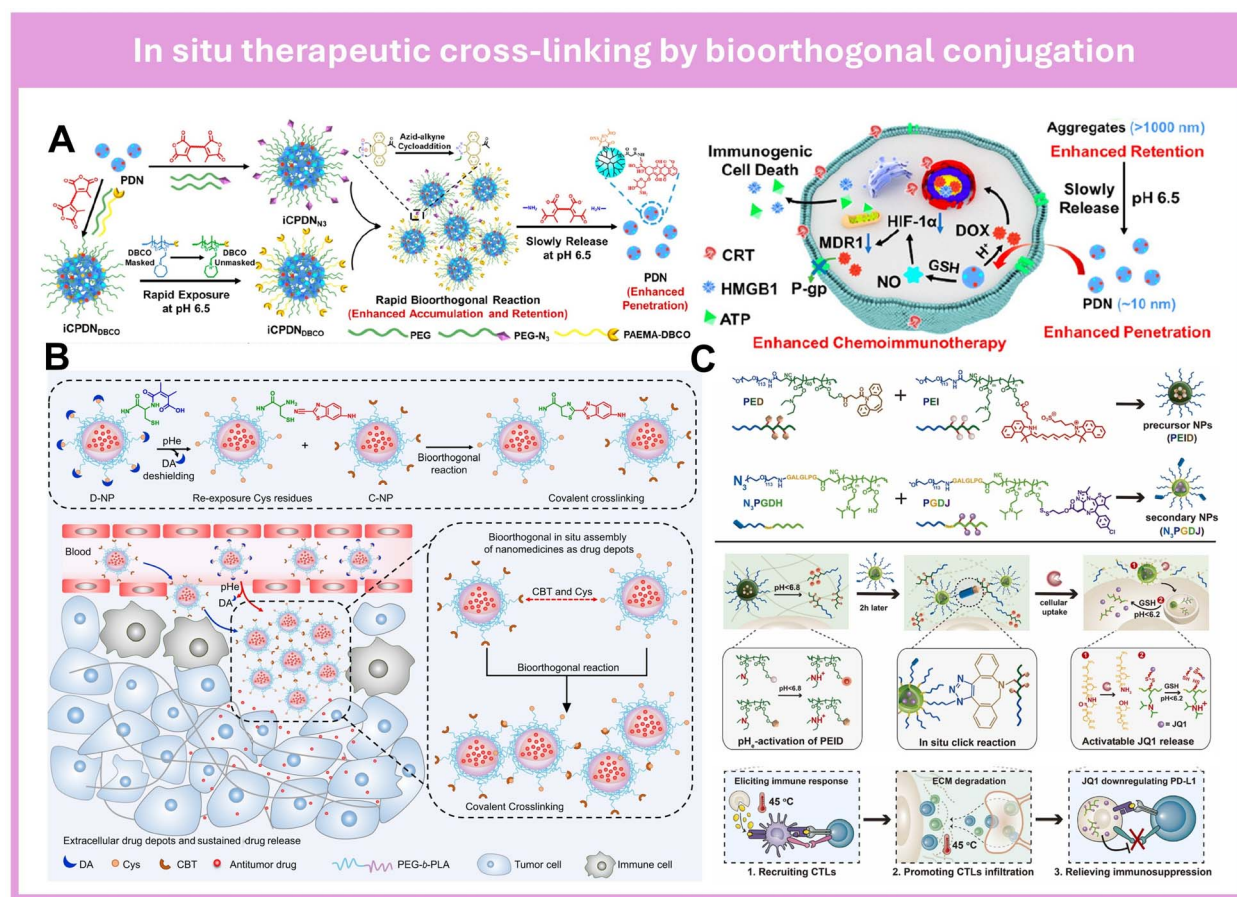


Fig. 9 (A) Schematic illustration of a self-adaptive MOF (ZIF-90@P-A NPs) enhancing intracellular copper(I) levels via sodium ascorbate-mediated reduction in the tumor microenvironment. Reprinted with permission from ref. 99. Copyright 2023 American Chemical Society. (B) Schematic diagram of DNA-mediated lysosomal degradation strategies. Reprinted with permission from ref. 103. Copyright 2025 American Chemical Society. (C) Schematic diagram of in-cell click-formed PROTACs (CLIPTAC) which split the conventional heterobifunctional structure into two clickable fragments. Reprinted with permission from ref. 104. Copyright 2016 American Chemical Society. (D) Schematic illustration of ENCTACs, an enzyme-activated strategy for selective protein degradation and precise immunomodulation in the tumor microenvironment. Reprinted with permission from ref. 105. Copyright 2025 John Wiley and Sons.

intracellular IEDDA click ligations with JQ1-TCO to form heterobifunctional protein degraders, therefore realizing the depletion of protein-of-interest. However, such click formation of therapeutics (*e.g.*, PROTACs, chemotherapeutics, *etc.*) still lacks the selectivity of the disease area, which may induce off-target side effects towards normal tissues.<sup>104</sup> Very recently, Xing *et al.* introduced a novel approach of target protein intervention, which is named Enzyme-Activated Orthogonal Proteolysis Chimeras (ENCTACs) to selectively degrade the protein-of-interest and precisely manipulate the immunomodulation only within the tumor microenvironment (Fig. 9D). The affinity warhead targeting the epigenetic protein BRD4 (JQ1) was modified with a 2-cyanobenzothiazole clickable moiety, and the CRBN E3 ligase binding warhead was caged with a short peptide, which could be specifically cleaved by tumor-specific enzymes, like cathepsin B. Subsequently, the exposed cysteine

on the CRBN binding warhead was covalently conjugated with JQ1 by the thiol-based clicking reaction, forming the heterobifunctional BRD4 degrader *via* a linker with a luciferin-based structure. More importantly, the intratumoral degradation of BRD4 induces the suppression of downstream PD-L1 expression, achieving outstanding immunotherapeutic performance by PD1/PD-L1 immune checkpoint blockade. Intriguingly, such clickable fragments of ENCTACs demonstrate improved permeability and protein degradation efficiency compared with conventional heterobifunctional degraders, suggesting that the bioorthogonal strategy for *in situ* drug formation also elicits great promise to boost immunotherapy within deep tissues. The ENCTAC treatment demonstrated significant tumor regression and improved immune cell infiltration in the tumor-bearing mice model, underscoring the great potential of this enzyme-activated bioorthogonal approach for antitumoral



**Fig. 10** (A) Schematic illustration of a cascade pH-responsive nanoassembly, utilizing SPAAC click chemistry to rapidly form clusters, enhancing the retention time within the tumor microenvironment. Reprinted with permission from ref. 108. Copyright 2022 American Chemical Society. (B) Schematic presentation of thiol-based click chemistry enabling *in situ* nanomedicine assembly for extracellular drug delivery and combined chemoimmunotherapy. Reprinted with permission from ref. 109. Copyright 2022 Springer Nature. (C) Schematic illustration of two acidity-responsive nanoparticles designed to enhance T lymphocyte infiltration and improve antitumor immunotherapy. Reprinted with permission from ref. 110. Copyright 2022 Elsevier.

immunotherapy in living systems.<sup>105</sup> Although orthogonal chemistry provides an effective strategy for *in situ* drug synthesis from clickable fragments, the kinetic discrepancies and distinct biodistribution patterns resulting from the sequential administration of different reagents must be carefully addressed. Moreover, it remains uncertain whether the reactive fragments generated by click chemistry can be fully shielded from drug activity during systemic circulation, particularly in the context of immune-activated PROTAC design. To address these challenges, the development of multi-responsive “dual-lock” molecules may further enhance drug selectivity to ensure potent activity precisely at the lesion site.

Furthermore, benefiting from *in situ* crosslinking reactions of bioorthogonal chemistry, the immunomodulating agents constructed as nanosystems could also undergo the selective click reaction within the disease area to promote their therapeutic efficacy and prevent the non-specific cytotoxicity toward normal tissues. Compared with direct click-conjugation of small molecular fragments, nanoparticles serve as an ideal drug delivery platform by enhancing the stability and mitigating the unfavorable pharmacokinetic properties of clickable

therapeutic fragments. Typically, considering the limited drug delivery and insufficient active cargo exposed to pathologic tissues (such as hypoxic and acidic tumor tissues), the nano-scale delivery system should enhance the localized accumulation and prolong the retention time in the lesion site, thereby improving the on-target therapeutic performance.<sup>106</sup> Notably, bioorthogonal chemistry could induce rapid crosslinking between nano-sized composites to *in situ* form clusters with larger size,<sup>107</sup> which could confine the nanomedicine and loaded active cargos within pathologic tissues. In particular, Wang *et al.* developed a cascade pH-responsive nanoassembly, which could rapidly form the cluster by the SPAAC click chemistry to enhance the retention time within the tumor area (Fig. 10A). The released ultrasmall nanoparticles realized deeper penetration into the tumor, while it delivered DOX and nitric oxide to overcome the hypoxia-induced drug resistance. The therapeutics released from nanoassembly subsequently induced immunogenic cell death to mediate the reprogrammed immune microenvironment, enhancing the antitumoral efficacy of chemoimmunotherapy.<sup>108</sup> Similarly, Cao *et al.* applied the thiol-based click chemistry to construct the *in situ* assembly

of nanomedicine for extracellular drug delivery and combined chemioimmunotherapy (Fig. 10B). The tumoral acidic environment removed the caging group to expose the cysteine on the nanoparticle surface, subsequently conjugating with neighboring cyanobenzothiazole-modified nanoparticles to form drug depots. The assembly system allowed diverse drug loading (e.g., BB94, DOX, and NLG919/BLZ945), realizing the cocktail chemioimmunotherapy and inducing a promising antitumoral immune response.<sup>109</sup>

Moreover, such *in situ* click conjugation of the nanoplatform could also allow the multi-modality regulation of immune responses to achieve improved therapeutic efficacy. In particular, Hou *et al.* developed two sets of acidity-response nanoparticles to improve T lymphocyte infiltration and antitumoral immunotherapy (Fig. 10C). The first set of nanoparticles allowed the accumulation of the DBCO clickable precursor and indocyanine green within acidic tumor extracellular milieu. Subsequently, the near-infrared fluorescence guided the photothermal ablation of the extracellular matrix (ECM), allowing the better penetration of cytotoxic T lymphocytes (CTLs). The second nanoparticles were localized within the tumor area by the SPAAC click reaction between the pre-accumulated DBCO and the azide function group on the surface of the 2nd nanoparticles. The endocytic acidity induced the release of JQ1 inhibitors from the 2nd nanoparticles, potentiating the down-regulation of PD-L1 to realize the immune checkpoint blockade. Such a mixture of nanoplatforms synergistically realized the multimodalities of antitumor immunotherapy both *in vitro* and *in vivo*, overcoming the limitations of combating immune-excluded tumors.<sup>110</sup>

Collectively, these nanoparticles can undergo *in situ* aggregation to form a drug depot, providing promising strategies for precise drug enrichment and immune modulation within diseased sites, thereby enhancing local efficacy and safety. However, as exemplified by representative studies, such systems often rely on multiple nanoparticles with distinct compositions and surface modifications, which markedly increase inter-particle heterogeneity, synthetic complexity, and uncertainties *in vivo*. Therefore, future designs should emphasize the use of nanoparticles with uniform composition to achieve this *in situ* aggregation strategy, thereby improving their feasibility for practical applications.

### 4.3 *In vivo* bioorthogonal engineering for localized accumulation of immune theranostic agents

Benefiting from a deep understanding of bioorthogonal click chemistry and remarkable advancements in chemical biology, diverse bioorthogonal labeling strategies have been developed, offering novel insights and opportunities for *in vivo* chemical manipulation and molecular conjugation. Researchers have continuously engineered new molecular tools to incorporate clickable functional groups (e.g., azide, alkyne, TCO, tetrazine, DBCO, *etc.*) into fundamental biological building blocks, such as monosaccharides, lipids, and amino acids, to understand and manipulate basic cellular events.<sup>111</sup> Through endogenous biological processes (e.g., glycan metabolism, protein

translation or transcription, *etc.*), these bioorthogonal functional groups are integrated into cellular structures such as the plasma membrane, cytoskeleton, and intracellular proteins,<sup>112</sup> while the modifications maintain minimum interference with the normal cellular behaviors.<sup>113</sup> Notably, bioorthogonal engineering of cells within disease regions enables the selective retention of immune theragnostic agents<sup>114</sup> which were modified with complementary clickable moieties. Such strategies provide a powerful tool for the precise enrichment of theragnostic agents at pathological sites and effective regulation of immune systems, facilitating both targeted immunotherapy and real-time imaging for disease diagnosis and treatment.

In particular, Qin *et al.* developed the bioorthogonal engineering of lymphatic endothelial cells (LECs) to facilitate the accumulation of cancer nanovaccines in lymph nodes, boosting the enhanced antitumoral immune response by triggering innate immune signaling and potent T cell infiltration. The azide functional groups were decorated on the cell membrane of LECs, where the precursor DSPE-PEG-azide migrated to lymph nodes by albumin hitchhiking. The antigen-loaded liposomes acted as the nanovaccine and were modified with complementary DBCO functional groups, hence targeting the pre-labeled azide on LECs and accumulating the antigens and adjuvants within lymph nodes for improved immunotherapy.<sup>115</sup> Similarly, Bai *et al.* devised a strategy to reverse immunosenescence in tumor-bearing aged mice by harnessing bioorthogonal-mediated drug accumulation (Fig. 11A). Aging T cells within tumor-draining lymph nodes are metabolically labeled with a clickable lipid, DSPE-PEG2000-azides, while rapamycin-loaded micelles are functionalized with complementary DBCO groups. These systemically administered micelles further undergo *in situ* click conjugation within lymph nodes, selectively delivering rapamycin to rejuvenate CD8<sup>+</sup> T cells. This restoration of T cell function significantly enhances the response to immune checkpoint blockade in older mice. The click chemistry element enables precise spatiotemporal targeting and minimizes off-target effects, thereby boosting the immunotherapeutic outcome.<sup>116</sup>

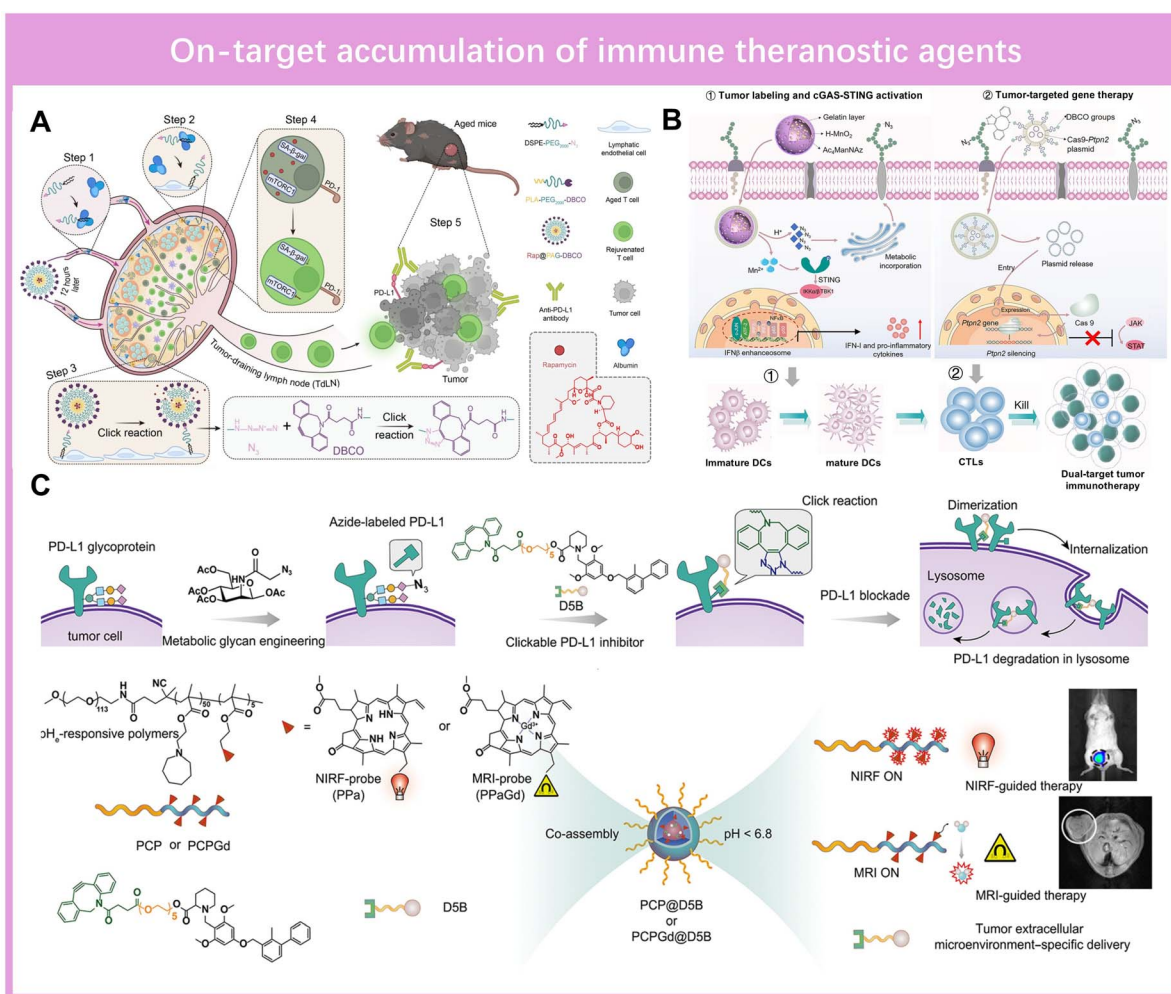
Distinctively, the bioorthogonal engineering of cells in the disease area could also be realized by the targeted glycan metabolism, to decorate the clickable functional groups on the plasma membrane. Yang *et al.* designed a tumor acidity-response delivery nanoplatform, which could selectively deliver the azide-modified monosaccharide (Ac<sub>4</sub>ManNAz) into tumor cells for bioorthogonal engineering (Fig. 11B). Accompanied by the delivery of manganese into carcinoma cells, the cGAS-STING pathway was activated to boost the upregulation of IFN-I and pro-inflammatory cytokines, realizing the initiation of innate immune response to turn “cold” tumors into “hot”.<sup>117</sup> Notably, the subsequent accumulation of CRISPR/Cas systems by the *in situ* click conjugation led to the silencing of protein tyrosine phosphatase N2 (PTPN2), synergistically activating the adaptive immune responses. Similarly, the glycan metabolism of bioorthogonal functional groups also allows the conjugation of small molecules for conducting immune theranostics. In particular, Hou *et al.* developed an immune theranostic platform by applying glycan metabolism labeling for covalently





illustrates improved therapeutic efficacy and precise spatiotemporal controllability. In particular, near-infrared (NIR) light-mediated immunotherapy has emerged as a highly promising strategy for precise and effective cancer treatment. Han *et al.* developed T-cell membrane biomimetic nanoparticles labeled with azide groups (N<sub>3</sub>-TINPs) for tumor targeting through immune recognition and *in situ* conjugation with glycan-metabolized complementary clickable handles (BCNs). Upon NIR laser irradiation, these nanoparticles generate localized photothermal and photodynamic effects, inducing tumor cell apoptosis and the subsequent release of tumor-associated antigens, which further amplify the immune response (Fig. 12A).<sup>119</sup> Similarly, Cui *et al.* applied the NIR-II aggregation-induced emission photosensitizer BODTPE as the NIR antenna to achieve photo-immune theranostics. The photosensitizer BODTPE was modified with PEG-DBCO to form a BODTPE-containing polymer (DBD), and then the polymer was mixed with lipid building blocks to form self-assembly nanoparticles

More importantly, the bioorthogonal toolbox could accumulate the functional compound within the disease area. Such compounds could act as the antenna to respond to the stimulus,<sup>123</sup> subsequently producing the cytotoxic radicals and components for combating disease progression and eliciting the immune response. The combinational immunoregulation

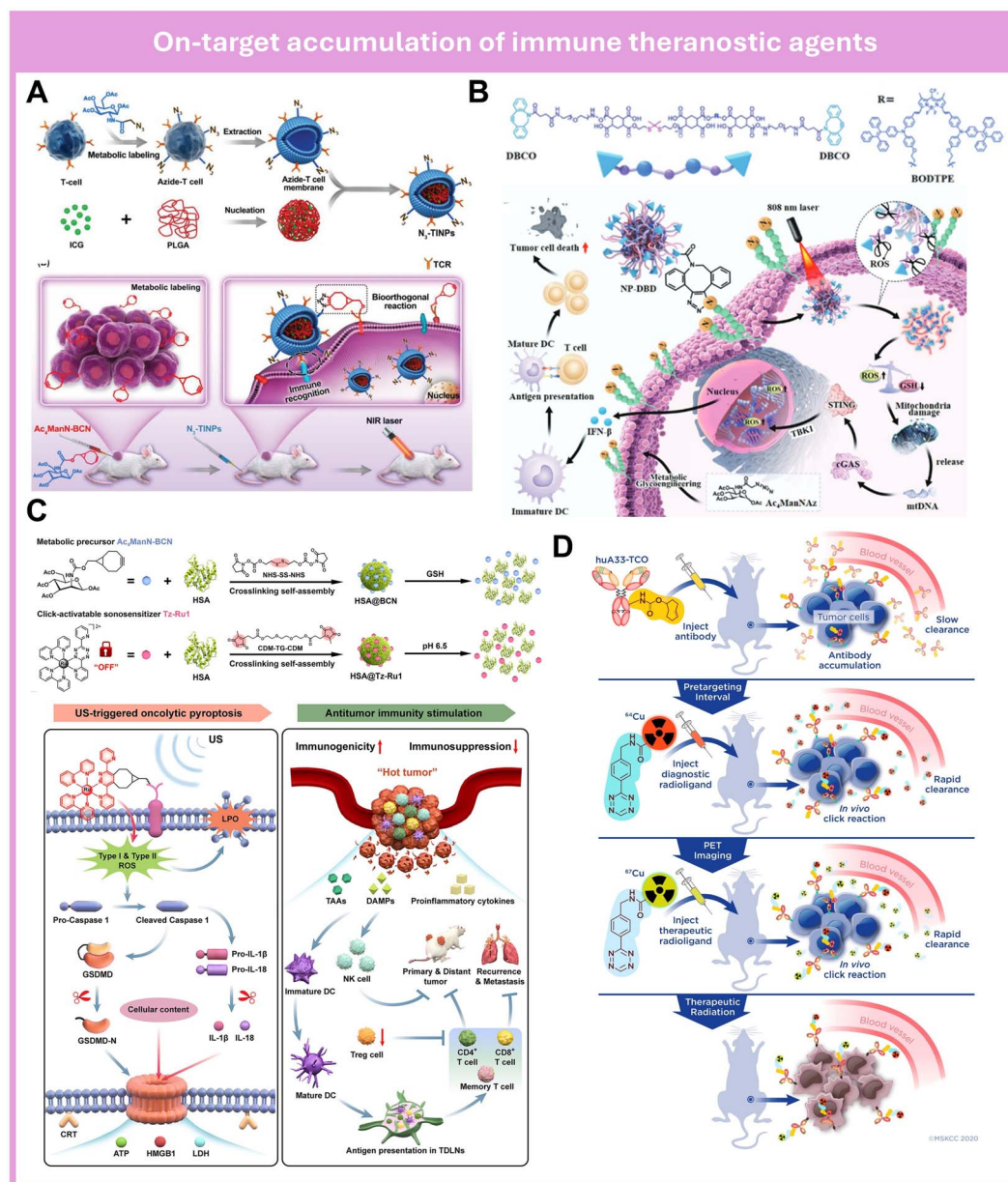


**Fig. 11** (A) Schematic illustration of the composition and mechanism of the tumor-draining lymph node (TdLN) delivery system. Reprinted with permission from ref. 116. Copyright 2025 American Chemical Society. (B) Schematic illustration of a tumor acidity-responsive nanoplatform for selective Ac<sub>4</sub>ManNAz delivery, enabling bioorthogonal tumor cell engineering. Reprinted with permission from ref. 117. Copyright 2023 John Wiley and Sons. (C) Schematic illustration of an immune theranostic platform leveraging glycan metabolism labeling to dimerize and degrade PD-L1, enabling immune checkpoint blockade for enhanced antitumor immunity. Reprinted with permission from ref. 118. Copyright 2024 The American Association for the Advancement of Science.



(Fig. 12B). These NIR-II signaling nanoparticles were covalently conjugated with glycan-labeled azide groups on the tumor cells. Under NIR irradiation, nanoparticles facilitate the production of reactive oxygen species (ROS), which contribute to tumor apoptosis, antigen release, and activation of the cGAS-STING pathway, thereby promoting dendritic cell (DC) maturation and adaptive immunity.<sup>120</sup> Moreover, the structural dissociation of NP-DBD upon NIR irradiation enhances the efficacy of photo-immunotherapies while simultaneously enabling real-time

fluorescence imaging, providing a theranostic advantage for tumor monitoring. Recently, diverse modalities of external stimulation (*e.g.*, ultrasound, X-ray, *etc.*) were developed to spatiotemporally control the immune theranostic agents for disease intervention. For instance, Xu *et al.* developed a bioorthogonal/ultrasound-mediated immune theranostic platform to activate oncolytic pyroptosis for boosted antitumor immunity (Fig. 12C). The tetrazine-functionalized ruthenium(II) sonosensitizers were covalently anchored onto the glycan-



**Fig. 12** (A) Schematic illustration of T-cell biomimetic nanoparticles for tumor targeting via *in situ* BCN conjugation, inducing tumor apoptosis and enhancing the immune response under NIR irradiation. Reprinted with permission from ref. 119. Copyright 2019 John Wiley and Sons. (B) Schematic presentation of the NIR-II AIE photosensitizer BODTPE as an NIR antenna for photo-immune theranostics, forming DBD via PEG-DBCO modification, which self-assembles with lipids into nanoparticles. Reprinted with permission from ref. 120. Copyright 2023 John Wiley and Sons. (C) PET/RIT theranostic platform using PRIT, where the huA33-TCO antibody targets tumors, followed by <sup>64</sup>Cu/<sup>67</sup>Cu radiolabeling by bioorthogonal click chemistry for imaging and therapy. Reprinted with permission from ref. 121. Copyright 2024 American Chemical Society. (D) Schematic of the MRI/NIRF theranostic system enabling multi-modal tumor imaging and treatment monitoring, enhancing PD-L1-targeted therapy. Reprinted with permission from ref. 122. Copyright 2020 National Academy of Sciences.

labeled cell membrane. Upon ultrasound irradiation, sonodynamic response from an activated sonosensitizer intensively disrupts the cell membrane with type I/II reactive oxygen species, thus promoting tumor cell pyroptosis, increasing antigen presentation, and reinforcing immune system activation. The synergistic integration of these external stimuli-driven modalities not only enables precise tumor targeting but also transforms tumors into *in situ* vaccine factories, ultimately enhancing antitumor immunity and improving therapeutic efficacy.<sup>121</sup>

Furthermore, benefiting from the development of *in vivo* bioorthogonal engineering for on-target labeling signaling molecules, these diverse chemistry tools provide great promise to achieve precise diagnosis and guide targeted therapies for potential clinical applications, meanwhile allowing the real-time monitoring of the patient's response to treatments. In particular, Keinänen *et al.* presented a theranostic platform integrating PET imaging and radioimmunotherapy (Fig. 12D). Using pre-targeted radioimmunotherapy (PRIT), a tumor-specific antibody (huA33-TCO) is administered first, followed by a radiolabeled small molecule ( $[^{64}\text{Cu}]\text{Cu-MeCOSar-Tz}$  or  $[^{67}\text{Cu}]\text{Cu-MeCOSar-Tz}$ ) by bioorthogonal click chemistry.  $^{64}\text{Cu}$  PET imaging enables real-time tumor localization, ensuring precise targeting before administering  $^{67}\text{Cu}$ , a  $\beta$ -emitting radionuclide for localized therapy. Dual-modal strategies optimize radio-pharmaceutical dosimetry, minimizing toxicity and improving treatment precision in colorectal and antigen-expressing cancers.<sup>122</sup> Distinctively, multimodalities of the imaging moiety could also be assembled together to achieve comprehensive and precise visualization of immune responses and therapeutic outcomes *in vivo*. Wang *et al.* presented an MRI/NIRF theranostic system integrating multi-modal imaging for enhanced tumor visualization and treatment monitoring. A PD-L1-targeted probe (APPGd-Cy7) consisted of an anti-PD-L1 peptide and bioorthogonal clickable handles, enabling MRI and near-infrared fluorescence imaging to precisely evaluate PD-L1 expression within the tumor area. Subsequently, a pH-responsive prodrug was utilized to facilitate tumor-selective accumulation *via* bioorthogonal click chemistry, enhancing PD-L1-targeted immune checkpoint blockade, controlled DOX release, and pyroptosis-induced immunogenic cell death. This combinatorial PD-L1-targeted chemo-immune theranostics not only effectively reprogrammed the immunosuppressive tumor microenvironment (TME) for eliciting potent tumor-specific immune responses but also potentiated multimodal diagnosis of malignant tumors to guide on-target therapy and report treatment outcomes.<sup>124</sup>

Collectively, these strategies highlight the promise of bioorthogonal chemistry in conjunction with signaling moieties, which could respond to the external stimuli, offering precise spatiotemporal drug activation, prolonged local retention, and amplified immune responses. Meanwhile, multimodal imaging from theranostic platforms greatly facilitates the monitoring of the *in vivo* circulation and lesion localization of immunotherapeutics. However, given the highly dynamic physiological environment, clinical practice requires not only accurate tracking of drug distribution but also real-time feedback on

therapeutic performance to guide treatment management. Therefore, future research may focus on integrating responsive “on-off” probes with immunotherapeutics into smart theranostic platforms, whereby imaging signals can be correlated with therapy-induced biomarkers to enable on-target evaluation and optimization of subsequent clinical interventions.

## 5. Challenges & conclusions

Over the past few decades, continuous progress in chemical biology has established bioorthogonal chemistry as a powerful toolbox for diverse biomedical applications, particularly in efficient immunotherapy and precise disease diagnostics. In this review, we systematically summarized widely employed and effective bioorthogonal reactions, encompassing a broad range of bioorthogonal conjugation and cleavage strategies. These reactions exhibited high efficiency and excellent reliability under physiological conditions, thereby enabling the development of diverse immune theranostic approaches.

Overall, using bioorthogonal chemistry could greatly boost the *ex vivo* construction of immunotherapeutic agents. Rapid and facile click reactions facilitated the fabrication of antibody-drug conjugates by covalently conjugating therapeutic agents to bioactive proteins. Similarly, bioorthogonal toolboxes have been leveraged to engineer next-generation vaccines and diverse cargo-loaded cells (*e.g.*, T cells, NK cells, DC cells *etc.*), paving new ways for innovative immunotherapies. Beyond mammalian systems, these strategies have also been extended to prokaryotes and viruses, leading to the development of immune activators that combat various disease progression. Furthermore, bioorthogonal chemistry could directly modulate *in vivo* immune activity to achieve effective therapeutic outcomes. On-target prodrug activation enabled the rapid and selective release of immunomodulatory agents at disease sites, thereby enhancing localized immune responses. Additionally, *in situ*, bioorthogonal ligation reactions could not only facilitate the localized synthesis of therapeutic molecules but also amplify the immunotherapeutic efficacy of designed nanoplateforms. More importantly, *in vivo* bioorthogonal engineering within diseased tissues enabled the precise accumulation of theranostic agents, allowing for highly specific disease diagnosis and real-time guidance for targeted therapy.

However, several challenges remain in the broader application of bioorthogonal chemistry in the development of immune theranostics, necessitating continuous advancements in innovative chemical design and deep biological understanding. So far, benefiting from its simple substrate and excellent reaction efficiency, the CuAAC reaction has been successfully employed for the *ex vivo* construction of targeted theranostic agents, which have stepped into clinical trials (European Clinical Trials no. 2013-003152-20).<sup>125</sup> However, the catalyst-free click reactions (*e.g.*, IEDDA, SPAAC, *etc.*), which were widely applied by researchers for *in situ* immunomodulation in living systems, still face significant limitations in their practical translation. In particular, the synthesis of their key substrates involves complex, multi-step procedures and high production costs, making them less amenable to large-scale industrial



manufacturing. Additionally, bioorthogonal molecular design for *in vivo* applications requires caution. The relatively large molecular size and high hydrophobicity of clickable substrates (*e.g.*, TCO, DBCO, *etc.*) may adversely affect their *in vivo* reactivity, biodistribution, and overall pharmacological performance. Moreover, careful attention should be paid to maintain a balance between substrate stability and reaction kinetics. In general, chemical groups with exceptionally high reactivity tend to exhibit limited stability under physiological or complex biological conditions.<sup>126</sup> As a result, linkers containing highly reactive bioorthogonal groups (*e.g.*, tetrazine) may undergo hydrolysis during systemic circulation, necessitating continuous structural screening and optimization to improve their *in vivo* stability. Besides, when bioorthogonal handles are incorporated into bioactive molecules, the reaction kinetics may be altered due to steric or electronic effects.<sup>127</sup> Therefore, it is also essential to evaluate the reaction performance directly in biological systems and to correlate the *in vivo* reaction efficiency with therapeutic outcomes. Despite the presence of various concerns, we have recently witnessed a significant milestone with the advancement of the first-in-class click chemistry-based cancer therapeutic (SQ3370) into a first-in-human dose escalation clinical trial (NCT04106492).<sup>128</sup> This achievement marks a pivotal step toward real-world clinical settings, and it is anticipated that more successful examples will soon follow, further validating the immense translational potential of bioorthogonal chemistry.

Besides, with the continuous advancement of chemistry and materials science, exogenous metal-based catalysts have gained increasing prominence in recent bioorthogonal system designs, as also illustrated in several examples throughout this review. Their long-term *in vivo* toxicity remains insufficiently characterized and warrants systematic evaluation. In this context, leveraging naturally derived bioorthogonal platforms (*e.g.*, bioorthogonal engineered cells, extracellular vesicles, detoxified bacteria, *etc.*) offers superior biocompatibility and represents a safer and more translationally favourable strategy for *in vivo* applications.

More importantly, most studies on bioorthogonal strategies for constructing immune theranostic platforms have predominantly focused on applications in oncology. While immunotherapy has indeed made significant contributions to clinical cancer treatment, it is important to recognize that the immune system plays a central role in maintaining overall health and homeostasis. Therefore, future research should broaden its scope to encompass other disease contexts, such as infectious diseases and autoimmune disorders, to expand the clinical utility and translational potential of bioorthogonal immune engineering.

Although there are still many challenges in current designs, we believe that the immune theranostics mediated by bioorthogonal chemistry have emerged as a new era of biomedical science and practical medicine, attributed to their attractive and diverse functions. This review aims to inspire the broader scientific community to explore next-generation immune-modulating strategies through the integration of convenient bioorthogonal chemistry tools, thereby maximizing the

therapeutic potential for smart immune theranostics and propelling their clinical translation.

## Author contributions

Conceptualization: S. H. Liu and B. G. Xing; writing of the original draft: S. H. Liu and C. Y. Hua; validation: S. H. Liu, C. Y. Hua, and B. G. Xing; review and editing: S. H. Liu, C. Y. Hua, P. C. Yuan, X. N. Li, and B. G. Xing.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

No primary research results, software, or code have been included, and no new data were generated or analysed as part of this review.

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