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Outlook

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The Future of Bioorthogonal Chemistry

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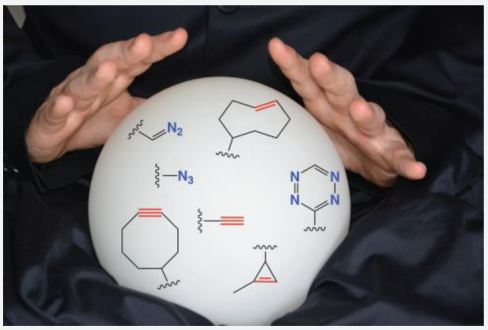
ABSTRACT: Bioorthogonal reactions have found widespread use in applications ranging from glycan engineering to in vivo imaging. Researchers have devised numerous reactions that can be predictably performed in a biological setting. Depending on the requirements of the intended application, one or more reactions from the available toolkit can be readily deployed. As an increasing number of investigators explore and apply chemical reactions in living systems, it is clear that there are a myriad of ways in which the field may advance. This article presents an outlook on the future of bioorthogonal chemistry. I discuss currently emerging opportunities and speculate on how bioorthogonal reactions might be applied in research and translational settings. I also outline hurdles that must be cleared if progress toward these goals is to be made. Given the incredible past successes of bioorthogonal chemistry and the rapid pace of innovations in the field, the future is undoubtedly very bright.



INTRODUCTION

Although chemists have been making molecules that interact with life since the dawn of modern chemistry, the actual chemical reactions used to assemble the molecules were kept as far away from life as possible. They were performed in organic solvents where water, and often oxygen, were to be avoided. Impurities were anathema. This all changed with the introduction of bioorthogonal chemistry by Bertozzi and co-workers.[1−3] The concept is elegant. Can we design reactions that are so selective they can be performed reliably even in a complex biological environment? These reactions must proceed efficiently in the presence of the multitude of functional groups found in living systems such nucleophiles, electrophiles, reductants, oxidants, and of course the solvent of life water. Simultaneously, these reactions should have a minimal impact on the biology itself. The transformation bioorthogonal chemistry triggered in the field of chemical biology was monumental. Suddenly, reactions that previous generations performed in refluxing toluene, were now being done in an aqueous mixture of proteins and sugars. Cancer cells and zebrafish replaced round-bottom flasks.[4,5] Bioorthogonal reactions have already made a tremendous scientific impact, helping us understand glycosylation in cells and animals,[6] providing tools for conjugating functional groups to therapeutically relevant proteins such as antibodies,[7] and enabling the assembly of molecular imaging agents in vivo to detect disease.[8]

The concept of bioorthogonal chemistry has inspired a generation of chemical biologists to think about how classic organic reactions can be performed in concert with living systems and how such reactions could lead to the development of tools to help understand biology. I think one of the greatest contributions of bioorthogonal chemistry has been its ability to challenge our imagination regarding the kinds of reactions that can be performed in living systems and how this enables us to ask extremely interesting and ambitious questions. Can pharmaceuticals be synthesized inside humans?[9] Can we co-opt bioorthogonal reactions to detect metabolites in situ?[10] How many orthogonal reactions can be performed simultaneously?[11]

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Over the last several years, our ability to combine chemistry and biology has accelerated through improved tools and resources. Therefore, I believe there are numerous future prospects for how bioorthogonal chemistry will have an increasing impact on chemical biology and medicine. In this short Outlook, I will describe my opinion of the future of bioorthogonal chemistry and explore what I believe are some outstanding opportunities in the field. I also outline many of the challenges that will need to be overcome for some of these opportunities to be realized.

THE DEVELOPMENT OF NEW BIOORTHOGONAL REACTIONS

Undoubtedly there will be continued development of new bioorthogonal reactions. Bioorthogonal chemistry has encouraged

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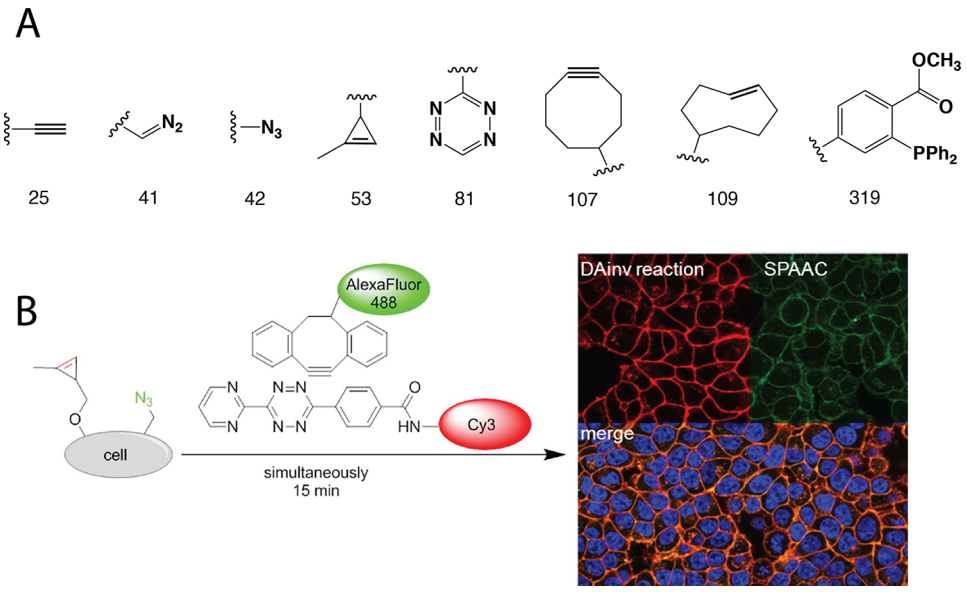

Figure 1. (A) Examples of bioorthogonal functional groups and their approximate molecular weights. (B) Mutually orthogonal bioorthogonal reactions for metabolic imaging. Tetrazines and cyclooctynes can selectively label cyclopropenes and azides, respectively. In this instance, the application is to visualize metabolic incorporation of two different sugars onto cell surface glycans. Image reproduced from ref 30.

chemists to consider how a vast number of organic transformations might be adapted to work in living systems. In just the last year alone, there has been the introduction of several new bioorthogonal reactions.[12−15] However, while there are a multitude of possible reactions that could be developed into bioorthogonal processes, it is worthwhile pointing out some of the desired properties of new bioorthogonal reactions that would significantly advance the field. For instance, the continued development of very rapid bioorthogonal reactions is desirable. Rapid reactions are useful because they, in principle, allow one to perform bioconjugations on a practical time scale using a lower concentration of reactants. This is important from a cost perspective. It also may not be practical to achieve high concentrations of a reactant, for instance, when working with proteins or attempting to perform reactions in living cells and animals. Previous work has made notable gains in improving the rate of bioorthogonal reactions, perhaps most notably through the development of tetrazine ligations, which have reported second order rate constants often exceeding 1000 M^{-1} s^{-1}.[16,17] For comparison, Staudinger ligations or strain-promoted cycloadditions typically have reported rates between 1×10^{-3} and 1 M^{-1} s^{-1}.[4,18,19] However, rate constants greater than 10 000 M^{-1} s^{-1} would be extremely useful. While there have been reports of very rapid reactions with rate constants approaching or exceeding these levels, the reactants themselves are often prone to degradation through side reaction, calling the "orthogonality" of these reactions into question. Development of more stable reactants that can still react very rapidly or the development of entirely new reactions will be highly welcome. There have been some recent inroads in these directions. Triazines have been explored as more stable alternatives to tetrazines, though the reaction rates are modest in comparison.[20] Additionally, researchers have shown that modified strained *trans*-cyclooctenes can avoid cycloisomerization back to the *cis* isomer,[21,22] a phenomenon that occurs frequently in the presence of thiols and essentially renders the reagents inactive.

Another area where there is room for future improvement is the size and physical properties of the reaction partners. One of the great advantages of the classic azide-cyclooctyne bioorthogonal reaction is the unobtrusive size of the azide functional group. Azides have been shown to be readily incorporated into analogs of biological metabolites, oftentimes with minor effects on overall function. This feature has enabled applications in metabolic imaging of glycans[4,23] and the incorporation and modification of reactive unnatural amino acids.[24,25] On the other hand, the cyclooctyne reactive group is a bulky probe that is hydrophobic and thus might accumulate nonspecifically in membranes and other cellular structures. Future reactions that use two coupling partners that are both of a lower molecular weight comparable to azides would be advantageous, as the reactive functionalities would be expected to have minimum impact on the molecules that they are appended to (Figure 1A). Recent studies exploring smaller bioorthogonal handles such as cyclopropenes[26,27] and diazo groups are promising directions toward this goal.[28]

There has also been growing interest in developing new bioorthogonal reactions that are orthogonal to preexisting bioorthogonal reactions.[11] For instance, several investigators have shown the use of tetrazine/dienophile reactions in parallel with azide/cyclooctyne reactions (Figure 1B).[27,29−31] With careful design of precursors and considering relative rate constants, reactions can be designed to be mutually orthogonal. The benefits of such selectivity are several. For instance, mutually orthogonal reactions enable one to perform metabolic imaging of two different processes by introduction of two differently tagged metabolites. Another exemplary application is the introduction of two reactive unnatural amino acids which can then be tagged using orthogonal chemistries.[32] Such an approach can be used to introduce FRET probes site-specifically on a protein to potentially monitor dynamics. It will be intriguing to see how many different bioorthogonal reactions can be reliably performed simultaneously. It will also be important to understand what the unique applications of such an approach would be and how to implement multiplexed bioorthogonal conjugations for answering biological questions.

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ASSEMBLING BIOACTIVE COMPOUNDS FOR IN SITU DRUG DELIVERY

The ability to stitch together organic molecules in vivo enables applications that involve the assembly of bioactive small molecules in situ. An exciting future goal might be the in situ assembly of pharmaceuticals at the site of their action.[9] The concept that therapeutic molecules might predictably self-assemble within living systems has been discussed for decades. There have been some notable examples, including the seminal work of Rideout, which demonstrated that aldehyde and hydrazine fragments could assemble into bioactive hydrazones within cells.[33] Since bioorthogonal reactions are robust methods for assembling molecules together, it stands to reason that they might have application toward in situ bioactive small molecule assembly. There is some prior art that suggests this might be feasible. First off, based on the strength and success of fragment based approaches for drug discovery,[34] there has been significant effort in using bioorthogonal chemistry, particularly azide–alkyne cycloadditions, to stitch together drug fragments in situ. Remarkably, it has been found that protein targets can be used to catalyze the formation of their own inhibitors by templating bioorthogonal reactions between reactive fragments that bind in close proximity to one another (Figure 2A).[35] Potent inhibitors for

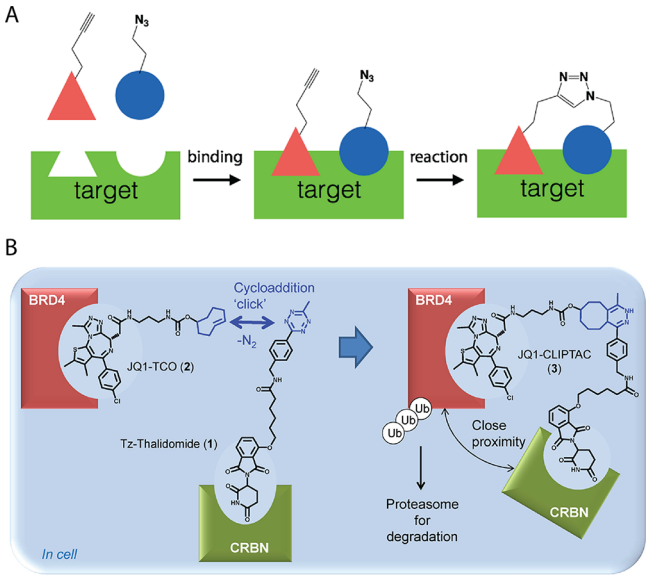

Figure 2. Assembling bioactive molecules in situ using bioorthogonal reactions. (A) A target biomolecule such as an enzyme can bind to two fragments bearing bioorthogonal functional groups. If the fragments are bound such that the functional groups are brought into close proximity to one another, reaction occurs, and a tighter binding compound is formed. This method has been used for in situ discovery of enzyme inhibitors, but it will be interesting to see if such reactions can take place within living cells and in vivo. (B) Researchers have used bioorthogonal reactions to form druglike molecules inside cells. Here, a heterobifunctional compound is formed by tetrazine ligation which enables the degradation of BRD4 through the ubiquitin pathway. Figure reproduced from ref 39.

enzymes such as acetylcholinesterase, carbonic anhydrase, and HIV protease have been discovered by screening libraries of compounds bearing reactive handles and analyzing what products are formed through templated reaction.[36−38]

In principle, templated synthesis of inhibitors might be extended beyond a tool for drug discovery and be used to trigger drug formation in vivo in response to specific protein targets.[9] There are, of course, several hurdles to realizing this vision. In some notable previous examples of in situ click chemistry, the templated thermal azide–alkyne cycloadditions were sluggish, and reagents were incubated over lengthy time scales.[36] For these reactions to work in vivo, the rate of product formation should be much faster, ideally within hours at physiological temperature. Fortunately, the development of biorthogonal reactions with highly tunable reaction rates[39] should enable control over the rate of fragment assembly such that product would form in short enough time scales to be therapeutically meaningful. Other hurdles include the complexity involved in having to optimize pharmacokinetics and delivery for two reagents versus one. However, the benefits could be enormous since bioactive compounds would in principle only be accumulating at high concentrations in cells that have appreciable concentration of protein targets. This would likely mean lower side effects due to off target engagement and potentially the ability to have a larger therapeutic window, which could be very valuable in the case of delivery of cytotoxic chemotherapeutics.[33] Additionally, the concept of delivering two smaller agents that later assemble to form a larger bioactive compound would represent a novel drug delivery strategy and potentially have advantages in terms of the ability of the smaller precursors to pass through cell membranes and the blood–brain barrier.

Recent work has made progress toward using bioorthogonal reactions to assemble functional therapeutics. There has been recent excitement in proteolysis inducing chimeras (PROTACS), which are heterobifunctional drugs that can trigger the degradation of proteins by linking a target protein with the cellular ubiquitinating machinery. However, the required bifunctional compounds are often of high molecular weight (800–1000 Da) and can have less than desirable pharmacokinetics for penetrating cell membranes or accumulating in the central nervous system. Recently, Astex Pharmaceutical has shown that tetrazine bioorthogonal reactions can be used to stitch together functional PROTACS for degrading oncology targets BRD4 and ERK1/2 within living cells (Figure 2B).[40] They were also able to demonstrate that in situ assembly of two small molecules within a cell showed greater efficacy than simply adding the preassembled molecule. However, a major challenge that remains to be addressed is how to selectively trigger formation within the cell versus outside the cell. In the described approach, bioorthogonal assembly would also rapidly take place in the bloodstream. Templated chemistry or perhaps small molecule targeting to specific cell types might alleviate this problem and lead to future translational applications.

BIOORTHOGONAL UNCAGING REACTIONS

A recent innovative direction for the coupling of bioorthogonal reactions to biological activity has been the use of bioorthogonal chemistry to uncage substrates, a strategy that has been termed "click to release."[41] This is an area of accelerating excitement, and applications are continuously expanding. The concept is similar to the well-known uncaging of small molecules in response to light. However, when using light as a stimuli, there is limited depth penetration. Therefore, optical techniques are often restricted to research applications or translational applications that do not require significant depth penetration or systemic activation. In contrast, the use of bioorthogonally reactive small molecules to trigger uncaging offers the potential to activate on the systemic level and in theory could be translatable to patients. Uncaging is typically done by using a reactive

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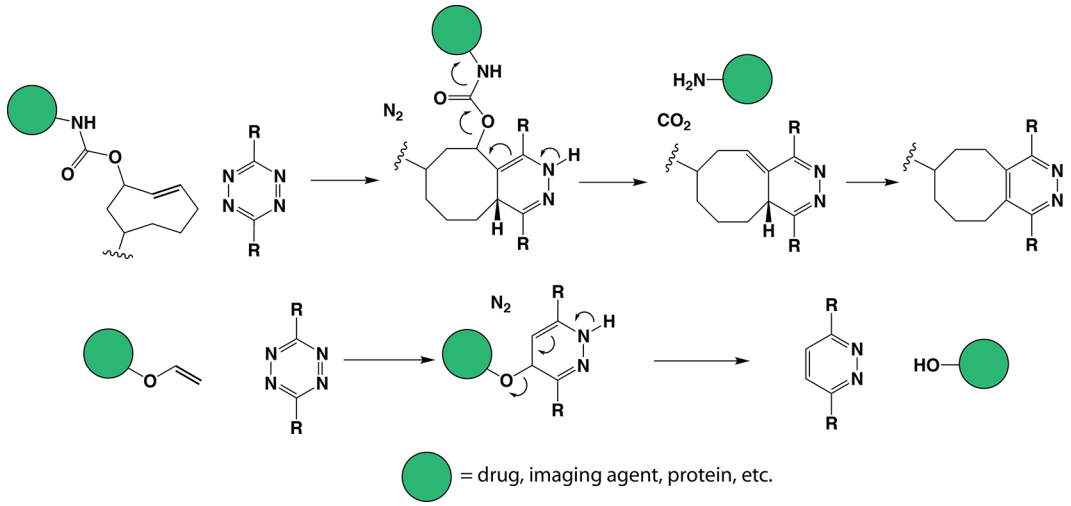

Figure 3. General examples of bioorthogonal uncaging reactions. Dienophiles act to mask functional groups such as amines and alcohols. Upon bioorthogonal reaction with tetrazine, the functional group is released. This enables the uncaging of drugs, imaging agents, and even enzymes.

handle to mask an amine or alcohol (Figure 3). A prominent example is the use of the highly strained alkene *trans*-cyclooctene to mask an amine, which is then released upon reaction with a tetrazine. Several investigators have already applied "click to release" strategies to trigger the formation of drugs in vitro and in vivo.[42−45] Furthermore, researchers have shown that, by incorporating a masked lysine residue through site-specific unnatural amino acid incorporation, bioorthogonal reaction can be used to trigger protein activation.[46,47] This strategy provides a mechanism to pharmacologically activate proteins in live animals and could reveal insights on the effects of protein activity on physiology. Additional work has shown that bioorthogonal masks can be used to activate fluorophores for imaging to detect biomolecules such as RNA.[48] Recent studies have even shown the ability to release gases such as carbon monoxide, indicating that bioorthogonal prodrugs of gasotransmitters are on the horizon.[49] I imagine that the use of bioorthogonal strategies to uncage biologically relevant molecules will continue to expand and see continued translation in vivo, potentially to humans. To aid applications, future studies may focus on improving the rate and yield of the release reaction, and recent efforts are promising in this direction.[50]

BIOORTHOGONAL REACTIONS THAT LEAD TO NATURAL LINKAGES

Most bioorthogonal reactions result in the formation of unnatural linkages. This makes sense since one would expect that exotic, abiological functional groups would have less chance of interacting with biological systems and thus contribute to the chemoselectivity of the reaction. There has been an interest in using bioorthogonal reactions to create analogs of natural compounds such as sugars, lipids, and nucleic acids.[51−53] However, the presence of unnatural linkages such as triazoles does cause concerns regarding whether the compounds formed are truly analogous in function. Thus, a future avenue of exploration would be the development of bioorthogonal coupling reactions that can be performed in vivo selectively but result in the formation of native linkages such as amides, esters, phosphodiesters, etc. A closely related class of reactions are the native chemical ligation, traceless Staudinger ligation, and associated coupling reactions that lead to amide bond formation.[54−57] The native chemical ligation involves the coupling of N-terminal cysteines to thioesters to form a native cysteine linkage. Unfortunately, these reactions have been for the most part limited to use in in vitro settings with purified components. However, one can imagine that the next generation of reactions will have appropriate selectivity and kinetics to be feasible for use in living cells and perhaps even in vivo. Strategies that template reactants could be one way to improve rates and selectivity, transforming chemoselective ligations into bioorthogonal reactions that can be performed in living systems.[58] Researchers could also take advantage of elegant reactions that use abiotic functional groups that come together to rapidly form peptide linkages.[59] For instance, recently our group has built on selective peptide forming ligations to synthesize bioactive lipids in living cells.[60] With such tools at our disposal, it seems likely that we would be able to use bioorthogonal coupling reactions to form native molecules inside living cells and test the effect of forming these molecules on cellular function. This would have tremendous benefits over genetic approaches, where pleiotropic effects and enzyme promiscuity can obscure observations and make it unclear if the activity observed is actually due to a change in concentration of the molecule of interest. As previously discussed, assembling molecules in situ would represent a novel mechanism of cellular delivery. The ability to shuttle in two molecules of lower molecular weight, which assemble at the site of action, could bypass physiological barriers such as cellular membranes.

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IN SITU FORMATION OF BIOORTHOGONAL HANDLES

Currently, bioorthogonal functional groups must be synthesized using standard organic chemistry methods and then

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introduced onto a biological molecule or metabolite, which is then introduced to the living system. This exogenous application of functional groups suffers occasionally from drawbacks including delivery issues, which are potentially problematic for in vivo applications. It would be very interesting if organisms could be engineered to synthesize bioorthogonal handles in situ. Past work from the Schultz lab has demonstrated that organisms can be engineered to synthesize unnatural amino acids, which can subsequently be incorporated into proteins site-specifically.[61] Would it be possible to do this but in a manner that the unnatural amino acid contained a reactive handle such as an azide, tetrazine, or strained dienophile? One ripe area for exploration might be the biosynthesis of strained cyclopropenes, dienophiles that are known to be useful bioorthogonal handles for reaction with tetrazines. Cyclopropenes are found in natural products, for instance, in the fatty acids malvalic acid and sterculic acid.[62] Unfortunately, the full details regarding the biosynthesis of cyclopropene fatty acids are still unclear, though future studies are likely to illuminate the pathway.[63] In the meantime, another approach might be to express enzymes specifically engineered to synthesize bioorthogonal handles. Recent work from the Arnold lab has demonstrated that enzymes can be engineered to catalyze the synthesis of cyclopropenes.[64] Adapting such systems to function in living cells might enable the in situ production of strained dienophiles which can then be incorporated into biological molecules and subsequently labeled.

IN VIVO CHEMISTRY

The ultimate frontier for performing reactions in a biological context would be conducting reactions in living animals and ultimately humans. While performing bioorthogonal reactions on and within living cells has become almost a routine phenomenon, the ability to conduct highly efficient reactions inside multicellular organisms remains nontrivial. Zebrafish are excellent model vertebrate organisms since developing zebrafish are optically transparent, and therefore visualization of bioorthogonal reactions through the use of fluorescent imaging probes is feasible.[5] Furthermore, reagents can be delivered either by injecting compounds directly into zebrafish embryos or bathing developing zebrafish in reactive precursors. However, performing bioorthogonal reactions in mammalian model organisms such as mice presents a slew of daunting challenges. First and foremost are optimizing the pharmacokinetics of the reagents to be reacted. There has to be some matching between the rate of the reaction and the lifetime of the precursors at the desired site of action.[8] For this reason, very rapid reactions are likely to find future application, since in many cases the attainable concentrations achievable are in the micromolar range, and lower still when one considers tracer imaging agents.[39] Pharmacokinetics also dictates whether the reagents even make it to the desired site of action. High doses of reagents have little benefit if a reaction desired should occur at a tumor but the reagents never make it there. In vivo chemistry is complicated by the fact that there are two reagents that will need to be delivered. While this may offer some benefits in terms of triggering the formation of drugs or bioactive compounds (see above), it also presents a number of challenges. For instance, the dosing, pharmacokinetics, stability, and toxicity will need to be optimized for not one but two reagents. However, despite these challenges, recent innovations and encouraging preclinical animal results suggest that these issues are likely surmountable, and one would expect to see applications potentially entering the clinic.[45,65,66]

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Ultimately, I imagine that bioorthogonal chemistry will go beyond research applications and preclinical testing and will be translated to applications in humans. What are some of the emerging applications that we might see translated initially? Arguably, one of the most developed applications is for pretargeted imaging or therapy using reactive monoclonal antibodies (Figure 4). The rationale for pretargeted approaches, which were pioneered by Goodwin and Meares, is compelling.[67] Monoclonal antibodies are excellent affinity ligands that show exceptional selectivity for homing to tissues expressing target antigens and achieving high signal to background. However, monoclonal antibodies also are cleared very slowly, often taking days for clearance in mice and longer still in humans. This long lifetime of monoclonal antibodies can present problems with trying to use antibody drug conjugates, particularly if the conjugate is a radiopharmaceutical used for imaging or therapy. In the case of imaging, the most commonly used radioisotopes are short-lived radioisotopes such as ^{18}F, which has a half-life of just under 2 h. Unfortunately, labeling of antibodies with ^{18}F is not useful since one needs to wait for days to allow sufficient antibody to clear to achieve appropriate target to background ratios for imaging antibody accumulation. By this time, the vast majority of the radionuclide will have decayed and imaging would not be possible. In a pretargeting approach, one would instead deliver a nonradioactive antibody conjugate bearing a bioorthogonal handle. This antibody will be allowed to accumulate and clear after which point a small molecule chaser with a much quicker clearance rate will be delivered. The chaser would possess the radionuclide and a complementary bioorthogonal handle, enabling reaction to the pretargeted antibody. Because the chaser is a small molecule and clears readily, appropriate target to background ratios in principle can be achieved quickly. Similar benefits can be achieved with therapeutic radiopharmaceuticals to avoid systemic toxicity and potentially maximize dose delivery to the target.

There have already been some outstanding developments toward achieving pretargeting imaging using bioorthogonal reagents.[65,66,68−70] Past work has shown promise using a variety of long-lived and short-lived radioisotopes. Most of these studies utilize fast tetrazine ligations, since very rapid kinetics is likely necessary to achieve efficient in vivo reaction with tracer quantities of imaging agent. However, while early work is encouraging, there are still several potential challenges

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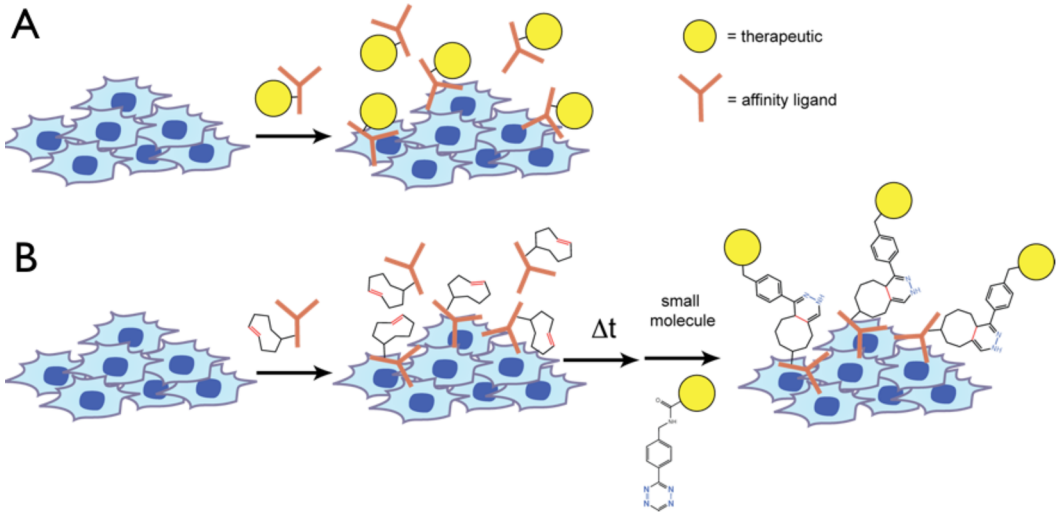

Figure 4. Bioorthogonal pretargeted therapy. Cartoon depicting the direct versus multistep labeling approach. (A) Target cells (blue) are exposed to an affinity ligand directly modified by a chemotherapeutic or imaging agent. The affinity ligand binds, but an excess of long circulating affinity ligand persists, contributing to toxicity or background signal. The latter problem prevents the use of short-lived radioisotopes (e.g., fluorine-18) with commonly used affinity ligands such as antibodies. (B) In the multistep approach, an affinity ligand directly connected to a nontherapeutic bioorthogonally reactive element (*trans*-cyclooctene) is delivered. Again, background is initially high, but this background is inactive. After time has passed to allow for clearance, a small molecule that reacts with the affinity ligand (tetrazine) and possesses a chemotherapeutic or imaging agent is delivered. The small molecule reacts rapidly with available *trans*-cyclooctene. The molecule also clears rapidly due to its small size thus lowering background side effects or signal.

that will need to be overcome in the future for such techniques to be translated to the clinic. Perhaps most restrictive is the current emphasis on targeting extracellular targets.[8,68] If the target antibody internalizes, then it will be more difficult for the bioorthogonal reaction to take place, as one of the reactants will be trapped within the cell. It would be simpler if the reactions were to take place extracellularly, but this presents the challenge of choosing appropriate targets that do not internalize once bound by antibodies, even after many hours. Additionally, it will be necessary to further improve the stability of the reactive partners while maintaining high rate constants. Recent studies have shown that highly strained alkenes that are often used in such bioorthogonal strategies may be prone to side reaction in physiological media.[21,26] Despite these challenges, current efforts by several clinical and commercial groups suggest that future improvements and clinical translation might lie ahead.

CONCLUSIONS

Bioorthogonal chemistries have enabled researchers to perform controlled chemistry in the presence of biological functional groups and in situ within living cells and organisms. Since its introduction, the toolkit of bioorthogonal reactions has steadily expanded and will definitely grow larger. New reactions will enable new opportunities and applications, particularly as kinetics, stability, and reactive handle size are further optimized. It seems likely that future studies will further emphasize the use of bioorthogonal couplings to trigger the formation of biologically active components, whether by direct in situ synthesis of drugs or through triggered drug release. Finally, continued applications in vivo will take place, and ultimately bioorthogonal reactions will find their way into the clinic. The predictions I have outlined are obviously just a small part of the future of bioorthogonal chemistry. Indeed, I am confident that some of the most exciting applications will come as a surprise and be driven by the need to address pressing biological and medical problems. Given the flexibility and power of the bioorthogonal approach, controlling chemistry within biological systems may soon only be limited by our imaginations.

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Notes

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