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The Emerging Role of Click Reactions in Chemical and Biological Engineering

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Introduction

A new paradigm encompassing several distinct chemical reactions and, more importantly, a generalized approach to molecular design and synthesis has been rapidly adopted in the fields of chemical synthesis, biotechnology, materials science, drug discovery, surface science, and polymer synthesis and modification. *Click* chemistry focuses on highly efficient reactions that reach quantitative conversion under mild conditions and require only facile separations. Key to this approach is the desire to achieve molecules and materials with the desired properties, behavior, and characteristics rather than a target molecular structure. Click reactions are ideal candidates for broad implementation and development in chemical and biological engineering. In this Perspective, the authors discuss the tenents of click chemistry, its adoption across several fields, and three of the most widely implemented click reactions in these areas including the original Cu catalyzed alkyne-azide cycloaddition reaction, the thiol-X family of click reactions and the Diels–Alder reaction.

The Click reaction paradigm and its history

The extraction of value from any given chemical synthesis process is a forte of the chemical engineering field and involves a complex underpinning in fundamental transport processes, reactor design, reaction kinetics, separations, materials cost, and many other factors. That said, an often overlooked part of the overall process is the selection of a well-suited combination of reactions that ultimately lead to the desired product. A highly efficient, orthogonal reaction that proceeds to quantitative yield from stoichiometric reactants at ambient conditions dramatically simplifies the process design and minimizes the cost ensuing from various aspects of the reaction, particularly in regards to the separation steps that are required. The ability to implement reactions at ambient or near-ambient conditions with mild solvents also has distinct advantages, including the general enhancement of the safety and environmental compatibility of the overall process. Reaction orthogonality, i.e., the ability to conduct a specific, targeted reaction without competition from side reactions that might otherwise consume the reactants, dramatically reduces the number of steps required by preventing the need for protection and deprotection steps, limiting the difficulty and number of the separation and purification steps that are required, or even by improving the efficacy of biological products for which impurities and side reactions can be particularly harmful. Ultimately, it is difficult to underestimate the potential value of highly efficient, high-yield chemical reaction processes in the fabrication and purification of various products in the chemical and biological engineering fields, including specifically block copolymers, modified proteins, drugs, surface modifications, and chemically altered materials of numerous types.

Sharpless and coworkers[1] recognized this incredible need and opportunity, and in 2001 launched the *click* reaction paradigm that focused on identifying, creating, and implementing reactions of just this type. Just a decade later, in 2011 alone, more than 1,500 articles were published that developed or implemented click chemistry. A relatively small but increasing fraction of this work is being conducted within the chemical and biological engineering fields. Uniquely, the click reaction concept not only focuses on the ability to produce specific molecular structures by certain, highly efficient reactions, but it also impacts the molecular design stage where the goal of the overall process is focused on the achievement of molecular or material performance. Ultimately, the goal is to identify molecular structures that achieve the desired characteristics, behavior, and performance of a compound or material, while being capable of being produced through these highly efficient, robust reactions that are simple to employ and lead to quantitative yields. This comprehensive consideration of not

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only the chemical reaction steps, but the overall process has long been strength of the chemical engineering approach to reaction and process design. However, the click concept adds to this approach an even greater emphasis on the selection of ideal reactions and benign conditions.

In particular, broadly, a click reaction process is considered to be one for which the reaction and/or process:

- Starts with readily available reactants;
- Yields a single, stable product from a stoichiometric mixture of reactants;
- Leads to quantitative yield of the product;
- Necessitates either no separation or minimal, facile separation to isolate the product;
- Is orthogonal to other reactions;
- Proceeds readily and rapidly at mild conditions including the atmosphere, temperature, and solvent selection (e.g., water is particularly desired as a reaction solvent);
- Forms either no byproducts or nontoxic ones; and
- Is stereospecific.

Processes that embody the click reaction paradigm are considered to be those that implement reactions with these attributes with the goal of forming molecules that achieve the targeted performance and behavior. To achieve these criteria, the original click reaction definition referred to click reactions as if they were ''spring-loaded'', which inherently implies a certain degree of exothermicity.[1] This need arises due to microreversibility, and the cost of using excesses of reagents to shift equilibrium in batch processes. Consider an equimolar coupling reaction, as often needed in block copolymer formation or protein modification, either with the formation of a small molecule (i.e., acid-amine condensation) or without (i.e., a cycloaddition or urethane formation) occurring at 1 M. In either case to achieve an equilibrium conversion of 70% conversion requires only a Gibbs free energy of 4-5 kJ/mol at 300 K, whereas an equilibrium conversion of 99% requires a Gibbs free energy of more than 23 kJ/mol. While high-equilibrium yields can sometimes be achieved by other process aspects such as either precipitation of a product or release of a gaseous small molecule, it is clear that thermodynamically the search for click reactions begins with reactions which are relatively highly exothermic.

In assessing the impact of click chemistry it is essential to examine the past. The question of whether click reactions are new is easily answered—in fact, most reactions now classified as click reactions are quite old, although nearly all have found newfound interest and recent fundamental enhancements, approaches, and implementations. The glaring exception is the copper-catalyzed azide-alkyne cycloaddition, discovered in 2002.[2,3] However, the uncatalyzed azide-alkyne reaction is a 1,3-dipolar cycloaddition, a reaction mechanism discovered and systematically explored across a wide range of reactants by Rolf Huisgen in the 1960s,[4,5] and it should be noted that the azide-alkyne reaction is itself even older, discovered in 1893 by Arthur Michael.[6] Other reactions such as the eponymous Michael addition and Diels—Alder reactions date to the 1890s and 1928,[7] respectively. The thiol-ene and thiol-Michael reactions emerged out of the question of why thiols undergo anti-Markovnikovian addition under some conditions, and both reaction mechanisms were proposed by Kharash in the 1930s and 1940s, respectively.[8,9] Excellent early reviews were written well in advance of any identification of the unique common features of these reactions.[10,11]

Given that most of the chemical reactions labeled as click reactions are not recent, an obvious question is whether the click reaction concept is new. A number of fields clearly do not make use of the principles of click chemistry. Sharpless's original case was that pharmaceutical chemistry is currently the foil of click chemistry, as it often pursues target molecules that are inherently difficult to synthesize and is seldom efficient in producing such molecules.[1] In looking at the requirements for fuels including even highly specialized jet fuels, specific molecules are seldom targeted or mentioned, whereas requirements for properties are abundant (e.g., flash point and heat of combustion).[12] However, despite being extremely efficient, petroleum chemistry is not click chemistry as it relies on specific catalysts and separations optimized for each step in a multistep progression. Biochemistry while also highly efficient does likewise. Solid-phase synthesis is capable of producing large molecules with highly specific structures in a modular nature. However, each step requires a large excess of reagents to achieve complete conversion, and the process is quite wasteful. In fact, the implementation of the solid phase support, which dramatically limits process yields by only using the substrate interface as the functional reactor volume, is used in deference to the difficulty of the separations that are required after each synthesis step. Only in such a heterogeneous reaction where the large stoichiometric reactant excess is removed after each step by dilution from the reactive solid substrate would this inefficient reaction be feasible. Clearly, these work-arounds for inefficient reactions have significant drawbacks, independent of the targeted molecular structure.

Ruling out a few fields still leaves many areas of applied synthesis, and it is hard to imagine that chemical engineers and chemists have been unintentionally using more difficult chemistry than required, especially given that click reactions have existed for decades. Indeed attributes of click chemistry have been implemented across an array of fields. A focus on using efficient *regio* and *enantio* selective reactions is shared with the concept of the atom economy—that is using reactions that maximize the number of atoms of the reactants retained in the product. This idea was put forward by Trost in 1991.[13] Green chemistry with its focus on reducing the use and generation of hazardous chemicals shares an interest in using reactions with benign byproducts, avoiding the use of protecting groups, and eliminating the generation of large amounts of waste by chromatographic separations and volatile solvent use.[14] Finally, statements such as the following attributed to Sir John Cornforth are encountered (as quoted in Ref. 15):

''The ideal chemical process is that which a one-armed operator can perform by pouring the reactants into a bath tub and collecting pure product from the drain hole''.

Hence, while many tools and themes of click chemistry have existed for an extended period, it is only very recently that all of the components have been put together in a neat package that has seen broad implementation. It has been argued that the merit of click chemistry is how a simple set of rules has led to new approaches to chemical problem solving.[16] In this Perspective, the authors will attempt to show how the click approach has enabled improved solutions to old problems, as well as novel molecules and materials that were not previously achievable or at least not practically feasible.

The Click Reaction Families

As indicated, numerous reactions have been classified as click reactions, although many identified as such do not regularly achieve all of the desired attributes of the click reaction description. Reactions such as the ruthenium catalyzed azide-alkyne cycloaddition,[17] azide-sulfonyl cyanide cycloadditions,[18] benzyne-azide cycloadditions,[19] and others[1] have been identified as click reactions and meet some or all of the criteria. Here, however, we choose to focus on three of the most broadly implemented click reactions that are particularly relevant to chemical and biological engineering. These three comprise the thiol-X family of reactions, Diels—Alder reactions, and the Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC). The thiol-X reaction family includes the radical mediated thiol-ene and thiol-yne reactions as well as the highly efficient and rapid base-catalyzed thiol Michael addition reaction. These reactions have been widely implemented in forming and modifying polymer materials and surfaces, and they are also well suited for producing and modifying biological substrates, such as peptides and sugars. The Diels—Alder reaction is a highly specific (4+2) cycloaddition reaction between a diene and a dienophile, with a rich history of diverse implementation. As a potential advantage and disadvantage, depending on the desired outcome, the reaction is thermoreversible at high temperatures for certain chemical substrates. The most recently and prominently identified click reaction is the CuAAC reaction which leads to the formation of a triazole ring structure through a cycloaddition reaction between an azide and an alkyne. This reaction has become ubiquitous in polymer and biological substrate modification and in the formation and modification of block copolymers. The drawback of necessitating the relatively toxic Cu(I) catalyst can also be addressed by creating highly strained alkyne substrates[20–22] in what has been referred to as copper-free click chemistry. These three reactions represent significant opportunities in traditional chemical engineering, materials and surface science, and biological engineering and are discussed here in that light.

Thiol-X reactions

The characterization of various thiol (often called mercaptans as well) reactions as click reactions was first proposed by Schlaad and coworkers[23] in 2007 for the radical-mediated thiol-ene reaction, and has now been broadly applied to various thiol reactions including those with enes, ynes, epoxies, alkyl halides, and isocyanates.[24] Chemically, considering the electron structure and density of the sulfur atom as well as the thiol functional group, when compared with alcohols and amines, thiols are generally thought of as soft nucleophiles. The nucleophilic thiolate anion and the electrophilic thiyl radical that form during these various reactions are highly reactive species that give rise to the breadth of thiol reactions considered to be in the thiol-X click reaction family. Interestingly, it is noted that the use of thiol-chemistry has historically had a pronounced industrial implementation, especially in polymeric materials development. In particular, several sulfur reactions were historically used to catalyze the crosslinking reactions of various thermoplastics, most notably in the vulcanization of rubbers. Several recent reviews have highlighted the potential and more recent developments within the thiol-X[24,25] and thiol-ene[26–29] click reaction family.

Interestingly, in examining the scope of click reactions in which the thiol participates, it is this same broad applicability and potential for facile reactions of the thiol with a variety of substrates that also represents the most significant downside of considering the thiol family of reactions as click reactions. Here, the potential for thiols to react with a variety of chemical substrates limits their orthogonality, i.e., the potential for side reactions with other, undesired chemical functional groups. The same aspects that make them highly efficient and rapid in reacting with one substrate may limit their ability to react only with that substrate. That said, the conditions under which each of these reactions occurs, e.g., radical-mediated, base-catalyzed, etc., may be selected to be mutually exclusive and focus the thiol reaction to occur with only a single other functional group. In addition to the potential for a lack of orthogonality, two other significant disadvantages arise in considering the implementation of the thiol family of click reactions. First, again related to the reactivity of the thiol, preformulated compositions that contain both the thiol and secondary functional group (e.g., the ene in thiol-ene reactions) are inherently relatively unstable, with a limited shelflife. Finally, low-molecular-weight thiols or higher molecular weight compounds with low-molecular-weight impurities possess a distinct and offensive odor. The implementation of higher molecular weight thiols reduces or eliminates this issue by lowering the vapor pressure of the reactants.

The relative reactivity of the thiol does give rise to at least one significant advantage of this reaction relative to nearly any other of the click reactions—it is extremely rapid. Complete conversion may take from a fraction of a second up to several minutes to achieve at ambient conditions, depending on the initiation conditions and the specific reactants. One unique benefit of the thiol reaction family for consideration in biological applications is the natural existence of the thiol-containing amino acid, cysteine. The ability to incorporate a thiol at specific peptide or protein positions and undertake selective functionalization is unique to the thiol. Additionally, several of the thiol-X family of reactions may also be photoinitiated, which gives rise to spatiotemporal control of the reaction which yields significant benefits for various surface modification and lithography applications. This opportunity for spatiotemporal control of the reaction has been unique among the click reaction family until very recently, when it became possible to photoinitiate the CuAAC reaction as well.[30–32]

Figure 1 provides a summary of the thiol-X reaction family where it is clear that the thiol is capable of reacting with a large range of substrates, nearly all of which under the right catalyst and reactant conditions are click reactions. The two most common thiol click reactions are the radical-mediated thiol-ene reaction and the base-catalyzed Michael addition reaction with their mechanisms briefly summarized in Figure 2. Here, the radical-mediated process has commonly been initiated by light exposure in combination with a radical photoinitiator, although redox and thermal radical initiating systems have also been used. This reaction was used to form crosslinked polymer films for optical adhesives and

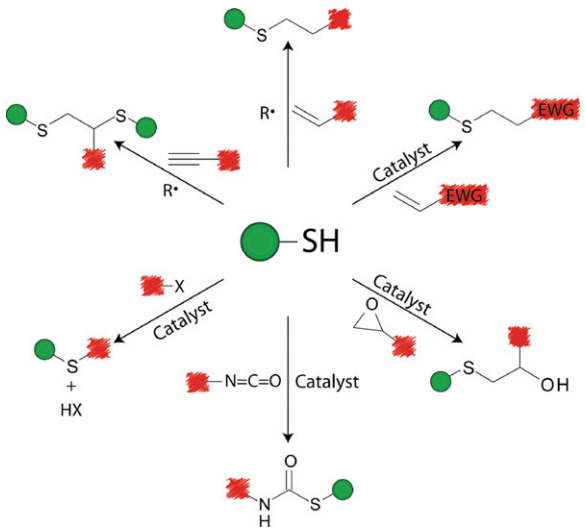

Figure 1. The thiol-X reaction family encompassing the thiol functional group's capability of reacting broadly with a range of chemical substrates.

The catalysts necessary for many of the reactions are unique and the reaction type, even in the presence of multiple possible substrates, can be controlled by the type and concentration of the catalyst.

coatings, for microfluidic devices and for hydrogel-based biomaterials because of its unique ability to overcome oxygen inhibition and to form nearly stress-free films. It has also been used to functionalize peptides,[33] sugars, and surfaces using free thiols that are readily attached to silicon or gold surfaces or through the amino acid cysteine in proteins and peptides. The Michael addition reaction, which can be base or nucleophile-catalyzed,[34] has also been used to form biomaterials, including degradable hydrogels that incorporate functionalized peptides[22,35,36] as well as in the formation of crosslinked polymer films.[37]

The Diels–Alder reaction

In its broadest definition the Diels–Alder reaction is a [4+2] cycloaddition occurring between a conjugated diene, capable of assuming a *cisoid* conformation, and a double bond (termed the dienophile, see Figure 3). Oftentimes the dienophile is electron deficient, the mechanism concerted, and the reaction reversible, although none of these characteristics are necessary. The Diels–Alder reaction has a number of excellent attributes. As both diene and dienophile can contain heteroatoms the Diels–Alder reaction is extremely wide in scope.[38] Moreover, the reaction is atom conservative, which eliminates the removal of a byproduct, and waste disposal. Furthermore, the reaction is largely immune to solvent effects, and the reactants are typically nonreactive toward alcohols, amines, acids, carboxyl, and many other functional groups eliminating the need for protection/deprotection steps. Catalysts are not necessary, although Lewis acids can act as catalysts.[39] Finally, the reaction is extremely rapid in water, showing an increased rate of nearly 1,000 times in some cases, and it is insensitive to oxygen.[40,41] Due in part to these attributes the Diels–Alder reaction has been the focus of many experimental studies examining both its synthetic application and mechanism. An overwhelming number of reviews are available, and the reader is referred to only a selected few.[38,42,43]

Despite a number of advantages, the Diels–Alder reaction is used with a few caveats. While efficient, modular, and wide in scope the reaction is not necessarily regio or stereo selective, although trends do emerge (Figure 4). For example, both *endo* and *exo* products can be produced by the reaction of cyclic dienes. Typically, the exo isomer is thermodynamically favored while the endo isomer is the kinetically favored product.[44] In such cases the reaction initially produces the endo product, and eventually the exo product predominates. However, such behavior does not always occur because some Diels–Alder reactions are reversible, and the retro-reaction occurs before a large

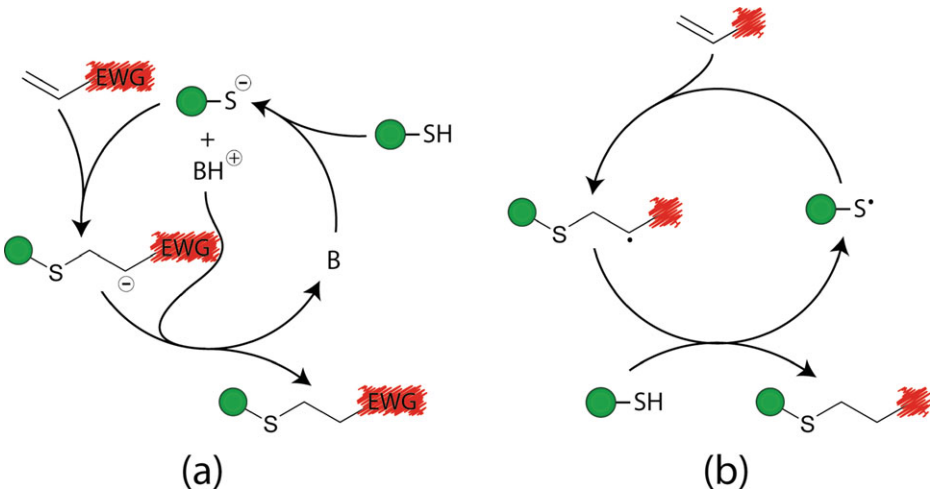

Figure 2. Mechanisms of the two most common thiol-X reactions.

(a) the base-catalyzed Michael addition of a thiol and an electron deficient vinyl group, which can also be nucleophile-catalyzed, and (b) the radical-mediated thiol-ene reaction where the radicals are generated by conventional free-radical processes including through redox, thermal, or photochemical methods. Both reactions yield a single product with addition of the thiol across the double bond to form the thioether adduct.

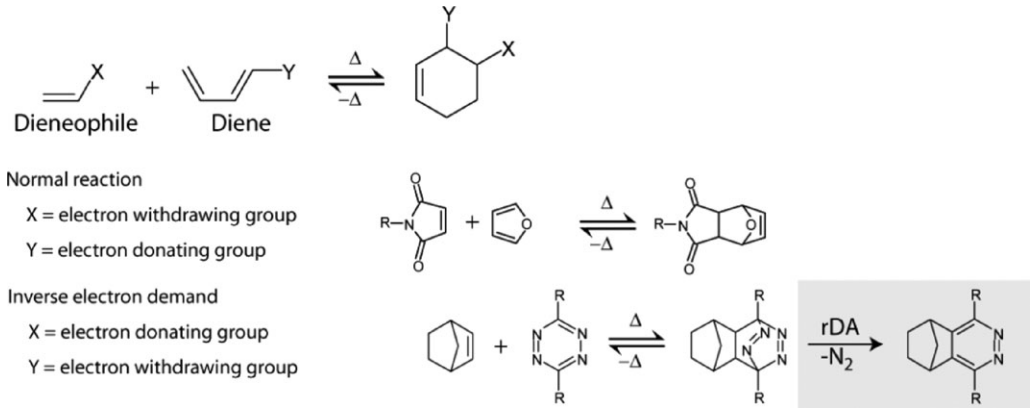

Figure 3. Two types of Diels—Alder reactions are commonly encountered.

In the normal reaction the dieneophiles possess electron-withdrawing groups and the diene electron-donating groups, as in the case of maleimide and furan. In the inverse reaction the diene is electronically activated while the dienophile is electron poor (norbornene and tetrazine shown). In both cases the reactivity is predicted by the energy differences of the HOMO and LUMO of the reactants, which are controlled by the induction effects of neighboring groups.[38,51] As shown in the gray box, in some cases retro-Diels—Alder reactions produce products other than the reactants and can eliminate gaseous species that irreversibly drive the reaction forward.

concentration of the exo product can form. In recent applications such as crack healing[45,46] such behavior is quite desirable as bond breakage and reformation allows for material healing at interfaces. However, the correct reassembly of complex structures, such as dendrimers, may not occur making the retro-reaction a significant liability for many applications.[47] For permanent chemical structures consistent with the click reaction paradigm, a more exothermic Diels–Alder reaction need be chosen (all Diels–Alder reactions are entropically disfavored, and, thus, the retro-reaction always occurs at elevated temperatures[38]). Several adducts are quite irreversible, dicyclopentadiene is ''cracked'' to cyclopentadiene by heating at its boiling point of at 170°C, while tri-cyano acrylate and fulvene adducts turn over rapidly near ambient temperature.[48] Reactions can also be driven forward by the elimination of a gaseous small molecule such as carbon monoxide, which can occur by a retro-reaction, to products other than the initial reactants (Figure 3).[49] Interestingly, due to hydrophobic effects, the reaction also exhibits some dependence on the solvent polarity.[43] Typically, this effect is of little consequence, but it has been noted that a solvent change to dimethylformamide aids the depolymerization of hydrogels.[50]

The 1,3-Dipolar Cycloaddition and the Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC). The 1,3-dipolar cycloaddition (or, Huisgen reaction) is a [3+2] cycloaddition that occurs between a 1,3-dipole and a dipolarophile (double or triple bond). The 1,3-dipolar cycloaddition and Diels—Alder reaction have much in common. Both are broad in scope,[4,5] atom conservative, largely free from solvent effects, orthogonal to many reactants, and retain the stereo chemistry of the reactants. Again, regioselectivity occurs for some reactants, and reactivity is related to the HOMO─LUMO gap.[52,53] Dipolarophiles like dienophiles are typically activated by electron-withdrawing groups, but again inverse electron demand reactions can occur. Figure 5 shows the 1,3-dipolar cycloaddition mechanism.

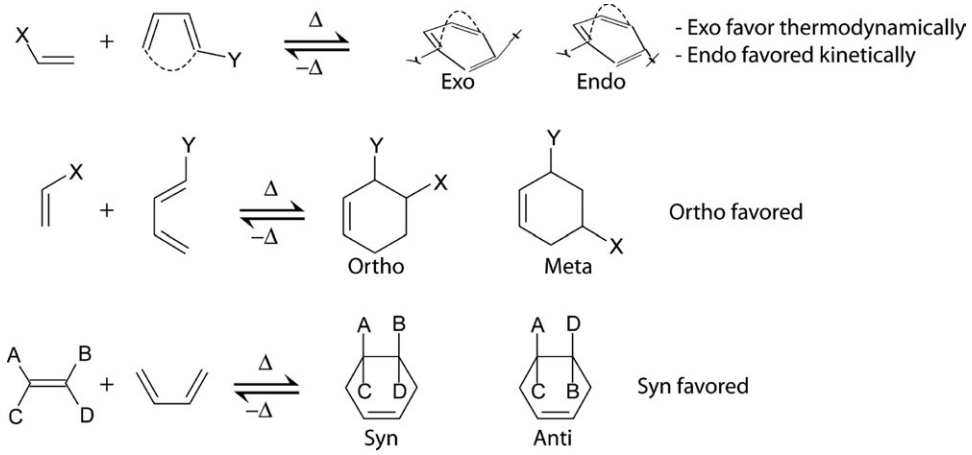

Figure 4. The Diels–Alder reaction can produce a complex array of regio and stereo isomers, although electronic and steric effects typically favor one isomer over the other.

Given the breadth of the Diels–Alder reaction, many exceptions are encountered.

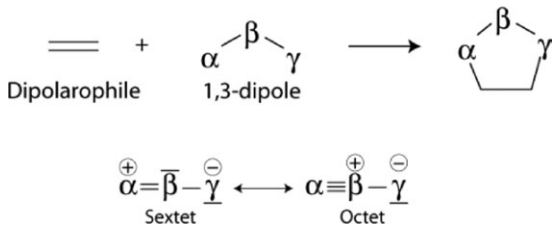

Figure 5. 1,3-dipolar cycloadditions are [3+2] cycloadditions between a pi bond (double or triple bond, with or without hetero atoms) and 1,3-dipoles.

1,3-dipoles are ambivalent species that can be represented by both sextet and octet formulas. The character of the former allows the [3+2] cycloaddition to occur.

While a wide range of 1,3-dipoles can undergo the reaction, fewer species such as azides,[1,20–22,54–56] nitrile imines,[57,58] nitrile oxides,[59] and a few others[60] have been successfully used as click reactants as many dipolar species are prone to rearrangement and hydrolysis. Azides have attracted much attention as they are relatively stable and largely invisible to many other functional groups; hence, possessing a high degree of orthogonality. Thus, azide-alkyne coupling reactions can be readily incorporated into reaction schemes without the need for protection/deprotection schemes. Unfortunately, in the absence of the Cu(I) catalyst, most alkynes have sluggish reaction rates and only proceed at temperatures greater than 100°C. Strained dipolarophiles such as norbornenes,[61] cyclo-octynes,[54] dibenzocyclooctynes,[55,56] and more so strained perfluoro cyclooctynes,[20–22] do give a rapid reaction at ambient temperature in a Cu-free reaction. These types of reactions have been quite successfully used for *in vivo* labeling, but the synthetic difficulty of synthesizing these groups compared to that of synthesizing terminal alkynes has largely limited their use in other areas.

Combined with the stability issues concerning azides, the azide-alkyne cycloaddition was seldom used outside the specific synthesis of triazoles until the discovery of the copper-catalyzed azide-alkyne cycloaddition (CuAAC).[2,3] Given that the discovery of the CuAAC reaction corresponded almost exactly to the emergence of the click chemistry paradigm, it is not surprising that the two became nearly synonymous. The CuAAC reaction displays a rate increase of approximately 10^7 over the uncatalyzed reaction, which allows the reaction[62] to succeed in a number of traditionally difficult reactions such as polymer–polymer coupling, which is useful for block copolymer formation.[63,64] Combined with the orthogonality and ease of incorporating azides and alkynes, this behavior lead to a modular ''lego-like'' approach to many synthetic routes.[62] The CuAAC reaction appears to be quite different from the 1,3-dipolar cycloaddition, and the two names should not be used synonymously. While a variety of azides participate in the Huisgen reaction, only terminal alkynes participate in the CuAAC reaction. This behavior is typically ascribed to the necessity of first forming copper-acetylide which then undergoes a stepwise reaction.[65] Thus, the CuAAC reaction is a *formal* cycloaddition, the exact mechanism of which to date is somewhat unclear, possibly due to the existence of multiple competing mechanistic pathways.[66–68] Regiospecificity, which is often observed for the uncatalyzed reaction, and depends on the nature of the neighboring group, is strictly observed in the CuAAC reaction and only 1,2,3-triazoles are produced.[2,3] Electronic effects, which dictate the reaction rate and regiospecificity in the uncatalyzed reaction, may be severely reduced, or even absent in the CuAAC reaction. There are reports that electron rich azides react faster, but little quantitative data is available.[69] Catalysis is attributed to only Cu(I), although a copper(II) acetate and other cuprous salts have been noted to catalyze the reaction, perhaps due to oxidative coupling reactions that produce Cu(I) (e.g., the Eglinton reaction).[68] More typically, Cu(I) is added directly as a copper salt,[3] or formed *in situ* via reduction[2] or comproportionation reactions.[65] Typical reductants include sodium ascorbate in aqueous systems and triethylamine in organic media.[70] However, electrolytic reactions[71] and photochemically generated radicals[30,31] can be used to spatially control the Cu(I) generation. Ruthenium catalyzed azide-alkyne (RuAAC) reactions are also known,[17] but such catalysts are not stable against air and water and far less frequently encountered. In principle, given the right pair of azide and alkyne both CuAAC and 1,3-dipolar reactions should be reversible, but despite several efforts searching for reversible systems, none have yet been realized.[72]

Click Reactions to Polymer Modification and Coupling

One of the most powerful and enabling aspects of click chemistry is in the control of polymer architecture and structure through selective and targeted coupling reactions. Click chemistry has been used to couple homopolymer segments into block copolymers, form idealized dendrimer structures rapidly of high generation, and to functionally modify polymers as a means for altering their chemomechanical behavior.[73–76] In fact, in one demonstration Hawker and coworkers[74] were able to combine sequential CuAAC and thiol-ene coupling reactions so as to achieve the efficient synthesis of a sixth generation dendrimer in a single day.

The problem of polymer ligation that is necessary in the synthesis of controlled architecture polymers, particularly in several approaches to block copolymer and star polymer formation,[75] is one that is ideally suited for click reactions. In ligating high-molecular-weight polymers together, the terminal end groups that must be coupled are present in miniscule concentrations and yet, because of the difficulties of separating uncoupled polymers from coupled polymers, high-yield, stoichiometric reactions of the two polymer substrates are generally required. Additionally, a high concentration of side chains or other reactive species also exist in the polymer chain and thus orthogonality of the reaction is critical. For all of these reasons, click chemistry has been frequently employed, as illustrated in Figure 6, for achieving polymer ligation and block copolymer formation,[75,77] as well as polymer functionalization and click-mediated crosslinking.[78]

One of the most significant implementations of click chemistry in polymers and materials has been in the development of new approaches to biomaterials, enabled by the advent of click chemistry. In fact, even in the formation of

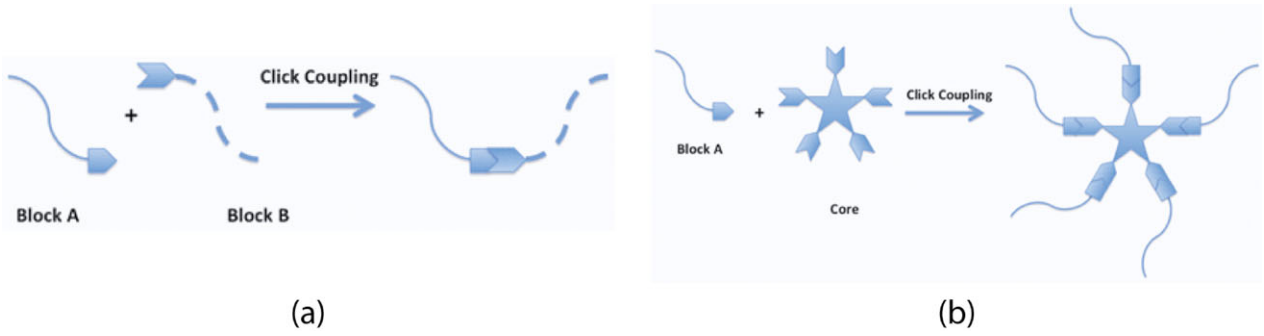

Figure 6. Ligation strategies employing click reaction chemistry for forming (a) block copolymers, and (b) star polymers.

Successful reaction strategies for achieving ligation must function well despite a highly dilute reactive functional group concentration, stoichiometric reactants, and the presence of numerous other reactive moieties that must not participate in the reaction.Each of the click reactions discussed here, including Diels–Alder, thiol-X and CuAAC, have been employed for polymer ligation and functionalization.

block copolymers, significant efforts have focused on methodologies for fabricating block copolymers that are functional biomaterials. Recently, Hawker and coworkers[79] have created well-defined block copolymers from degradable units, including poly (lactic acid) or PLA, that form self-assembled nanoparticles. As the degradable units are cleaved, controlled release of compounds from within the nanoparticle can be achieved with intimate control of the release profile achieved by manipulation of the block copolymer characteristics. Click reactions of peptide oligomers have also been used to control the peptide architecture, forming cyclic RGD peptides via the thio-ene reaction.[33]

Hydrogels for both controlled drug release and tissue engineering constructs have been formed by thiol-ene,[80] thiol-Michael addition reactions,[36] and alkyne-azide click reactions,[22,35] including reactions in which cysteine-terminal peptide segments were used as the crosslinking agents. In such systems, biofunctionality is readily imparted by the selection of the peptide unit. The choice of an enzymatically cleavable peptide was used to produce hydrogels with tunable degradation and controlled release.

Furthermore, the ability to perform multiple, sequential click reactions (i.e., ''double clicking'')[22,35] is also of great significance and has recently been used with sequential CuAAC (or copper-free azide-alkyne cycloaddition) and thiol-ene reactions. Anseth and coworkers used the copper-free azide-alkyne click reaction to initially form a hydrogel and subsequently used a thiol-ene coupling reaction to photopattern the attachment of an additional ligand.[22] The ability to control hydrogel structure and biochemical functionality in 3-D, including the potential for peptide coupling through the thiol-ene reaction, provides control over cell attachment, proliferation and migration in 2-D and 3-D cell constructs.

Click reactions in surface modification

Self-assembled monolayers (SAMs) consist of a single layer of amphiphilic molecules bound to a surface. As the surface density of the molecules increases, highly ordered and often quasi-crystalline arrays form[81] and spatial control of SAM formation is readily accomplished, using simple techniques such as photolithography[82] and microcontact printing.[83–85] A variety of SAM chemistries are available,[86–89] although the most common are the reaction of alkyl-thiols with gold surfaces,[88,90,91] and chloro- or alkoxysilanes with hydroxyl functionalized surfaces.[92–95] The combination of a highly exothermic formation reaction and multiple bonds connecting silicon atoms makes silane SAM formation quite irreversible and stable under most conditions. Since both of these SAM chemistries produce high quality, dense films, both approaches have also been used in combination with subsequent click reactions to perform either patterned or unpatterned surface modification.

While modification of surface properties can be achieved simply by the formation of a SAM bearing simple alkyl or other functional groups, improved properties are most often realized by subsequently and selectively functionalizing SAMs with specific chemical entities, such as targeted chemical functional groups or large molecules such as proteins, enzymes, or electrochemically active species. Very few molecules are required to modify the surface, and as bulky groups attached to the silane sterically hinder the formation of a dense monolayer, nonreactive molecules are added to ''dilute'' the surface and form a mixed monolayer. This approach allows valuable molecular species to be efficiently used. Although, proteins often nonspecifically adsorb to surfaces, covalent attachment is typically preferred, and two strategies exist (1) the chemabsorptive molecule and the desired species can be coupled before SAM formation, or (2) the SAM can first be formed and the desired species grafted to it. As silanes react readily with atmospheric water, alcohols, and acids, synthetic routes coupling them to many targeted molecules are difficult or impossible. Thiols are far more orthogonal, but often require protection and deprotection steps, or other lengthy synthetic procedures.

The increase in efficiency accomplished by the click philosophy of simple, modular, and efficient reactions is perhaps best highlighted by the case of RGD-peptide functionalized SAMs. These SAMs are composed of both ethylene glycol functionalized molecules that resist nonspecific cell adhesion, and others bearing the peptide motif RGD which promotes cell adhesion.[96,97] The size, shape, density, and nature of the cell's focal adhesions as directed by the RGD peptide significantly influence the cell's fate. As illustrated in Figure 7, Whitesides and coworkers prepared a GRGD

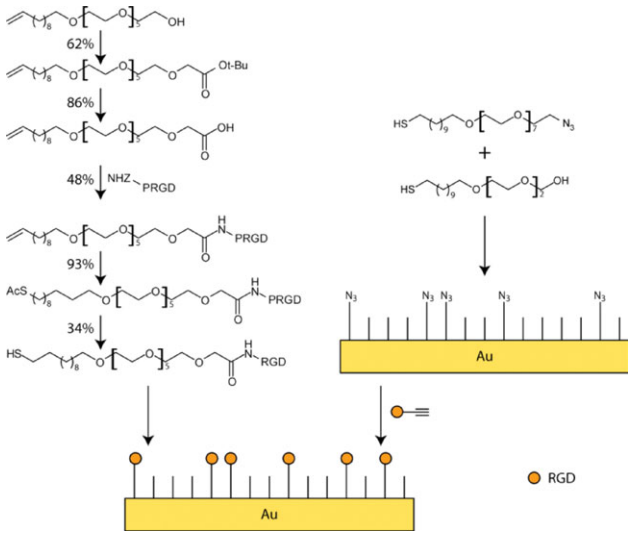

Figure 7. Two strategies for preparing an RGD functionalized SAM are shown.

On the left a six-step procedure for preparing a thiol functionalized RGD molecule is shown. More than 60% of the material prepared in the first four steps is lost when the thiol, arginine, and aspartic acids groups are deprotected. At the right a two-step version where an azide functionalized surface is first prepared using a commercial azide functionalized thiol. In the second step a peptide containing an alkyne is coupled to it using the CuAAC reaction.

peptide coupled to a hexaethylene glycol alkane thiol in a five-step synthesis with a final yield of 8% (ignoring both the efficacy and number of steps involved in synthesizing the peptide) which was then used to prepare the SAM directly.[96] Using the click philosophy, a similar surface can be prepared in two steps. First a mixture of a commercially available azide functionalized thiol is used to prepare a SAM on a gold surface. Second, a peptide sequence bearing a nonnatural alkyne functionalized amino acid that is easily incorporated in automated peptide synthesis routine is then coupled to the azide groups of the SAM via the CuAAC reaction.[98] A similar approach where a thiol functionalized surface is prepared from 3-mercapto-propyulrimethoxy silane and is then coupled to maleimide functionalized fibronectin has proven successful for studying how adhesive locations affect cell shape.[99] An alternative approach using the Diels—Alder reaction has also been used to couple RGD and cyclic RGD to SAMs and study the influence of each on focal adhesion formation.[97]

Arguably, similar approaches using nonclick coupling reaction to modify SAMs have been demonstrated. Under irradiation aryl azides decompose to nitrenes that readily couple with amines. This approach has been demonstrated for coupling ferrocene to a gold surface, and readily allows photochemical patterning of the reaction with 2 μm resolution and near 90% coupling efficiency in some cases.[100] Unfortunately, several products are produced depending on the substitution of the aryl azide, the wavelength of irradiation, the dose of irradiation, the solvent, and the amine concentration. These products undergo further photochemical reactions, and a large yield of any particular species may never be achieved. While tolerable for a liquid phase reaction, such side products cannot be removed from a surface. Coupling can also be accomplished, using more conventional carbodiimide chemistry popularized in peptide synthesis. Activated esters can be generated, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and n-hydroxysulfosuccinimide. This process has been explored for coupling polylysine to SAMs. However, this approach is pH sensitive, requires at least a two-step process, and multiple iterations may be required to achieve high conversion.[101] Ironically, perhaps the best alternative to click chemistry for coupling reactions to SAMs is the noncovalent interaction between streptavidin and biotin which has a dissociation constant of approximately 10^{-15}, and which is relatively independent of pH, solvent, and temperature.[102] This approach has been successfully used to conjugate large biomolecules such as IgG's and DNA.[103] However, streptavidin is 60 kDa in mass and high-surface densities may not be achieved. Interestingly, while noncovalent, the biotin-avidin conjugation approach has much in common with the covalent approaches implementing click chemistry.

Click reactions avoid many of these problems and have been successfully applied to the preparation of SAMs bearing other molecules such as DNA,[104] dyes,[105] saccharides,[106] ferrocenes,[107,108] and vegetable oils.[109] It has even been argued that application of the click approach to preparing cytochrome c oxidase models on functionalized SAMs played a key role in elucidating several mechanistic features.[110] Functionalizing SAMs via coupling is not necessarily limited to the CuAAC reaction as the inverse electron demand Diels—Alder reactions of quinones and cyclopentadiene[111] and the thiol-ene reaction[30,109] have both been used. Click chemistry can also be applied to surfaces other than SAMs. A nonexhaustive list includes: graphitic surfaces functionalized with iodide azide;[112] silica particles reacted with bromo-plasma, followed by sodium azide;[113] alkyne functionalized cotton fibers;[114] azide modified Merrifield peptide synthesis beads;[114] azide functionalized silica gel;[115] thiol[22] and alkyne[30] functionalized hydrogels; HPLC columns;[116] and ene functionalized aluminum.[109] Finally, many of the patterning techniques developed for SAM formation can be accomplished using click chemistry, including microcontact printing,[117] dip-pen lithography,[118] and photolithography.[56]

Click reactions in pharmaceuticals design and synthesis

Click chemistry was largely borne based on the difficulties faced in drug synthesis. Sharpless's chief recognition was that despite the extremely large number of potentially useful molecules available (10^{63}), molecules that were extremely difficult to synthesize were often pursued as drug targets rather than those that could be efficiently made.[1] For example, the total synthesis of pacitaxel, a potent anticancer drug, requires between 40 and 50 synthesis steps and is only an academic demonstration as the best yield is 0.4%.[119–121] Semisynthetic pathways starting with a closely related natural product, 10-deacetylbaccatin, or substrates prepared by genetically engineered organisms are viable alternatives, but still require numerous reaction steps.[122,123] As a result paclitaxel is now commercially produced by extraction from

renewable biological sources or other largely nonsynthetic processes.[121]

Consequently, click chemistry approaches to drug synthesis have emphasized compounds that are simple to make, but still achieve the sufficient structural diversity required to produce useful molecules. These approaches have taken many forms. Lexicon Pharmaceuticals produced a library of 200,000 species in 25–50 mg quantities using the click approach and automated liquid handling equipment.[124] Each library was produced in one or two steps from key building blocks prepared on multigram levels. Key click reactions used included the ring opening of epoxides and aziridines, and the 1,3-dipolar cycloaddition of azides with β-ketoesters, and 3-aminoazetidines. This approach led to the discovery of a peroxisome proliferator-activated receptor γ (PPAR-γ) agonist. Another screening approach successfully produced an inhibitor of protein tyrosine phosphatases, which may prove useful for preventing type II diabetes.[125] In this method methyl 4-azidobenzoylformate was reacted with a library of 56 alkynes using the CuAAC reaction. The most potent product showed a ninefold increase over the starting azide. An azide group was then added to this species, and it was reacted with the original 56 alkynes to produce a second generation library, and the most potent inhibitor of this generation was 400 times more active than the original azide. Other workers have tried to modify successful antiviral drugs like zanamivir in hopes of staying ahead of rapidly evolving viruses such as avian influenza virus (AIV, H5N1). Such attempts have been successful in producing species nearly as active as the starting drugs, but that are much more readily produced.[126] *In situ* library screening, which eliminates the need for purification of library compounds has been demonstrated for both the CuAAC and epoxide ring opening reactions.[127,128]

In an alternative vein, rather than relying on an iterative cycle of synthesis and screening, Lewis et al. developed a target guided synthesis for an inhibitor of the enzyme acetylcholinesterase (AChe). AChe contains two binding sites that inhibit activity of the enzyme. Inhibitors that function by occupying either one or both sites are known. To form a new bivalent inhibitor, 49 pairs of azides and alkynes similar to the known inhibitors tacrine and phenanthridinium were chosen as reactants. It was hypothesized that noncovalent interactions between the enzyme and the inhibitor fragments would produce the correct orientation and proximity to accelerate the 1,3-dipolar cycloaddition. The adduct produced would then be a more potent inhibitor, as it would occupy both sites. Such a strategy leverages the orthogonal reactivity of azides and alkynes that allow them to react in the presence of other functional groups. Furthermore, the typically sluggish kinetics of the reaction is an advantage as only the enzyme mediated reaction can occur. This study produced one inhibitor pair that was not only several times more active than its constitute parts, but is the most potent inhibitor identified to date.[129]

Other Applications of Click Chemistry

The formentioned difficulties in conjugating synthetic polymers pale in comparison to those faced when attempting to selectively functionalize or conjugate natural materials such as proteins, RNA, DNA, and viral capsids. In the preparation of natural-synthetic hybrids many functional groups are present whether the environment is *in vivo* or *in vitro*. Furthermore, considerations of toxicity and denaturation are paramount, as retaining functionality is crucial in applications such as labeling, polymer-drug hybrids, and functional materials. Despite these particularly strict requirements a number of click reactions have been successfully implemented.

Cysteine is the second least abundant amino acid and because of this feature, it is often a good choice for site-specific conjugation using the thiol–Michael reaction.[130] Typically, maleimides are used for this reaction as their electron deficiency makes them potent reactants with either naturally occurring cysteine residues or those added by recombinant techniques. Maleimides are advantageous as they are largely invisible to groups other than thiols, and such techniques have been used to functionalize viral capsids with fluorophores,[131] porphyrins,[132] pacitaxel,[133] and MRI contrast agents.[134] Similarly, photochemical initiated thiol-ene reactions have been used to pattern cysteine-functionalized peptides in hydrogels,[22] and pattern cell adhesion.[35] Reviews highlighting other important cysteine based reactions, such as disulfide formation, and applications are available.[135,136]

Another use of click reactions is in bioorthogonal labeling. These reactions are carried out in living cells or animals and provide insight into the interactions within and between cells.[137] As in bioconjugation, orthogonality to reactions with water, amines, and other commonly encountered biological functionalities is critical, although site-specificity is not. The ability to functionalize glycans and other biomolecules with azides has proven quite successful, and these azides appear to be nearly invisible to the systems they are inserted into. A number of reactants using 1,3-dipolar cycloadditions reaction have been used for labeling.[20–22,54–56,61,137,138] Such techniques have provided insight into the distribution of glycans in developing zebrafish embryos.[139] Notably, a number of Diels–Alder reactions have also been used.[137,138] Interestingly, despite the expected toxicity of the Cu(I) catalyst, live cells have been successfully labeled using the CuAAC reaction by incorporating a ligand that both accelerates the reaction and prevents the Cu(I) from detrimentally interacting with cells.[140]

Finally, while the primary focus of this Perspective has been on large and often polymeric species, the utility of click reactions in small molecule synthesis should not be overlooked. Click reactions allow easy access to species with a diverse range of functional groups, which in turn allows rapid and systematic variation of end properties. For example, the CuAAC reaction has allowed the systematic study of the effect of several functional groups on the melting temperature, thermal stability, and carbon dioxide solubility of a series of ionic liquids prepared from triazole species.[141] The CuAAC reaction has also been used to prepare functional vinyl monomers for radical polymerizations. Like acrylates and styrenics, these vinyl-triazole monomers allow access to a wide range of chemical functionality, and over 50 monofunctional monomers alone have been prepared and polymerized.[142–144] It should also be noted that click reactions can even be used to beget click reactants. For example,

sulfides prepared by the thiol-ene or thiol-halide reactions can be reduced to thiols using a variety of approaches.[145–149]

Summary and the Future

In the decade since it was initiated, the click reaction paradigm has dramatically shifted the manner in which chemical synthesis is approached. Its impact on research and development in materials science, surface science, molecular discovery, and bioengineered systems is difficult to overstate. However, its implementation in practical commercial products has been more limited, due at least in part to limited adoption of this approach within the chemical and biological engineering fields. The development of comprehensive chemical design and production processes that employ the click chemistry philosophy and reactions has the potential for significant advancements with respect to capabilities, simplicity, functionality, environmental impact, and value. With its focus on achieving the desired performance or properties in as simple and effective a manner as possible, rather than on producing a specific molecular structure, the click chemistry concept is one which has the potential for changing the way that we discover, synthesize, produce, and employ useful chemicals and materials. With the vast effects of reaction selection on the overall chemical process schematic and with the importance of reaction engineering and separations within those chemical process schemes, the chemical and biological engineering fields benefit greatly from embracing the click chemistry concept as these fields are ideally suited to take advantage of this paradigm shift in chemical synthesis and convert it into practical, highly beneficial outcomes for all.

Acknowledgments

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