Teaser Click chemistry is set to develop new potential molecules and biomaterials for treatment of various diseases, drug delivery and diagnostics, respectively.

Mechanistic applications of click chemistry for pharmaceutical drug discovery and drug delivery

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The concept of click chemistry (CC), first introduced by K.B. Sharpless, has been widely adopted for use in drug discovery, novel drug delivery systems (DDS), polymer chemistry, and material sciences. In this review, we outline novel aspects of CC related to drug discovery and drug delivery, with a brief overview of molecular mechanisms underlying each click reaction commonly used by researchers, and the main patents that paved the way for further diverse medicinal applications. We also describe recent progress in drug discovery and polymeric and carbon material-based drug delivery for potential pharmaceutical applications and advancements based on the CC approach, and discuss some intrinsic limitations of this popular conjugation reaction. The use of CC is likely to significantly advance drug discovery and bioconjugation development.

Introduction

For a decade, CC has provided researchers with the ability to synthesize novel materials. CC was developed to help meet the demand of modern-day chemistry research, mainly drug discovery. It uses pairs of functional groups that rapidly and selectively react (click reaction) with each other in ecofriendly, mild, aqueous conditions as well as in organic solvents. The selection of each click reaction is based on its selectivity, reactivity, biocompatibility, and stability. The goal of this new synthetic technique was to generate powerful, selective, and modular 'blocks' that can be applied reliably in both small- and large-scale production settings. Since its initial conception, the application of CC in various fields has increased drastically [1,2].

Sharpless *et al.* [3] provided a stringent set of criteria for the processes or reactions to fit into the context of CC: 'modular reaction, wide in scope, produce high chemical yields, provide only inoffensive byproducts that could be simply separated by non-chromatographic methods (like crystallization or distillation) or no need of re-purification, with readily available starting materials, should occur in mild reaction condition with benign solvent (mainly water), are stereospecific (although not necessarily enantioselective) and product must remain stable under physiological conditions.' These characteristics are achieved via a high thermodynamic force,

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usually >20 kcal mol^{-1}. Each reaction proceeds at a fast rate to completion and is highly selective (also referred as 'spring loaded').

Among the reactions that satisfy the 'click criteria', the Huisgen Cycloaddition has remained the premier example of a click reaction because of its synthesis simplicity, kinetic stability, and tolerance for a variety of functional groups and reaction conditions. Here, we discuss the application of CC in the context of drug discovery and DDS, as well as highlighting the basic mechanisms of each reaction discussed [4]. We also highlight applications of CC with key recent examples in the context of drug discovery and drug delivery (especially polymeric and carbon-based materials).

Elements of click chemistry

Copper-catalyzed alkyne-azide click chemistry

An alkyne–azide reaction occurs between an organic azide and a terminal alkyne in the presence of copper [Cu(I)] to form a 1,4 disubstituted 1,2,3 triazole (Fig. 1), which is in contrast to the noncatalyzed reaction, which proceeds at higher temperatures to form a mixture of 1,4- and 1,5-triazole regioisomers [5]. The rate of this reaction is $>10^7$ times that of the conventional reaction, which means that it proceeds efficiently at room temperature. However, the mechanism of the alkyne–azide click reaction mediated by copper (CuAAC) remains difficult to establish owing to the involvement of several reaction intermediates and despite the widespread use of copper-catalyzed cycloaddition reactions. Initially, there is formation of several Cu-acetylide intermediates, depending on the reaction conditions utilized [6]. In the next step, nitrogen of azide displaces one Cu of the Cu-acetylide intermediate and coordinates the organic azide; the nucleophilic carbon on the copper acetylide then reacts with the electrophilic nitrogen [N (3)] on the azide. In the final reaction step, ring contraction of the metallocyte occurs, followed by dissociation upon protonation of the product to reproduce the catalyst [5,7]. Unlike CuAAC, the ruthenium-catalyzed 1,3-dipolar azide-alkyne cycloaddition (RuAAC) results in 1,5-triazole, and both terminal and internal alkynes participate in the reaction.

Hein and Fokin *et al.* [8] developed an alternative to the need for oxygen-free conditions for the use of Cu(I) by using Cu(II) salts, such as copper (II) sulfate pentahydrate or copper(II) acetate, in the presence of mild reductants, such as sodium ascorbate, hydroquinone, and Tris (carboxyethyl)phosphine [9]. Reducing agents not only reduce Cu(II) to active Cu(I) for a reaction, but also reduce any dioxygen present and, thus, decrease any oxidative byproducts. One reason for the interest of material scientists in this cycloaddition reaction is that azide and terminal alkynes are easy to install, and are stable under standard conditions [10]. Their tolerance for oxygen, water, common organic synthetic conditions, biological molecules, a large number of solvents and pHs, and reaction conditions of living systems, such as the reducing atmosphere inside cells, hydrolysis, and so on, make them practical substrates for drug discovery and drug delivery [5]. However, the toxicity of Cu(I) has limited the use of this particular reaction for *in vivo* applications, as discussed below.

Bioorthogonal copper-free click chemistry

The potential toxicity of Cu(I) can be overcome by improving the rate of the Huisgen alkyne-azide cycloaddition without using copper as a catalyst. Bertozzi *et al.* [11] documented an approach using a family of strained alkynes, 'cyclooctynes', to allow strain-promoted cycloaddition of alkynes and azide (SPAAC) and Staudinger ligation. The mechanism of SPAAC is based on the introduction of ring strain into the alkyne molecule, which is then released in a transition state during the reaction (Fig. 2). The angle strain of the triple bond, along with the ring strain present in cyclooctyne, contributes to a significantly faster reaction rate. In the ligation reaction, the intermediate aza-ylide undergoes an intramolecular reaction with an ester, forming an amide bond [12,13].

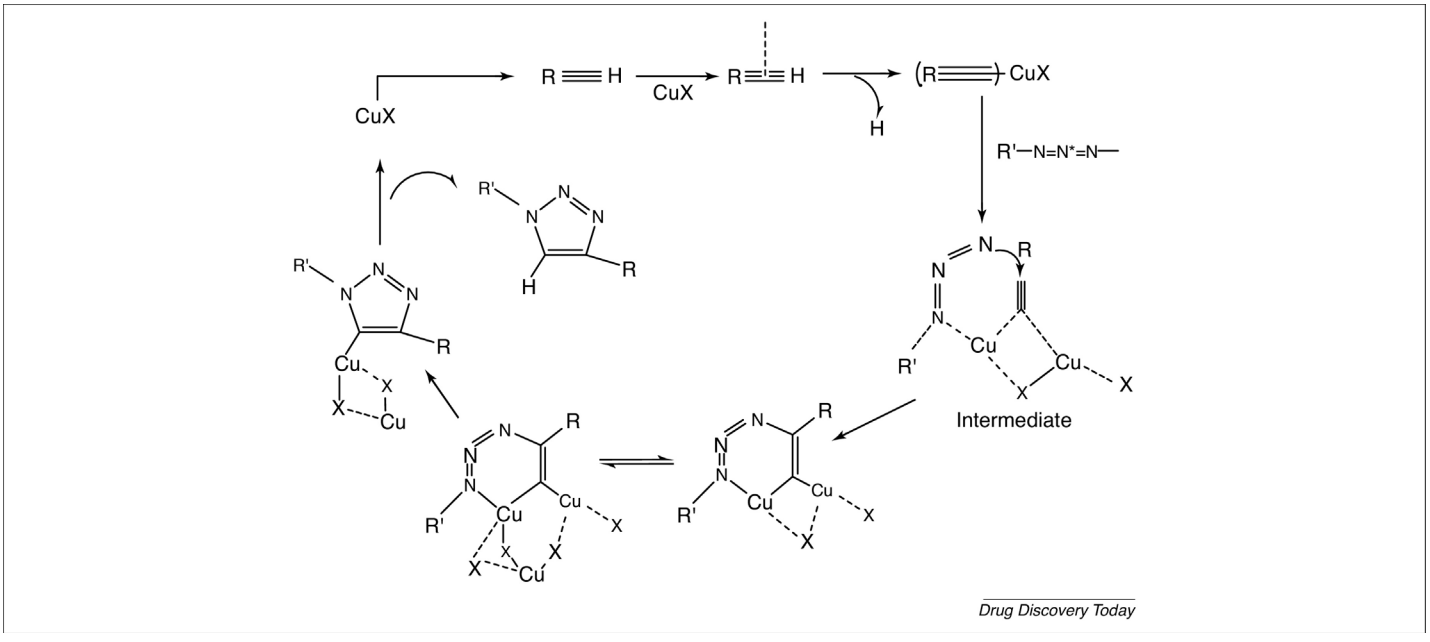

FIGURE 1

Mechanism of copper (Cu)-mediated azide-alkyne click reaction.

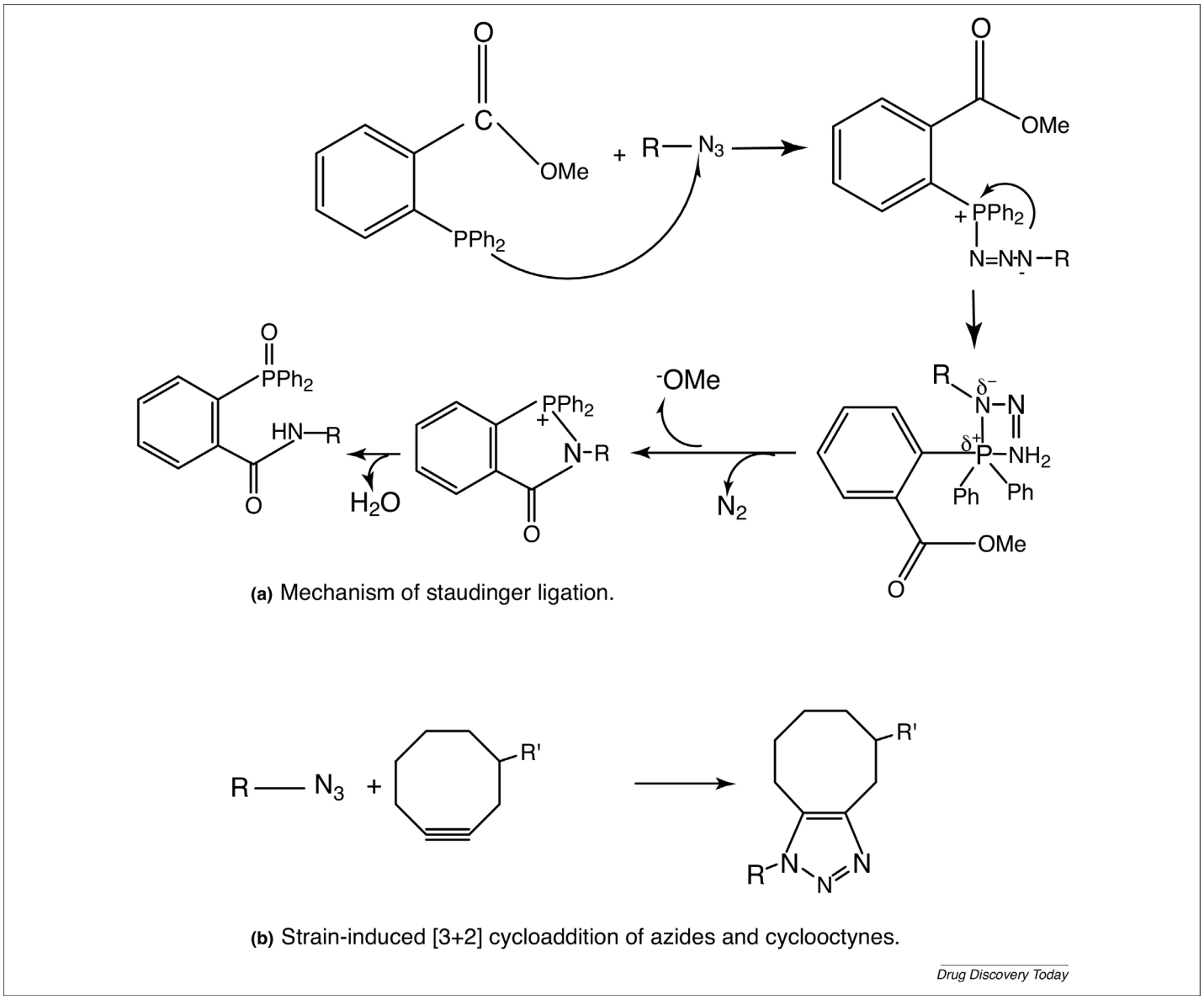

FIGURE 2

Examples of cycloaddition reactions. **(a)** Mechanism of Staudinger ligation. (b) Strain-induced [3 + 2] cycloaddition of azides and cyclooctynes.

Through a series of studies, these authors demonstrated that the reaction rate could be tuned by the substituent on the cyclooctyne group. Being an alkyne and azide cycloaddition reaction, it can lead to a mixture of stereoisomers. Several other related systems have been investigated by using dibenzocyclooctynes or oxanorbornadienes [14,15]. The second-order rate constant for the cycloaddition reaction between a cyclooctyne derivative and a benzyl azide in aqueous CD_3CN is 0.0012 M^{-1} s^{-1}, which is lower than that of a typical Staudinger reaction (0.0025 M^{-1} s^{-1}) [16]. Nevertheless, this version of the strain-promoted cycloaddition ligation reaction has been successfully used to label glycoproteins both *in vitro* and *in vivo* on cellular surfaces, without noticeable cytotoxicity [17]. McKay *et al.* reported another strain-promoted cycloaddition of alkynes and nitrones (SPANC), which represents another useful tool for chemical biology because of their fast reaction kinetics (k_2 up to 60 M^{-1} S^{-1}), biological stability and compatibility, and flexibility of substitution of groups on both the carbon and nitrogen atoms of the nitrone dipole [18].

Diels–Alder cycloaddition

Sharpless *et al.* identified the reaction between a diene and dienophile as a 'click reaction'; this reaction is also known as Diels–Alder [4 + 2] (DA) cycloaddition (Fig. 3A). It results in highly selective transformation, and proceeds faster in water than in organic solvents owing to hydrophobic effects [3,19]. In addition to being high-yielding and allowing the control of regio- and stereoformation under reagent-free conditions, it also produces thermoreversible products and, thus, can be used for diverse applications. The temperature used to form cyclized products and noncyclized starting materials can control the Diels–Alder and the reverse retro-Diels–Alder reaction.

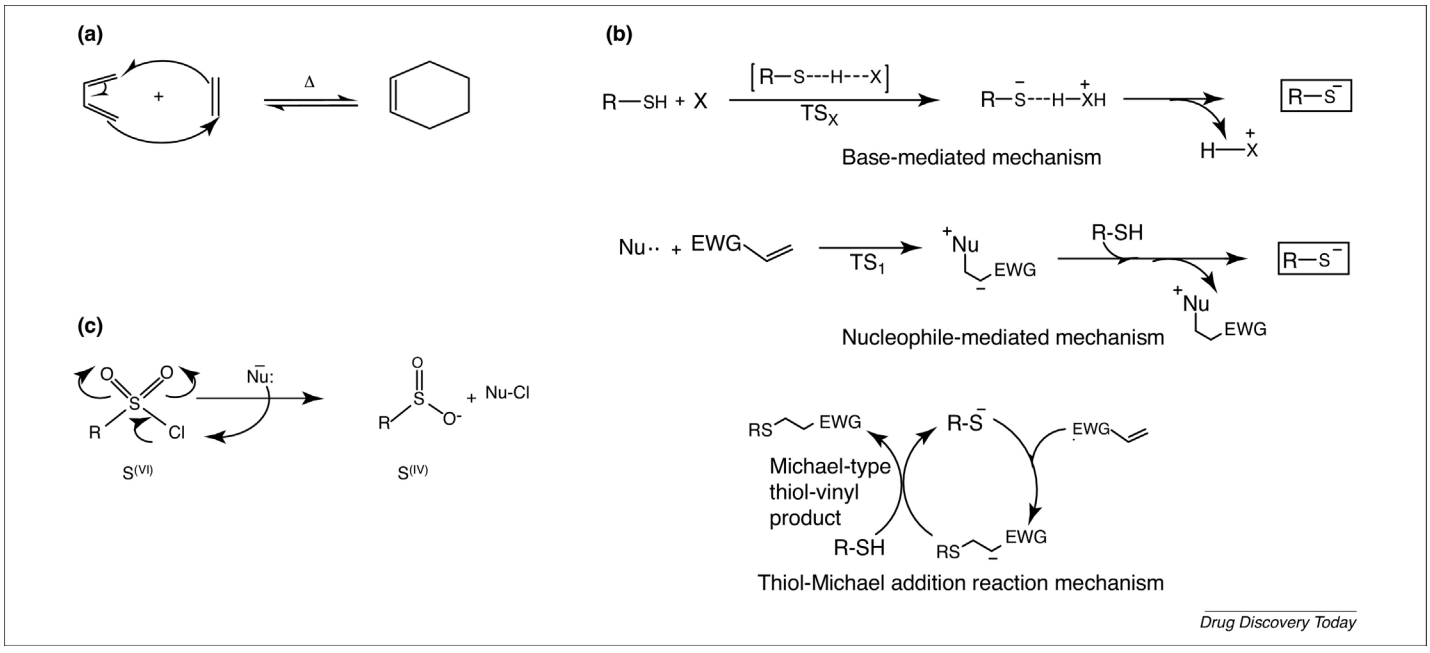

FIGURE 3

(a) General mechanism of Diels–Alder and retro Diels–Alder reactions. **(b)** Basic mechanism of thiol-ene reaction. **(c)** Basic reaction mechanism of Sulfur (VI) fluoride exchange.

The variety of diene and dienophiles that can be used enables the tuning of the thermoreversibility of the reaction; for example, furan and maleimide undergo reversion at approximately 110 °C, whereas anthracene and maleimide products a temperature above 200 °C. Milder reaction conditions have also been demonstrated recently for the coupling of polymers by cyclopentadiene and activated RAFT end groups [20]. This thermoreversibility of the Diels–Alder reaction has led to fabrication of materials with 'self-healing' properties, including gels and polymeric resins [21]. Given its fast kinetics, the ligation reaction of tetrazine can be particularly useful in situations in which rapid reactions are required for tracking dynamic biological events, as well as for the labeling of less-abundant biomolecules.

Thiol-ene click chemistry

Thiol-ene reactions have also received significant attention in material science and are based on thiol and alkene groups and are catalyzed using a radical source. Although thiol-ene chemistry has been commonly considered a click reaction, owing to the high reactivity of the thiol group to a diverse range of substrates, it falls short of click behavior because it is not truly orthogonal. In addition, what makes the reaction more reactive and efficient also makes it more susceptible to simultaneous reactions owing to the high reactivity of thiols. Therefore, reaction conditions must be chosen carefully to control the possibility of simultaneous reactions in a given chemical, biological, physical, and electrical environment.

Owing to the high reactivity of thiols, thiol-ene click reactions have advantages over other click reactions owing to their fast kinetics; thus, high conversion can be achieved in approximately 1–10 s. There are two basic types of chemistry that encompass thiol-click chemistry: the reaction is proceeded first by radical chain reactions and, second, by nucleophilic reactions (Fig. 3B) [22]. Series of thiol-click chemistries have been identified, including thiol-ene [23,24], thiol-maleimide [25], thiol-yne [26], thiol-epoxy [27], thiol-isocyanate [28], thiol-para-fluorostyrene [29], and thiohalide [30] reactions. After Schlaad *et al.* first introduced the radical-mediated thiol-ene reaction as a potential click reaction, many thermal, redox, and photochemical methods have been used to generate radicals to initiate the thiol-ene reaction. In particular, photo-initiation has garnered much attention because it allows both spatial and temporal control over the progress of the reaction. The mechanisms under such conditions comprise three steps that are similar to radical polymerization: initiation, propagation and termination. The reaction proceeds at equivalent rates in an ideal thiol-ene environment and yields homogeneous and uniform products, a feature that is unusual for typical photochemical reactions [31].

Sulfur (VI) fluoride exchange

Recently, Sharpless *et al.* [32] reviewed the basic principles of sulfur (VI) fluoride reactivity to reintroduce this class of compound to their maximum potential. Sulfur (VI) fluoride exchange (SuFEx) has the potential to aid the discovery of novel diagnostics, drugs, and other therapeutics, even those that are reactive within the human body. Similar to other click reactions, many essential features of sulfur (VI) fluoride reactivity were discovered some time ago; however, interest in this area has faded over the years, despite the rich literature relating to RSO_2F and $ArSO_2F$ compounds driven by the accessibility and dye properties of sulfur (VI) derivatives of benzene, naphthalenes, and anthracenes available from coal tar [33].

The basic chemistry of SuFEx involves interplay between the unique hydrogen-bonding requirements of the fluoride ion and the thermodynamic and kinetic properties of fluoride bonds to sulfur (VI) and silicon centers (Fig. 3C). As illustrated by Sharpless

and his team, understanding of the distinctive stability–reactivity pattern of sulfonyl fluorides depends mainly on five factors: resistance to reduction; thermodynamic stability; exclusive reaction at sulfur; special nature of the fluoride–proton interaction; and closely related functional groups. Thus, there are compelling reasons to pay attention to the SO_2F group, given that click reactions find their most facile applications in biological, medicinal, and materials chemistry [32,34].

Click chemistry and drug discovery

In recent years, the arduous process of lead discovery and optimization has been aided by combinatorial chemistry, which is dependent on the reliability of individual reactions used to construct a new network of chemical bonds. Click chemistry has greatly facilitated the overall drug discovery process by providing easy access for the synthesis of building blocks for new molecular entities (NMEs). Although it has by no means replaced existing methods for drug discovery, it has complemented and extended them by aiding lead discovery and optimization (Table 1). The use of CC aids structure-based design and improves combinatorial chemistry techniques and, through the choice of appropriate building blocks, it can provide derivatives or mimic traditional pharmacophores, natural products, and drugs [3,35]. Given the large dipole movement, triazole linkage is known as an aggressive pharmacophore and binds strongly to various proteins in diverse ways [36]. In an explorative study, Massarotti *et al*. analyzed the X-ray crystal structure of complexes of 1,2,3-triazoles and DNA or proteins (protein–triazole) to understand the pharmacophoric role of the triazole ring. In addition, the role of triazole was studied to understand its ability to inhibit cytochromes, its metabolic stability, and the overall aqueous solubility of the compounds [37]. The authors concluded that triazole pharmacophores can: (i) participate in C-H hydrogen-bonding interactions; (ii) act as intercalating agents via π-π stacking interactions; (iii) act as hydrogen-bond donors with N2 and N3 atoms; (iv) be metabolically inert (1,4-triazoles) or active (1,5-triazoles); (v) substitute an amide without changing the binding pose; (vi) decrease aqueous solubility; (vii) have negligible inhibitory effects against CYP enzymes, which are more pronounced for 1,4-triazoles than for 1,5-triazoles; and (viii) produce *N*-oxide metabolites 1,5-triazoles, which can be reactive intermediates.

CC-based drug discovery can be classified into three types: (i) high-throughput screening (HTS); (ii) fragment-based drug discovery (FBDD); and (iii) dynamic template-assisted strategies in FBDD.

High-throughput screening

HTS involves the screening of large chemical libraries for activity against biological targets via the use of computerization, miniaturized assays, and large-scale data analysis, and is a well-established process for lead drug discovery in the pharmaceutical industry, as well as academia [38]. Many of the biochemical targets in recent drug discovery efforts are in families of enzymes, such as kinases, proteases, phosphodiesterases, phosphatases, transferases, and oxidoreductases. Despite the large number of genes and expressed proteins, molecular targets with drugs approved are still limited (324 targets), indicating that some targets remain intractable for modulation by low-molecular-weight compounds (i.e., small molecules), whereas others remain unapproachable by current technologies and, thus, not only are a great challenge, but also could have tremendous potential for drug discovery [39,40]. CC combined with high-throughput enzyme assay technologies, such as microarrays, have transformed lead finding and lead optimization in drug discovery. Small-molecule libraries synthesized by CC have been successfully used in generating unique inhibitors and activity-guided fingerprinting of important enzymes, which could lead to the identification and characterization of new subclasses of enzymes [41].

Fragment-based drug discovery

FBDD is based on the fact that the free binding energy of a protein ligand results from its molecular components. First, a small-molecule fragment that binds to the protein scaffold of interest is identified, after which starting fragments that bind to the protein scaffold are modified for greater binding affinity, which is further optimized to a lead structure [42]. Although many enzymes have multiple binding pockets, conventional inhibitor development generally focuses on the active site. However, in many cases, secondary or allosteric binding sites also have selectivity as well

TABLE 1

Review of click chemistry patents and their impact on drug discovery and chemical biology

Patent number	Click reaction type	Applications/inhibitor of target	Refs
US7375234	CUAAC	Stepwise Huisgen cycloaddition reaction catalyzed by copper(I) for regioselective ligation of azides and terminal alkynes to form triazoles	[35]
WO2011003365	CUAAC, RUAAC	Histone deacetylase inhibitors with branched structures	[36]
US2006110782	SPAAC	Cycloalkynes and heterocycloalkynes to label azide-containing biomolecules without Cu catalysts *in vitro* and *in vivo*	[37]
US8236949	Diels–Alder ligation	Reaction between 1,2,4,5-tetrazines and dienophiles in organic or aqueous media for preparation of various novel *trans*-cyclo-octenes and cyclopropenes	[38]
US20130323169	Thiol-ene	Ligation of drug molecule to free thiol group within a cellular recognition ligand	[39]
WO2011018613	Thiol-maleimide	Reaction using maleimides with stronger electrophilic leaving groups at 3- and/or 4-positions	[40]
EP2534138	CUAAC	Guanidino and carboxylic groups bearing compounds with 1,2,3-triazole ring as peptidomimetic integrin inhibitors	[41]
US20140018318	CUAAC	*N*1- and *N*2- carbamoyl-1,2,3-triazole-containing serine hydrolase inhibitors	[42]
US20150045393	CUAAC	Epoxide containing novel cysteine and protease and calpain inhibitors	[43]
WO2014089078	Sulfo-click chemistry	Generation of polymers containing sulfur (VI) $–SO_2–$ connectors via condensation of fluoro- and silyl-substituted monomers in presence of base catalyst	[44]
WO2015021134	Sulfo-click chemistry	Bcl-2 family proteins binding molecules identified by kinetic target-guided synthesis	[45]

as potency. In this scenario, owing to its highly modular and efficient reactive nature, CC is one of the most practical methods for the development of fragment-based inhibitors. The assembled products could be screened directly for inhibition without any need for purification because of the efficiency and water-compatible nature of the click reaction [43].

Dynamic template-assisted strategies in fragment-based drug discovery

To address the problem of the identification of low-affinity fragments and biologically efficient active linkages associated with FBDD, dynamic template-assisted strategies have been proposed by researchers. In all such approaches, there is a chemical reaction, reversible or irreversible/enzymatic or nonenzymatic, which is exploited for the best fragment combination [44]. In click reactions, azide and alkyne groups are combined onto fragments that interact favorably with the enzyme, and are selected to partially interact with the enzymatic binding site. Thus, cycloaddition between alkyne and azide connects the fragments to form the final inhibitor. This results in a gene/protein-specific pharmaceutical composition that contains the encoded combination of two or more bioorthogonally fragmented prodrugs that can be administered simultaneously or sequentially for *in vivo* self-assembly of the drug via target-driven mechanism. As a result, low-molecular-weight drugs with highly desirable pharmacological properties can be achieved and problems related to plasma level biodistribution can be minimized for clinical application [45,46]. Thus, with the help of CC, pharmaceutical compositions of closely related but fragmented prodrugs can be designed to interact specifically with molecular targets, such as protein or genes, resulting in personalized therapeutics.

Application of click chemistry for enzyme inhibitor synthesis

Enzymes are drug targets for many diseases, including cancer, Alzheimer's disease, diabetes, TB, and many currently incurable diseases and, thus, could have a significant role in curing such diseases [47]. With an increasing number of patients with such incurable diseases, CC is impacting the synthesis and development of compounds in a faster and more efficient manner, providing novel approaches for the screening of compound libraries. The highly modular and efficient reaction properties of CC have enabled synthetic chemists to build libraries of different classes of compound through fragment-based enzyme inhibitor development. We direct readers interested in the applications of CC for the development of enzyme inhibitors to a useful article by Thirumurugan and his colleagues for further detailed descriptions [48]. As a result of space constraints, we show only representative structures for each enzyme inhibitor (Figs. 4 and 6) and discuss only the key, most recently studies published.

Protein kinases (PKs) are a family of enzymes involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid

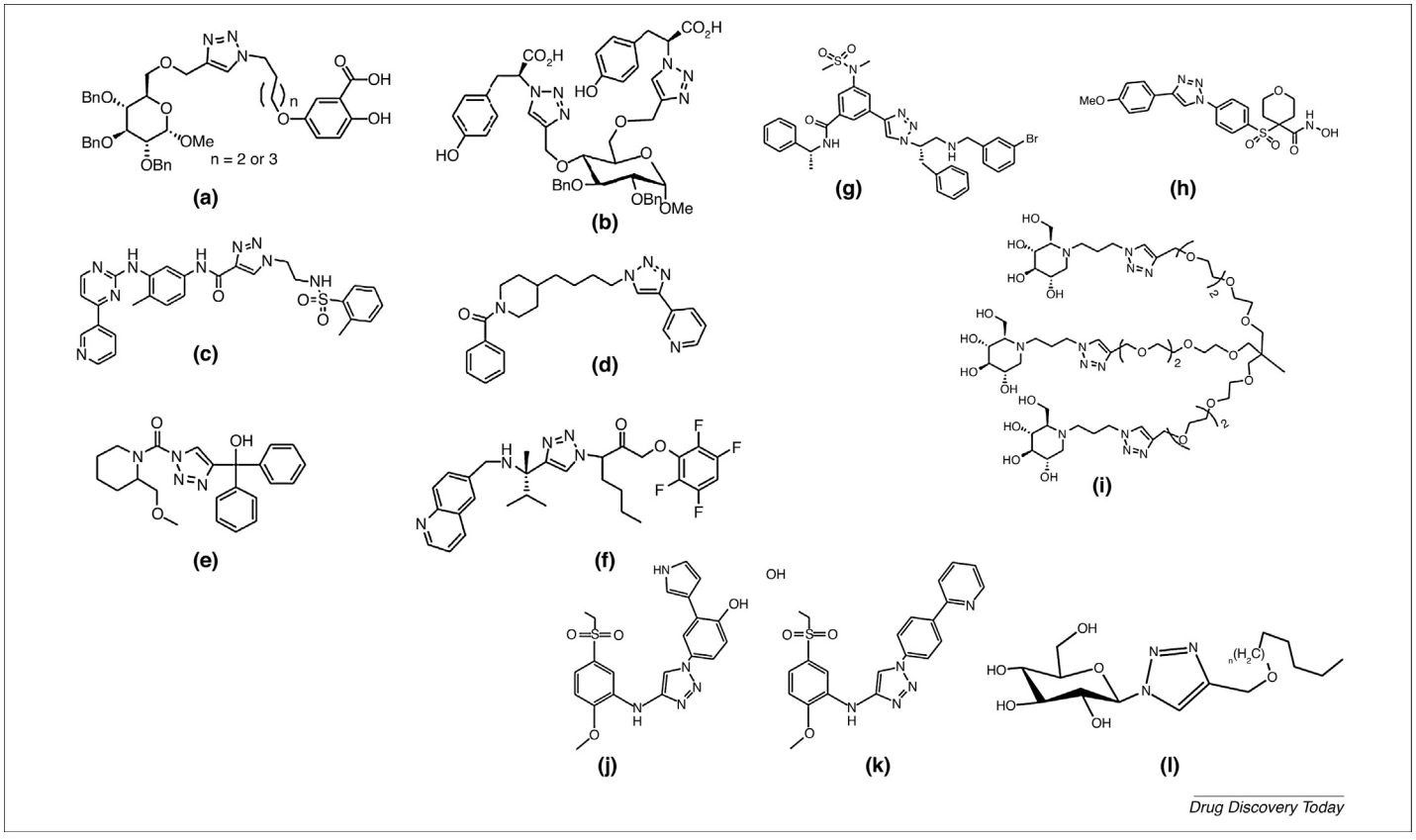

FIGURE 4

Examples of chemical structures of various inhibitors synthesized by click chemistry: **(a,b)** protein tyrosine phosphatase inhibitors**; (c)** protein kinase inhibitor; **(d)** transferase inhibitors; **(e)** serine hydrolase inhibitor; **(f)** cysteine protease inhibitors**; (g)** aspartic protease inhibitors; **(h)** matrix metalloproteinase inhibitors; **(i)** glycosidase inhibitor; **(j,k)** VEGFR2 kinase inhibitors; **(l)** triazolyl glycolipid for MRSA (PBP2a inhibitors). Reprinted, with permission, from [48] (a–i).

residues on these proteins. Many diseases, such as cancer and diabetes, are characterized by anomalies in a kinase or its expression level. Given their significant role in several signal transduction processes, drug discovery efforts have targeted PKs as primary drug targets [49]. Recently, the improved water solubility and decreased lipophilicity of kinase inhibitors was achieved by the addition of (1H-1,2,3,-triazol-1-yl)acetate or phosphonate to a basic 4-(4′-fluorophenyl)imidazole by CuAAC [50]. The kinase selectivity was influenced by the position of a carboxylic acid or tetrazole at imidazole C-2 and a heteroaryl group at imidazole C-5. The inhibition of p38α mitogen-activated protien kinase (MAPK), casein kinase 1δ (CK1δ) and Janus kinase 2 (JAK2) was achieved with IC_{50} in the nM range. A library of seven 1,2,3-triazole compounds was prepared from the oxazole vascular endothelial growth factor receptor 2 (VEGFR2) tyrosine kinase (TK) inhibitor complex 1Y6A, which contains an *N*-aryl-5-aryloxazol-2-amine (AAZ) [51]. A radiometric VEGFR2 kinase assay was used to estimate the inhibition concentration (IC_{50}) values of each compound. Out of the seven compounds, three had IC_{50} values of 6 MM, 40.1 MM, and 42 MM. To increase the anticancer efficacy of curcumin, a 24-monocarbonyl curcumin analog with a 1,2,3-triazole ring was synthesized by CuAAC. Its anticancer activity was confirmed on prostate cancer cells (PC-3 and DU-145 cells) and breast cancer cells (MCF-7, MDA-MB-231 and 4T1 cells) with a IC_{50} of 6–10 MM [52].

Protease enzymes catalyze the hydrolysis of peptide bonds and are currently classified into six broad classes: cysteine, aspartic, serine, threonine, glutamic acid, and metalloproteases. They are involved in multiple physiological functions, including digestion, blood coagulation, cell differentiation and growth, protein turnover, wound healing, cell signaling, immune response, and apoptosis. Imbalanced or unregulated activity of proteases leads to several pathological conditions, such as arthritis, stroke, Alzheimer's disease, aging, cancer, multiple sclerosis, and viral infections [53]. A new class of sulfoxythiocarbamate small-molecule inhibitors of heat shock protein 90 (HSP90) was prepared that, as a result of their sulfhydryl reactivity, targeted HSP90, destabilized oncoproteins, and inhibited cell proliferation. Mild electrophilic sulfoxythiocarbamate alkyne (STCA) selectively targeted cysteine residues of HSP90 with IC_{50} of 10 MM to form a thiocarbamate adduct [54]. Click reactions using the biotin azide and protease-mass spectrometric method was used to identify which cysteines were modified by STCA.

Serine hydrolases are one of the most prevalent and diverse enzyme classes and include lipases, amidases, esterases, thioesterases, proteases, and peptidases. They represents approximately 1% of all proteins in mammals and have vital roles in many pathophysiological conditions, including blood clotting, digestion, inflammation, the nervous system, and cancer [55]. Cravatt and colleagues developed triazole urea derivatives with ultrapotent *in vivo* inhibition of serine hydrolases. Recently, the same team used CC-based protein profiling to identify multiple classes of carbamate, including *O*-aryl, *O*-hexafluoroisopropyl (HFIP), and *O*-*N*-hydroxysuccinimidyl (NHS) carbamates, which react selectively with serine hydrolases across entire mouse tissue proteomes *in vivo*. The findings suggested a potential way to accommodate diverse structural modifications to produce serine hydrolases inhibitors with exceptional potency and selectivity [56,57].

Glycosidases catalyze the hydrolysis of glycosidic linkages and degrade diverse classes of biopolymers, including oligosaccharides and glycoconjugates [58]. Gouin and colleagues synthesized a set of 14 iminosugars and evaluated their ability to inhibit commercially available glycosidases. *In vitro* assays determined that these compounds inhibited those enzymes at MM concentrations, with aromatic aglycones displaying the highest inhibitory potency [59]. Glycolipids have been identified as potential drug candidates with anticancer, antiviral, and antimicrobial properties, and represent a pivotal class of cell surface components that participate in diverse molecular events *in vivo*. To increase the susceptibility of drug-resistant bacteria to β-lactam antibiotics, the azidos β-D-glucoside, β-D-galactoside, and β-D-mannoside were coupled with alkynyl lipids with increasing chain length by CuAAC to form triazolyl glycolipids [60]. The clicked derivatives showed the ability to disarm methicillin-resistant *Staphylococcus aureus* (MRSA) synergistically with β-lactam antibiotics that are currently in clinic use.

Despite growing applications of CuAAC for the synthesis of biological compounds, the usage of copper and reducing agents is still a matter of debate because of reports of copper radicals destroying or degrading peptides and/or protein complexes during typical CuAAC reactions [61,62]. To avoid these drawbacks, CuAAC cell compatibility has been improved by the use of either water-soluble ligands, such as bis-(L-histidine) or accelerated Cu(I) ligands, which allow low Cu(I) loading during a catalytic reaction [63]. Furthermore, copper wires have also been shown to catalyze a reaction without the need for an additional ligand or reducing agents [64,65].

In situ click chemistry

Over the past decade, novel strategies for the discovery of lead compounds based on CC have been investigated where the target moiety is actively involved in the synthesis of its own inhibitory compound. These fragment-based strategies, also called target-guided synthesis (TGS), are a subset of the conventional combinatorial approach, in which the biological target (either protein or DNA) is actively involved in the choice of ligands assembled from a pool of smaller fragments (Fig. 5). The slow reaction between inactivated alkynes and azides is vital in the application of this reaction to TGS. Sharpless and Finn used the alkyne-azide click reaction (AAC) in a template-directed formation of an enzyme inhibitor, the enzyme taking the conceptual role of the cucurbituril host. The use of AAC reactions in TGS has been termed 'CC *in situ*' [66]. The properties of this irreversible reaction, which make it well suited for lead discovery, include: (i) extremely thermodynamically favorable reaction; (ii) reaction does not involve any third-party participants, such as catalysts or other reagents; and (iii) bioorthogonality (i.e., both azides and alkyne are inert *in vivo* and can survive in biological conditions) [67].

The enzyme acetylcholinesterase (AChE) was selected as the first target for *in situ* CC because it is a vital component of neurological function, specifically in Alzheimer's disease, and for the structure of its active site. Sharpless and colleagues applied the *in situ* click approach to AChE to synthesize 98 potential inhibitors from 16 building blocks [68]. It was predicted that a triazole link made between two of these small-molecule inhibitors appropriately decorated with complementary alkyne and azide functionalities could form *in situ*, to produce a new bivalent inhibitor of the

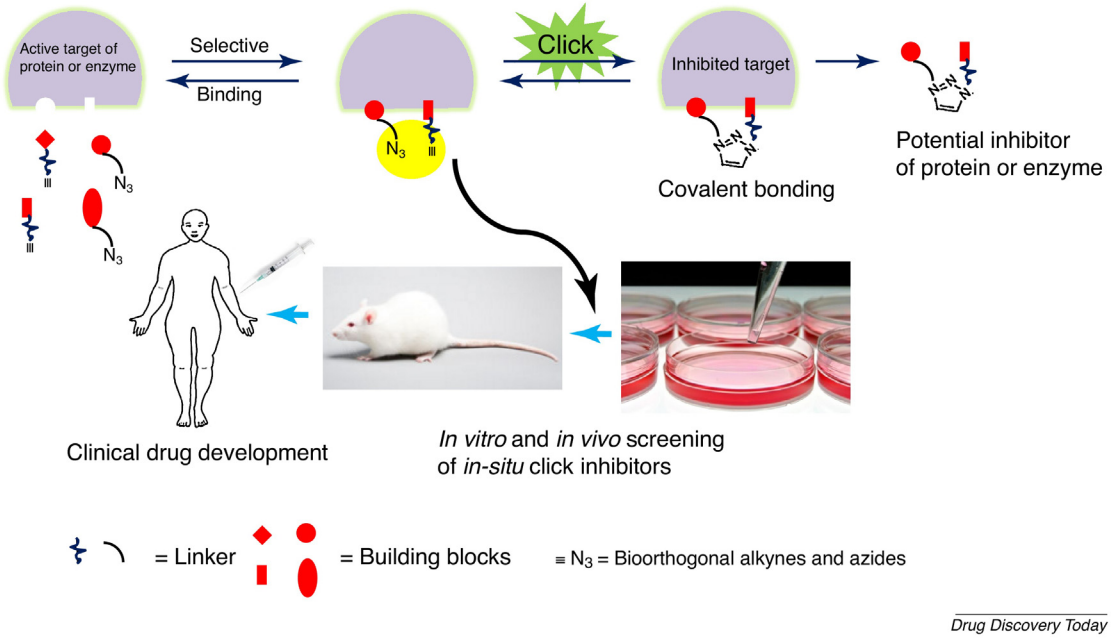

FIGURE 5

Schematic illustration of *in situ* click chemistry used to develop enzyme or protein inhibitors and drug development.

enzyme. Recently, Fokin *et al.* combined CuAAC and *in situ* CC for the synthesis of a highly potent and selective ligand for *Lymnaea stagnalis* acetylcholine binding protein (AChBPs). Their study showed that an *in situ* click reaction occurred at the subunit interfaces of the oligomeric protein, and, thus, this approach can be used as a tool for identifying novel candidates for nicotinic ACh receptor ligands [69]. Recently, automated docking using the program AutoDock with protein flexibility was used to design potent noncovalent inhibitors of AChE with a K_d of ~100 fM. This approach also enabled additional conformational flexibility in selected amino acid side chains of the target protein [70].

Huprine derivatives are considered the best AChE active-site ligands reported to date (270-fold more potent than tacrine) and are readily functionalized at positions 9 or 12 [71]. Recently, Renard *et al.* further reported the use of human AChE as an enzyme for the synthesis of new potent heterodimeric huprine-based inhibitors. Two huprine derivatives bearing a 2- or 4-carbon alkyne azide chain [(±)-9-HUPZm] were incubated in the presence of alkyne containing phenyltetrahydroisoquinoline (PIQ-An) derivatives of varying chain lengths. From the possible ten regioisomeric heterodimers, only the (±)-9-HUPZ4PIQ-A2 (anti-) and the regioisomeric mixture of (±)-9-HUZP4PIQ-A3 (syn and anti-) were observed after 2 and 7 days of incubation with m-AChE (IC_{50} = 0.4 nM and 1.2 nM, respectively) [72]. Lately, various novel acridones linked to 1,2,3-triazole derivatives were synthesized from the reaction of 2-bromobenzoic acid with aniline derivatives followed by a cyclization reaction to give acridone derivatives, which were later reacted with propargyl bromide followed by CuAAC and evaluated against AChE and butyrylcholinesterase (BChE) [73]. Of 14 compounds, only those having a methoxy and chlorine group as their side chains showed potent inhibitory activity (IC_{50} = 7.31 MM).

Aspartic proteases, which are found in plants, fungi, vertebrates, and HIV retroviruses, have a causative role in hypertension, AIDS, malaria, and Alzheimer's disease. Combining fragment-based linking and protein-templated CC (PTCC) for the first time, efficient triazole inhibitors for the aspartic protease endothiapepsin were prepared with an optimal IC_{50} value of 43 MM [74]. The advantage of this approach was the small amount of catalytic protein required to initiate and accelerate triazole formation from a suitably large library.

Histone deacetylases (HDAC) are a class of enzymes that have a crucial role in various biological processes, largely through their repressive influence on transcription and, thus, their inhibitors are attractive drug candidates [75]. Using *in situ* CC, Miyata *et al.* designed a library of HDAC8 inhibitors using CC containing a zinc-binding group (ZBG) that can attach to the central zinc ion of the enzyme. A potent inhibitor with a IC_{50} of 0.070 MM was identified, and its potency was revealed by the selective acetylation of cohesin in cells and growth inhibitory effects on T cell lymphoma and neuroblastoma cells (GI_{50} = 3–80 MM) [76].

Protein–protein interactions (PPIs) have an important role in most biological processes and, thus, they are also attractive targets for the development of novel therapeutics for the various diseases. Despite the hurdles to developing small molecules as PPI inhibitors, recent progress has seen some success in clinical trials [77,78]. Using a kinetic target-guided synthesis (KTGS) and sulfo CC approach, Manetsch *et al.* prepared a library of nine thio acids and nine sulfonyl azides leading to 81 potential acylsulfonamides (PPIMs), out of which the target protein assembled four PPIMs with K_i values in the nanomolar range [79]. Ohkanda *et al.* identified *in situ* PPI inhibitors by studying the template effect of the 14-3-3 protein on the chemical ligation of fusicoccin (FC) compounds containing an epoxide group and the pentapeptide QSYDC (H-Gln-Ser-Tyr-Asp-Cys-OH) [80]. 14-3-3 proteins are dimeric proteins with critical roles in the regulation of serine/threonine kinase-dependent signaling pathways via phosphorylation-dependent binding to a large number of ligand proteins and

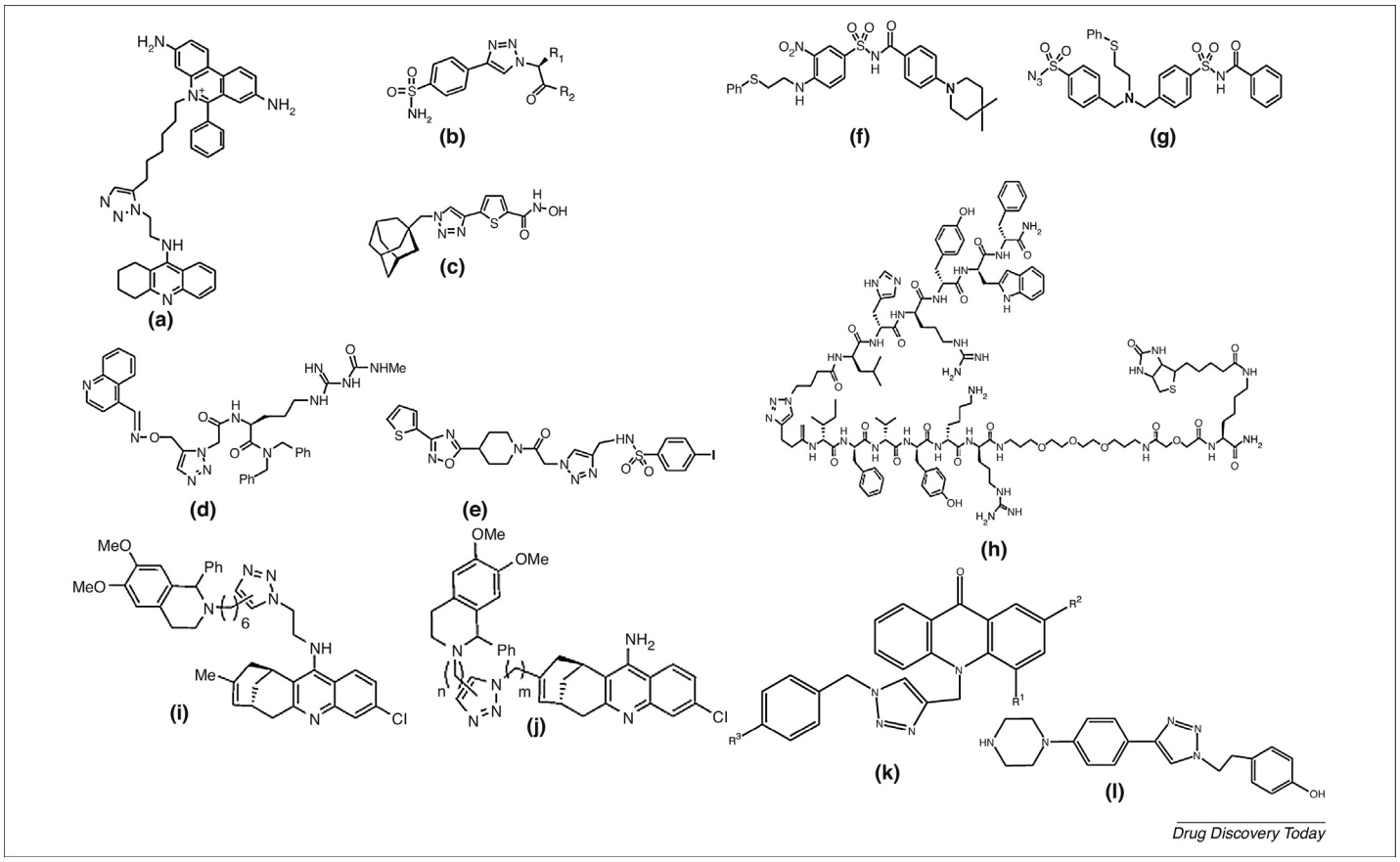

FIGURE 6

Examples of chemical structures of various inhibitors synthesized by *in situ* click chemistry: **(a,i,j)** acetylcholinesterase inhibitors; **(b)** carbonic anhydrase inhibitors; **(c)** histone deacetylase inhibitors; **(d)** chitinase inhibitors; **(e)** ethionamide repressor inhibitor; **(f,g)** protein–protein interaction modulators; **(h)** antibody-like protein-capture agents; **(k)** butyrylcholinesterase inhibitors; **(l)** aspartic protease endothiapepsin inhibitor. Reprinted with permission, from [48,72] (a–j).

have been implicated in cancer and neurological diseases. The fungal phytotoxin fusicoccin binds to a hydrophobic cavity of the phosphopeptide-binding pocket of plant 14-3-3 proteins and forms a stable ternary complex with phosphopeptide QSYpTV (H-Gln-Ser-Tyr-phosphoThr-Val-OH). The authors designed a library of various FC derivatives with different spacer arms containing epoxide ring and thiol-containing pentapeptides (QSYpTV and QSYDC) as the reactive groups and incubated them in the presence of 14-3-3 protein to form the corresponding conjugate product by reactions between the epoxide ring and thiol groups. The data suggested that the conjugation yield was higher (up to 200%) with longer space arms than with shorter spacer arms (37%). Thus, the authors suggested the importance of spacer length and, consequently, proper spatial arrangement for *in situ* CC and its application for the discovery of PPI inhibitors.

To overcome the conventional limitations of antibodies, Heath *et al*. used a novel approach to design linear and branched peptide multi-ligands of high affinity and specificity by combining target guided synthesis *in situ* CC with solid-phase peptide synthesis and coined the term 'Iterative Peptide *In situ* Click Chemistry' (IPISC) [81]. Antibodies can identify a range of molecules, such as nucleic acids, proteins, small molecules, pathogens, lipids, and carbohydrates, by the surface of their six peptide loops (three heavy chains and three light chains) and form a binding surface of approximately 1400–1900 Å for protein ligands. Crystallography studies shed light on the diversity of the antigen-binding site architecture, such as the presence of high-affinity binding of CDR-H3 loops in HIV 24 protein, lysozyme, and VEGF [82]. The IPISC process proceeds stepwise, starting from an anchor ligand that has a weak-binding (Kd = 500 MM) short hepta-peptide comprising non-natural D-amino acids and a terminal with an acetylene moiety. This was identified by standard one-bead-one-compound (OBOC) target screen for protein binding. The anchor ligand was then incubated with the bCAII enzyme and a peptide library of azide groups, and a suitable secondary ligand was conjugated to the anchor peptide using CuAAC to generate a bi-ligand (Kd = 3 MM).

In immunoprecipitation experiments, tri-ligand antibodies were found to be more efficient than monoclonal antibodies. For further detail on the spatial orientation of ligands with affinity and specificity for IPISC, please see [81]. Furthermore, the same approach was used to detect the anthrax toxin, a three-protein exotoxin secreted by *Bacillus anthracis*. Protein catalyzed capture (PCC) agent was developed by changing a bacterial peptide into a high-affinity bi-ligand via *in situ* click screening against a vast library of synthesized peptides. The bi-ligand was attached to an electrochemical ELISA assay through gold nanoelectrodes to measure the sensitivity of the bi-ligand. The assay showed a limit of

detection of 170 pg/ml in buffer with a good stability of several days at 65 °C as a powder. The binding affinity of the bi-ligand was Kd = 379 nM and 450 nM as detected by ELISA and surface plasmon resonance (SPR), respectively [83].

Click chemistry and drug delivery

Localized or targeted DDS have become the preferred choice of drug delivery scientists, whose primary goals are to minimize the adverse effects of a drug and to address the unmet and emerging needs for better patient compliance. Thus, modifications of the formulation or design of the drug molecules are not sufficient to maximize the clinical efficacy of targeted therapies. Given its mild reaction conditions and biofriendly nature, CC has been used for the synthesis of novel DDS, leading to products that can better meet the needs of individual patients. A literature search of Scopus on December 31 2016 using the keywords 'click chemistry' AND 'Drug Delivery' revealed a total of 635 publications, which included journal articles, reviews, preprints, and abstracts. Here, we discuss important aspects of CC with respect to polymeric and inorganic drug delivery carriers, highlighting not only its chemical features, but also its utility for DDS, its preparation and functionalization, and its use so far in therapy or diagnosis.

Polymer-based drug delivery systems

Despite not yet being commercialized, polymeric DDSs have shown the potential to treat a variety of diseases and are particularly effective against those with enhanced permeability and retention (EPR) effects, such as solid tumors or rheumatoid arthritis [84,85]. Polymer chemistry has benefited from the simplicity and versatility of CC and a variety of materials have been prepared, including terminal- and pendant-functional polymers; micelles; block co-polymers; and complex structures, such as graft, star, brush, dendritic polymers, gels, polymersomes, and polymeric nanoparticles (NPs) [86–88]. The number (more than 340) of polymer-related publications in Scopus with the keywords 'click' AND 'drug delivery' AND 'polymer' vouches for the impact of CC on the field of polymers for DDS; making it one of the largest area of CC research. Kowollik *et al.* further expanded the criteria of the 'Click' reaction in the context of polymer chemistry [89] to include equimolarity and large-scale purification. Here; we discuss the application of CC for the synthesis and functionalization of polymers for use in DDS (Fig. 7).

Talelli *et al.* used a prodrug approach that covalently coupled doxorubicin (DOX)-glucuronide to the core of polymeric micelles based on a PEG-b-poly[N-(2-hydroxypropyl) methacrylamide-lactate] [mPEG-b-p(HPMAmLac$_n$)] block co-polymer by CuAAC [90]. This system is specifically activated by human β-glucuronidase, an enzyme that is overexpressed in cancer cells. Drug-loaded micelles were 50 nm in diameter and released 40% of the drug payload after 5 days of incubation at 37 °C in the presence of β-glucuronidase. Using 'clip' and 'click' reactions, Freichels and co-workers highlighted a versatile pathway for the synthesis of functional amphiphilic and degradable co-polymers for biomedical applications [91]. A-methoxy-ω-hydroxy-PEG (Me-OHPEG) was modified with alkyne groups for CuAAC and with an activated ester along the PEG backbone bearing a molecular clip (O-succinimidyl-4-(1-azi-2,2,2-trifluoroethyl)-benzoate) for a 'clip' photoreaction. Poly [LA-co-GA-co-α(PEG-clip-TagF$_6$)εCL] was prepared in a series of

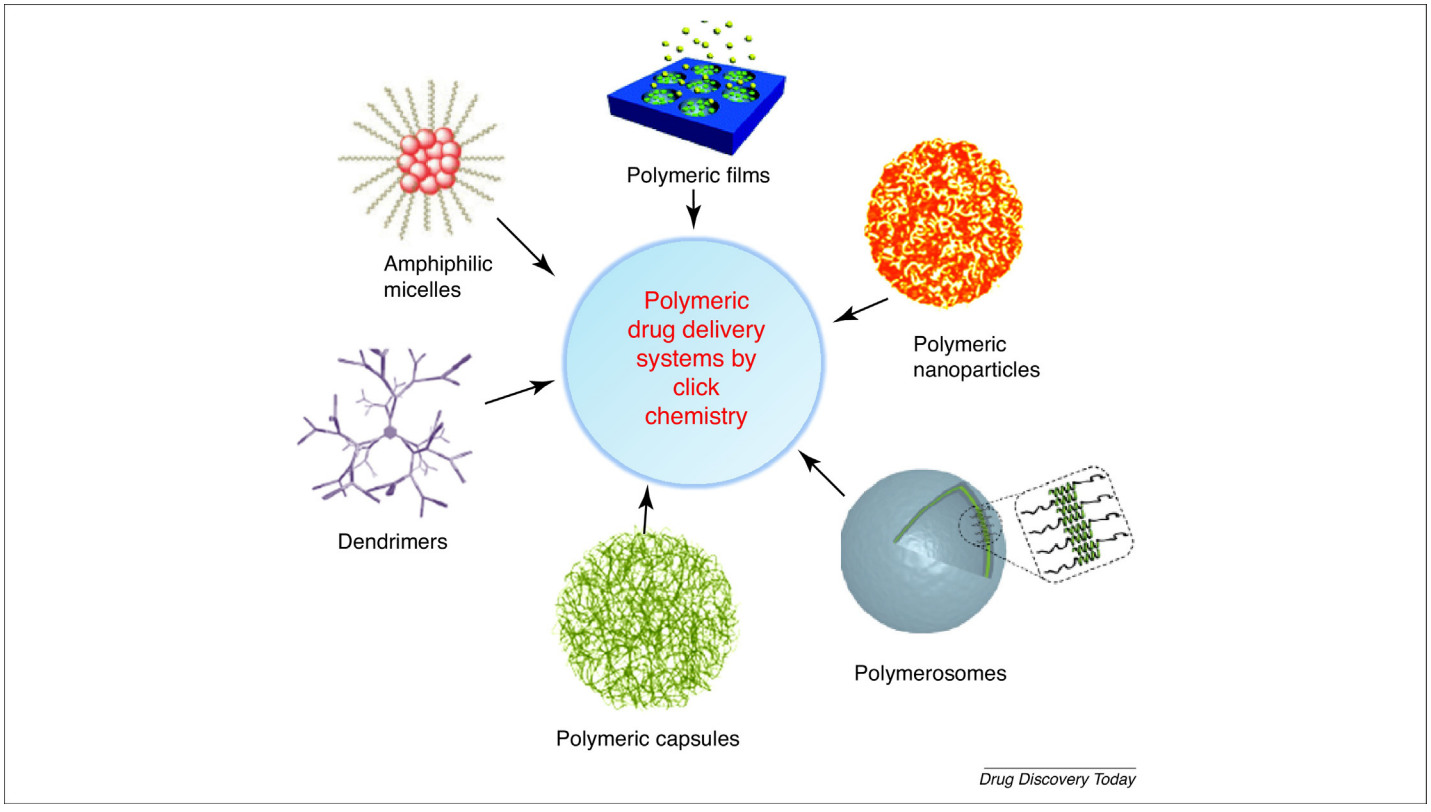

FIGURE 7

Click chemistry-based polymeric drug delivery systems.

steps, modified with an azido group using NaN_3 and connected with the alkyne-containing preformed polymer via CuAAC. This approach was further used to prepare mannosylated poly(ethylene oxide) with a PLGA backbone for the delivery of a glycoreceptor protein, concanavalin A (ConA) [92]. To evaluate the ability of mannosylated micelles to form a complex with a mannose receptor, ConA (a lectin that has a good affinity for mannose) was immobilized onto the gold-coated quartz crystals.

Peptide-based polymers can be applied for the design of new DDS, tissue engineering scaffolds, and novel biomaterials. The first approach to synthesizing peptide–polymers using CuAAC was carried out by Fan and colleagues [93], who synthesized peptidotriazoles by alternating triazole and amide linkages in the backbone of peptide.

Glycopolymers have been shown to act as multivalent ligands and, thus, enhance the binding affinity toward proteins, which is known as the 'glycoside cluster effect' [94]. Comb-shaped peptide–glycopolymer bioconjugates bearing an acid-labile β-thiopropionate linkage were prepared by combination of RAFT polymerization and thiol-ene CC [95]. As per the NMR data, a degradation study of the micelles formed in NaOAc/HAc buffer solution at pH 5.1 from the glycopolymer–peptide conjugates confirmed that the β-thiopropionate linkage between GSH and polymer was cleaved via hydrolysis after 16 days, which was in compliance with the report of the cleavage of β-thiopropionate linkages in a intracellular endosomal compartment (pH = 5.0–6.5) [96]. The rate of disassembly of the aggregates was slower because of this cleavage at acidic pH and, thus, sustained release of the encapsulated compound was achieved.

RNA interference (RNAi) is a cellular mechanism that regulates the expression of genes. Short or small interfering RNA (siRNA) is the most widely used RNAi tool for inducing short-term silencing of protein-coding genes. siRNA is a synthetic RNA duplex that is designed to specifically target a particular mRNA for degradation. The degradation and urinary excretion of the naked siRNA was investigated by conjugation with different-sized azido-modified PEGs (siRNA-PEG) using CuAAC CC [97], and siRNA coupled with 20-kDa PEG (PEG20k-siRNA) showed significantly prolonged blood circulation post injection (50% PEG20k-siRNA). A human lung cancer cell line (H1299) stably expressing enhanced GFP (eGFP) was used to analyze the gene-silencing efficiency of PEGylated siRNA. A series of galactopeptides was synthesized through ring-opening polymerization (ROP) of γ-propargyl-L-glutamate N-carboxyanhydride (PLG NCA) and γ-benzyl-L-glutamate N-carboxyanhydride (BLG NCA), deprotection of the benzyl group, and a subsequent click reaction with an azide-functionalized galactosyl group (PMLG-b-PLGA and PGLG-b-PLGA) [98]. A human hepatoma cell line, HepG2, was used to confirm the targeting efficiency of the system and release of DOX *in vivo* and *in vitro* through recognition of ASGP-R (an endocytotic receptor on the surface of HepG2). Chan and coworkers prepared polymeric dual-function micelles from a graft co-polymer of polylactic acid (PLA), 2-carboxytrimethylene carbonate, and PEG [P(LA-co-TMCC)-g-PEG-X] with X representing either azide or furan, and these were used to conjugate FLAG-dibenzylcyclooctyne peptides and trastuzumab-maleimide antibodies, respectively, via SPAAC [99]. Prepared dual-functionalized micelles showed co-localization of antibodies and peptides in a confocal microscopy study on a human ovary cancer cell line (SKOV-3luc cells). Inspired by the transport mechanism evolved by viruses that transfers nucleic acids and other biomolecules into specific host cells, a prodrug approach was used to design a novel target DDS using aptamer–polymer hybrids [100]. In this approach, cell-targeting aptamers were coupled with block co-polymers via CuAAC to synthesize aptamer–polymer hybrids (APHs) that carried a therapeutic payload (DOX) in an inactive state. Release of the DOX-containing hybrids was observed via coumarin dye after endocytosis. Tumorigenic human breast epithelial cells (MCF-7), which overexpress nucleolin on their cell membrane, were used as a target cell line. Nontumorigenic human breast epithelial cells (MCF-10A), which do not express nucleolin, were used as a negative control.

By metabolic engineering of the glycosylation pathway and thiol-ene CC, thiol-terminated four-arm PEG ($PEG_4$10K-SH) was conjugated to methacryloyl groups [101]. A mannosamine derivative, *N*-methacryloylmannosamine (ManMA), was synthesized as a precursor of cell surface sialic acid residues to deliver the methacryloyl groups to the surface of human cervical carcinoma (HeLa) cells via glycosylation pathway. Ren *et al.* reported two types of biodegradable drug-eluting stent (DES) material, one of which was the prodrug, paclitaxel (PTX) end-capped poly(lactic acid)-b-polyisobutylene (PTX-PLA-b-PIB) diblock co-polymer, and the other was a mixture of PIB-b-PLA diblock co-polymer and PTX. PTX-PLA-b-PIB was synthesized by ROP and CuAAC [102]. The solutions of PTX-PLA-b-PIB and PIB-b-PLA/PTX were coated onto the bare metal stents to form PTX-layered DES. *In vitro* data suggested the sustained release of PTX from the PTX-coated DES and PIB-b-PLA/PTX compared with individual prodrug or a PTX-containing block co-polymer mixture. In another innovative approach, clickable acid-responsive polymeric NPs were conjugated with an HDAC inhibitor, CI-994 (tacedinaline) [103].

Using CC, Burts *et al.* developed a 'brush-first' ring-opening metathesis polymerization (ROMP) method to prepare photodegradable brush-arm star polymer (BASP) NPs that simultaneously degrade and release DOX when irradiated with a 365-nm light source [104]. Polymerization of a norbornene-PEG-branchchloride macromonomer was carried out with NaN_3 to synthesize the azide-functionalized BASP (N_3-BASP), and DOX was functionalized with alkyne groups (DOX-NBOC-alkyne). Application of thiol-ene CC in the targeted delivery of chemotherapy was proven by preparation of amino acid-based core cross-linked star (CCS) polymers [poly(L-lysine)$_{arm}$poly(L-cysteine)$_{core}$] with peripheral allyl functionalities synthesized via sequential ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides (NCAs). Subsequent functionalization with a PEG-folic acid (FA) conjugate via a thiol-ene click reaction resulted in poly(PEG-b-L-lysine)$_{arm}$poly(L-cysteine)$_{core}$) stars with outer PEG coronas decorated with FA for targeting tumor cells [105]. *In vitro* studies of the breast cancer cell line MDA-MB-231 showed that more of these stars were internalized into cells when FA was present. It was reported that the coupling reaction primarily occurs between the hydroxyl group of the PEG and γ-carboxylic acid of FA, which allows the conjugated FA to maintain their affinity towards folate receptors expressed on tumor cells. Recently, by synthesizing a series of glycoclusters of cyclodextrin using CuAAC, Zhang *et al.* demonstrated the effective binding of these glycoclusters to dendritic cell-specific ICAM-3-grabbing nonintegrin (DCSIGN) at nanomolar

concentrations that inhibited the binding of HIV gp120 to DCSIGN [106]. Encapsulation of these glycoconjugates with a hydrophobic core resulting in a high loading capacity for anti-HIV drugs, suggesting the potential of this system in HIV drug delivery.

Hapuarachchige *et al.* reported a new strategy for a two-step, target-specific antibody and drug-loaded delivery method based on a bioorthogonal click reaction that utilizes enhanced internalization of drug conjugates via albumin nanocarriers [107]. Trastuzumab (an antibody) was modified with PEGylated azide and rhodamine, whereas albumin was conjugated with pegylated DBCO and PTX (drug) and Alexa Fluor 488. The system was effective in the HER2-overexpressing breast cancer cell line, BT-474. In a quest to find alternative rapid, selective, and easily accessible coupling reactions for potential biomedical applications, Pipkorn and co-workers applied a novel approach of CC based on the Diels–Alder reaction with 'inverse-electron-demand' (DA_{inv}) to prepare a highly efficient temozolomide (TMZ) bioshuttle, in which TMZ is linked with transporter and subcellular address molecules [108]. Furthermore, the authors prepared a cyclic RGD-bioshuttle-TMZ to target the $\alpha_v\beta_3$ integrin receptor in MCF-7 breast cancer cells; the IC_{50} value was 12 MM, indicating effective cell killing [109]. Devaraj *et al.* further investigated the [4 + 2] inverse Diels–Alder reaction and developed a predictable method for efficient *in vivo* click reactions using computational modeling and a design of a pharmacokinetically optimized polymer-modified tetrazines (PMT) and *trans*-cyclooctynes (TCO) [110]. Flow cytometry studies showed a high labeling efficiency for PMT10 and PMT40. This study demonstrated the efficient reactivity of TCO/Tz cycloaddition at both the cellular and whole-animal level when reaction-partner pharmacokinetics are optimized.

Inorganic drug carriers

As a result of their unique geometry and structure, and remarkable physical-chemical-electrical properties, carbon nanotubes (CNT), fullerenes, and graphene are highly sought-after materials for a range of applications, from nanoelectronics to nanomedicine [111,112]. Graphene forms a building block for other graphitic materials with different geometries, such as CNTs (rolled into different wall numbers, 1D), fullerenes (spherical, 0D) and graphite (layered, 3D). The main limitations to the use of graphene and graphene-like material for biomedical applications are their limited aqueous solubility and toxicity. Therefore, there is a need to improve their solubility and biocompatibility by using different techniques, including CC (Fig. 8). For more detail on the functionalization of graphene and graphene oxide (GO), please see [113].

First proof of the surface modification of CNTs by CuAAC was published by Li *et al.* Single-walled CNTs (SWCNTs) were reacted with *p*-aminophenyl propargyl ether to form alkyl-modified

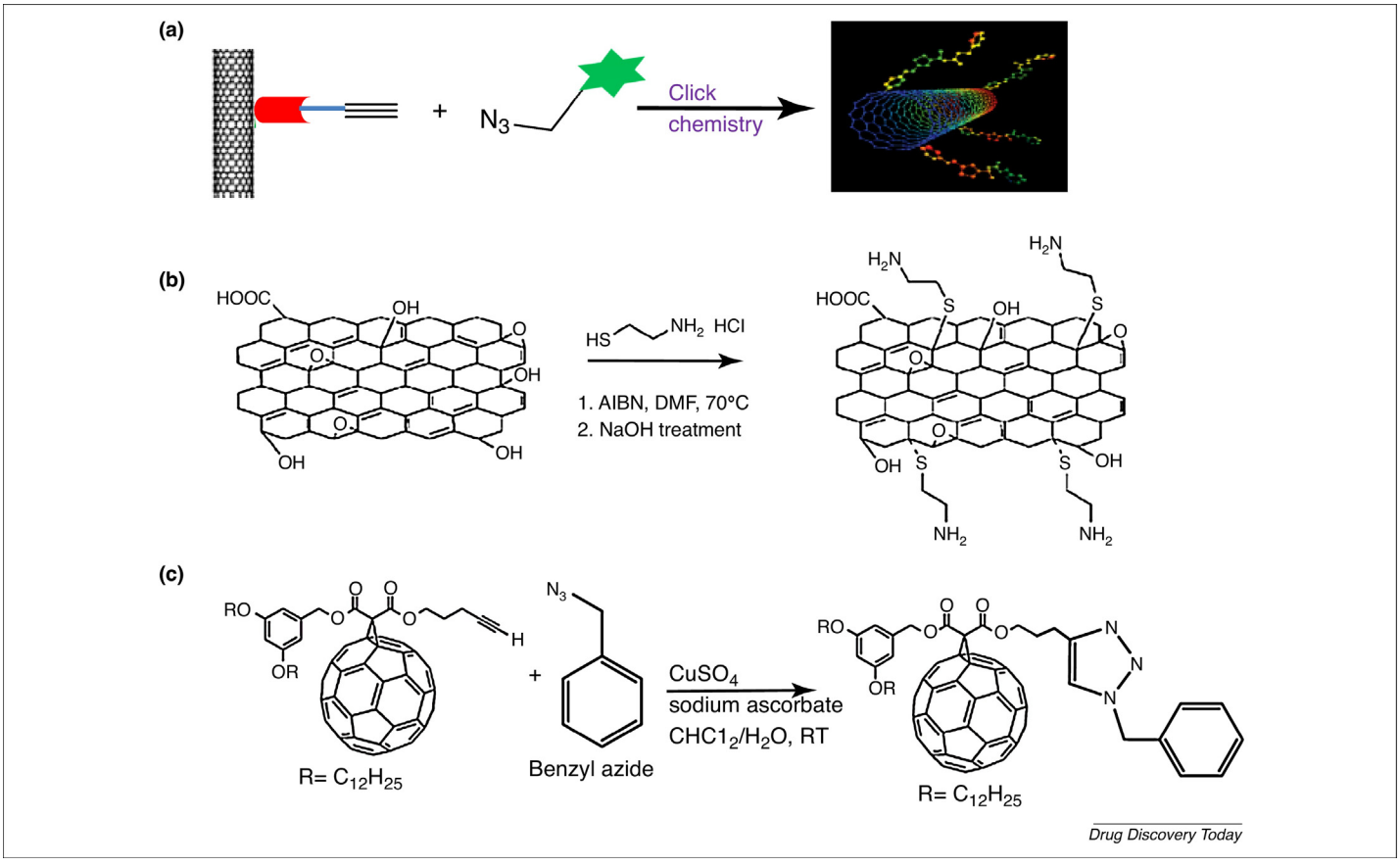

FIGURE 8

Schematic representations of inorganic click reactions. **(a)** Functionalization of carbon nanotubes by click chemistry. **(b)** Thiol-ene conjugation of graphene oxide (GO) and cystamine HCl. Reprinted, with permission, from [139]. **(c)** Conjugation of fullerene alkyne with benzyl azide by an alkyne–azide click reaction mediated by copper (CuAAC). Adapted, with permission, from [122].

SWCNT and were conjugated with azide-modified polystyrene using Cu(I) in dimethylformamide (DMF) [114]. Kumar *et al.* functionalized the SWCNTs with azide-modified amino acids using CuAAC and improved the organic solubility of the resultant material in Tetrahydrofuran (THF), $CHCl_3$, and CH_2Cl_2 [115]. Using a similar approach, the drug binding ability of nanocarriers towards guanine and modified nucleoside were proven using acyclovir as a model drug [116]. The higher binding affinity between the carrier and guanine-based drugs was found to result from the interaction with the β-cyclodextrin (CD) cavity and/or π-stacking on the CNT surface and/or the click-based 1,2,3-triazole ring and phenyl group of the linker.

To enhance the water solubility further for biological applications, Kawagoe *et al.* appended alkyne-modified thymidine and trimethylammonium nucleoside modules to azido-modified cellulose chain (Cel-N_3) by CuAAC. The resultant cellulose derivative showed a sheet-like structure with linear arrays at its both edges, which were able to wrap around SWCNTs because of the affinity between the nucleosides and the π-rich surface of SWCNTs. The resultant cellulose/SWCNT nanohybrid had a rod-like structure and effectively dispersed in water [117]. To optically trigger drug release, SWCNTs were incorporated into a PVA hydrogel to aid electron transport by CuAAC [118]. Using a similar approach, the drug release behavior of tetracycline was determined via electrostimulated drug delivery [119]. Furthermore, Bachl *et al.* used SPAAC to incorporate SWCNTs into a K-carrageenan-based bioactive hydrogel. The resultant hybrid structure had a significant antiproliferative effect on HeLa cells instead of additional cytotoxicity, as seen with native hydrogels [120].

The use of fullerene (C_{60}) for drug delivery was first demonstrated by Steinmetz *et al.* via the synthesis of derivatives of C_{60} (Buckyball) conjugated to virus NPs. Briefly, a propargyl-O-PEG-C_{60} derivative was conjugated to the azide-terminated capsid of bacteriophage Qβ by CuAAC (VNP-PEG-C_{60}). The resulting hybrid complex, VNP-PEG-C_{60}, remained soluble and stable in aqueous buffer medium for several months and was internalized in HeLa human cancer cell lines [121]. The development of clickable fullerene building blocks and their application in the preparation of variety of unique advanced materials and bioactive compounds has been explored by Nierengarten and Nierengarten [122]. In an advanced study, giant globular multivalent glycofullerenes were synthesized by CuAAC in which central C_{60} was covalently connected with 12 hexakis adducts of C_{60} to form the first tridecafullerenes to be reported so far. Each peripheral fullerene was endowed with ten monosaccharides, resulting in a total of 120 carbohydrates decorating the periphery of each molecule [123]. Tridecafullerenes are soluble in water and their ability to inhibit lectin-mediated viral infection processes was investigated in cellular studies. They were found to block Ebola virus infection efficiently in the subnanomolar concentration range. Recently, Magoulas *et al.* conjugated and evaluated pegylated fullerenes with DOX (PEG-C_{60}-DOX) for anticancer activity in a WST-1 test in MCF-7 cell line [124].

Yongzheng *et al.* also delivered anticancer drugs by grafting thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM) onto graphene sheets via CC. Azide-functionalized PNIPAM was reacted with alkyne-modified GO in the presence of CuBr and DMF to form a nanosized structure that easily dissolved in water and PBS at pH 7.4 with the aid of ultrasonication. Camptothecin (CPT) was loaded into PNIPAM-GO NPs, which showed high toxicity to A-5RT3metastatic skin tumor cells. The system proved to be an effective vehicle for anticancer drug delivery [125]. In a straightforward approach to improve the biocompatibility of CNTs in adult stem cells, azide-modified SWCNTs were functionalized with alkyne-modified oligo-lysine dendrons (Boc-K3-CNT). In a rat mesenchymal stem cell culture model, at concentrations above 20 Mg/ml, Fluorescein isothiocyanate (FITC)-modified Boc-K3-CNT (FITC- K3-CNT) showed reduced viability over a 48-h period [126].

To further enhance the application of DNA-grafted hybrid structures in biomedicine, especially for drug and gene delivery, alkyl-modified DNA was grafted onto azide-modified graphene by CuAAC [127]. The assembled DNA-graphene nanohybrids exhibited excellent integration and stability. Their DNA complexation capability and, thus, transfection efficiency of GO, was improved by conjugation with a polyamidoamine (PAMAM) dendron by CuAAC [128]. Their transfection efficiency and cell viability in HeLa cells were 51% and 80%, respectively. GO has been further modified by various polymers; inorganic particles, such as silver; and peptides to improve its antibacterial and antifouling activities [129–131].

Perspectives and limitations

CC has significantly impacted drug discovery and drug delivery for the synthesis of materials with desired kinetics. However, because of the stringent criteria on which CC is based, the approach does have some limitations. For example, these strict criteria restrict the chemical diversity of the reaction. One of the most discussed disadvantages of basic CuAAC is the use of copper as a catalyst and the associated potential toxicity. Although the human body requires copper to perform certain functions, a copper excess can have severe consequences [132]. Several studies have shown that copper can be toxic to cells at concentrations as low as 10 mM. Adverse effects include neurological disorders, hepatitis and kidney disease [133]. The reason for such toxicities is because copper can easily donate and accept single electrons to change its oxidation state, thereby enabling it to catalyze toxic reactions, such as the *in vivo* reduction of hydrogen peroxide to form hydroxyl free radicals [134]. Thus, to apply click reactions *in vivo*, the copper catalyst must be completely removed. Several researchers have reported some success in removing copper from the reaction. For example, Finn and colleagues showed that tris-(hydroxypropyltriazolylmethyl)amine-chelated Cu(I) prevents cell toxicity and has the advantage of faster click reactions compared with free Cu (I) [135]. Macdonald and colleagues used oxide-capped metallic NPs to quench Cu from the system. After performing a click reaction, the crude product was incubated with the NPs and filtered twice. Analysis showed that the copper concentration was reduced from 2026 ppm to 4.6 ppm [136].

Furthermore, azides, which are the prime reactants in CC-based cycloaddition, have the potential to explode. If the ratio of nitrogen atoms to carbon atoms in an organic molecule exceeds or is equal to one, then the molecule can be explosive and dangerous. Therefore, there is the need for extreme care when working with azides in the laboratory, which often results in increased operational costs [10]. A common problem with CC using alkynes is

alkyne homo-coupling, which occurs when an alkyne reacts with another alkyne instead of the azide. Some alkyne homo-coupling reactions require a Cu(I) catalyst, a Cu(II) catalyst, and the presence of oxygen to react, or an inert atmosphere [137]. However, most of these reactions can be minimized by using a sterically bulky base. Another challenge can be biocompatibility of the 1,2,3-triazoles, given that nothing specific has been reported regarding their biological pathways despite the fact that they were identified over a decade ago and many drug-like compounds have since been synthesized via CC. One of the most notable limitations for researchers is the availability of starting materials used to perform CC and, thus, the high cost of commercially available click reaction-related precursors. The high cost of non-natural amino acids for the synthesis of peptide-based molecules is also a problem. Despite the success and attention garnered by CC, this issue remains an obstacle to widespread research based on click chemicals in materials synthesis and drug delivery. However, more click precursors are likely to become available commercially at affordable prices in the future. A database of 16 million triazoles, called 'ZINClick', has been prepared from literature reporting alkynes and azides that can be synthesized within three steps from commercially available products [138]. The library, which can be accessed online via at www.symech.it/ZINClick, contains new 1,4-disubstituted-1,2,3-triazoles that are easily synthesizable and patentable.

Concluding remarks

The use of CC has led to the clinical transformation of medicine for the development of drugs for patients with unmet medical needs. Despite its limitations, CC is still one of the most versatile chemistry tools for diverse pharmaceutical applications. Its tolerance of standard biological conditions and of most diverse functional groups, and the high aqueous solubility of the covalent bonds formed between the click groups. have resulted in its use to synthesize many diverse delivery systems and molecules in a large number of libraries. A licensing agreement with the Script Research Institute will provide Sutro Biopharma access to 'CC' and a worldwide license to apply for the synthesis of novel therapeutic protein candidates by utilizing Sutro Biopharma's advanced biochemical protein synthesis platforms to incorporate one or more of the 'click' components into a peptide or protein chain (www.prnewswire.com/news-releases/sutro-licenses-click-chemistry-from-the-scripps-research-institute-159033425.html). With continuous research and invention of new chemical transformations and their applications that meet the 'click' status, there is great potential for the use of CC in drug discovery and drug delivery research. Generally, the time required for a drug to reach the market is 15–20 years; thus, given that CC was initially developed in 2001–2002, it is speculated that this highly versatile and efficient technique will soon be commercialized at a greater level and will fulfill the unmet therapeutic needs of many systemic and localized diseases. We also hope that it will help restart recently stalled innovative drug pipelines in the pharmaceutical industry.

Acknowledgment

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References

1 Canalle, L.A. *et al.* (2009) Copper-free clickable coatings. *Adv. Funct. Mater.* 19, 3464–3470
2 Zhang, Y. *et al.* (2009) Covalent layer-by-layer functionalization of multiwalled carbon nanotubes by click chemistry. *Langmuir* 25, 5814–5824
3 Kolb, H.C. *et al.* (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed. Engl.* 40, 2004–2021
4 Huisgen, R. (1963) 1,3-dipolar cycloadditions. Past and future. *Angew. Chem. Int. Ed. Engl.* 2, 565–598
5 Bock, V.D. *et al.* (2006) CuI-catalysed alkyne–azide 'click' cycloadditions from a mechanistic and synthetic perspective. *Eur. J. Org. Chem.* 2006, 51–68
6 Rodionov, V.O. *et al.* (2005) Mechanism of the ligand-free CuI-catalysed azide-alkyne cycloaddition reaction. *Angew. Chem. Int. Ed. Engl.* 44, 2210–2215
7 Such, G.K. *et al.* (2012) Synthesis and functionalization of nanoengineered materials using click chemistry. *Prog. Polym. Sci.* 37, 985–1003
8 Hein, J.E. and Fokin, V.V. (2010) Copper-catalysed azide-alkyne cycloaddition (CuAAC) and beyond: new reactivity of copper(I) acetylides. *Chem. Soc. Rev.* 39, 1302–1315
9 Wang, Q. *et al.* (2003) Bioconjugation by copper(I)-catalysed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* 125, 3192–3193
10 Brase, S. *et al.* (2005) Organic azides: an exploding diversity of a unique class of compounds. *Angew. Chem. Int. Ed. Engl.* 44, 5188–5240
11 Jewett, J.C. *et al.* (2010) Rapid Cu-free click chemistry with readily synthesized biarylazacyclooctynones. *J. Am. Chem. Soc.* 132, 3688–3690
12 Bach, R.D. (2009) Ring strain energy in the cyclooctyl system. The effect of strain energy on [3 + 2] cycloaddition reactions with azides. *J. Am. Chem. Soc.* 131, 5233–5243
13 Sletten, E.M. and Bertozzi, C.R. (2011) From mechanism to mouse: a tale of two bioorthogonal reactions. *ACC Chem. Res.* 44, 666–676
14 Jewett, J.C. and Bertozzi, C.R. (2010) Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev.* 39, 1272–1279
15 Friscourt, F. *et al.* (2012) Polar dibenzocyclooctynes for selective labeling of extracellular glycoconjugates of living cells. *J. Am. Chem. Soc.* 134, 5381–5389
16 Agard, N.J. *et al.* (2006) A comparative study of bioorthogonal reactions with azides. *ACS Chem. Biol.* 1, 644–648
17 Baskin, J.M. *et al.* (2007) Copper-free click chemistry for dynamic *in vivo* imaging. *Proc. Natl. Acad. Sci. U. S. A.* 104, 16793–16797
18 McKay, C.S. *et al.* (2010) Nitrones as dipoles for rapid strain-promoted 1, 3-dipolar cycloadditions with cyclooctynes. *Chem. Commun.* 46, 931–933
19 Rideout, D.C. and Breslow, R. (1980) Hydrophobic acceleration of Diels–Alder reactions. *J. Am. Chem. Soc.* 102, 7816–7817
20 Inglis, A.J. and Barner-Kowollik, C. (2010) Ultra rapid approaches to mild macromolecular conjugation. *Macromol. Rapid Commun.* 31, 1247–1266
21 Mauldin, T. and Kessler, M. (2010) Self-healing polymers and composites. *Int. Mater. Rev.* 55, 317–346
22 Hoyle, C.E. and Bowman, C.N. (2010) Thiol-ene click chemistry. *Angew. Chem. Int. Ed. Engl.* 49, 1540–1573
23 Becer, C.R. *et al.* (2009) Click chemistry beyond metal-catalysed cycloaddition. *Angew. Chem. Int. Ed. Engl.* 48, 4900–4908
24 Lowe, A.B. (2010) Thiol-ene 'click' reactions and recent applications in polymer and materials synthesis. *Polymer Chem.* 1, 17–36
25 Northrop, B.H. *et al.* (2015) Thiol–maleimide 'click' chemistry: evaluating the influence of solvent, initiator, and thiol on the reaction mechanism, kinetics, and selectivity. *Polym. Chem.* 6, 3415–3430
26 Lowe, A.B. (2014) Thiol-yne 'click'/coupling chemistry and recent applications in polymer and materials synthesis and modification. *Polymer* 55, 5517–5549
27 Carioscia, J.A. *et al.* (2007) Evaluation and control of thiol-ene/thiol-epoxy hybrid networks. *Polymer (Guildf)* 48, 1526–1532
28 Hensarling, R.M. *et al.* (2011) Thiol-isocyanate 'click' reactions: rapid development of functional polymeric surfaces. *Polym. Chem.* 2, 88–90

29 Becer, C.R. *et al.* (2009) Clicking pentafluorostyrene copolymers: synthesis, nanoprecipitation, and glycosylation. *Macromolecules* 42, 2387–2394
30 Lowe, A.B. and Bowman, C.N. (2013) *Thiol-X Chemistries in Polymer and Materials Science*. Royal Society of Chemistry
31 Lin, C.C. *et al.* (2015) Thiol-norbornene photoclick hydrogels for tissue engineering applications. *J. Appl. Polym. Sci.* 132, 41563
32 Dong, J. *et al.* (2014) Sulfur (VI) fluoride exchange (SuFEx): another good reaction for click chemistry. *Angew. Chem. Int. Ed.* 53, 9430–9448
33 Steinkopf, W. (1927) Uber Aromatische Sulfofluoride. *J. Praktische Chem.* 117, 1–82
34 Suter, C. (1944) *The Organic Chemistry of Sulphur*. J. Wiley & Sons
35 Kolb, H.C. and Sharpless, K.B. (2003) The growing impact of click chemistry on drug discovery. *Drug Discov. Today* 8, 1128–1137
36 Sharpless, K. (2005) Secret life of enzymes: an aggressive strategy for drug discovery. *Abstr. Pap. Am. Chem. Soc.* 229, U523
37 Massarotti, A. *et al.* (2014) Are 1,4- and 1,5-disubstituted 1,2,3-triazoles good pharmacophoric groups? *ChemMedChem* 9, 2497–2508
38 Simon, C.G., Jr and Lin-Gibson, S. (2011) Combinatorial and high-throughput screening of biomaterials. *Adv. Mater.* 23, 369–387
39 Inglese, J. (2010) A practical guide to assay development and high-throughput screening in drug discovery. *ChemMedChem* 5, 1398–1399
40 Mayr, L.M. and Bojanic, D. (2009) Novel trends in high-throughput screening. *Curr. Opin. Pharmacol.* 9, 580–588
41 Niphakis, M.J. and Cravatt, B.F. (2014) Enzyme inhibitor discovery by activity-based protein profiling. *Annu. Rev. Biochem.* 83, 341–377
42 de Kloe, G.E. *et al.* (2009) Transforming fragments into candidates: small becomes big in medicinal chemistry. *Drug Discov. Today* 14, 630–646
43 Salum, L.B. and Andricopulo, A.D. (2010) Fragment-based QSAR strategies in drug design. *Expert Opin. Drug Discov.* 5, 405–412
44 Schmidt, M.F. and Rademann, J. (2009) Dynamic template-assisted strategies in fragment-based drug discovery. *Trends Biotechnol.* 27, 512–521
45 Corbett, P.T. *et al.* (2006) Dynamic combinatorial chemistry. *Chem. Rev.* 106, 3652–3711
46 Herrmann, A. (2014) Dynamic combinatorial/covalent chemistry: a tool to read, generate and modulate the bioactivity of compounds and compound mixtures. *Chem. Soc. Rev.* 43, 1899–1933
47 Imming, P. *et al.* (2006) Drugs, their targets and the nature and number of drug targets. *Nat. Rev. Drug Discov.* 5, 821–834
48 Thirumurugan, P. *et al.* (2013) Click chemistry for drug development and diverse chemical-biology applications. *Chem. Rev.* 113, 4905–4979
49 Cheng, H.C. *et al.* (2011) Regulation and function of protein kinases and phosphatases. *Enzyme Res.* 2011, 794089
50 Seerden, J.-P.G. *et al.* (2014) Synthesis and structure–activity relationships of 4-fluorophenyl-imidazole p38α MAPK, CK1δ and JAK2 kinase inhibitors. *Bioorg. Med. Chem. Lett.* 24, 3412–3418
51 Vojtičková, M. *et al.* (2015) Ynamide Click chemistry in development of triazole VEGFR2 TK modulators. *Eur. J. Med. Chem.* 103, 105–122
52 Mandalapu, D. *et al.* (2016) Synthesis and biological evaluation of some novel triazole hybrids of curcumin mimics and their selective anticancer activity against breast and prostate cancer cell lines. *Bioorg. Med. Chem. Lett.* 26, 4223–4232
53 Quiros, P.M. *et al.* (2015) New roles for mitochondrial proteases in health, ageing and disease. *Nat. Rev. Mol. Cell. Biol.* 16, 345–359
54 Zhang, Y. *et al.* (2014) Sulphoxythiocarbamates modify cysteine residues in HSP90 causing degradation of client proteins and inhibition of cancer cell proliferation. *Br. J. Cancer* 110, 71–82
55 Bachovchin, D.A. and Cravatt, B.F. (2012) The pharmacological landscape and therapeutic potential of serine hydrolases. *Nat. Rev. Drug Discov.* 11, 52–68
56 Adibekian, A. *et al.* (2011) Click-generated triazole ureas as ultrapotent *in vivo*–active serine hydrolase inhibitors. *Nat. Chem. Biol.* 7, 469–478
57 Chang, J.W. *et al.* (2013) Proteome-wide reactivity profiling identifies diverse carbamate chemotypes tuned for serine hydrolase inhibition. *ACS Chem. Biol.* 8, 1590–1599
58 Kötzler, M.P. *et al.* (2014) Glycosidases: Functions, Families and Folds. *eLS*
59 Gouin, S.G. (2014) Multivalent inhibitors for carbohydrate-processing enzymes: beyond the 'lock-and-key' concept. *Chemistry* 20, 11616–11628
60 Hu, X.L. *et al.* (2015) Triazole-linked glycolipids enhance the susceptibility of MRSA to beta-lactam antibiotics. *ACS Med. Chem. Lett.* 6, 793–797
61 Ahmad Fuaad, A.A. *et al.* (2013) Peptide conjugation via CuAAC 'click' chemistry. *Molecules* 18, 13148–13174
62 Kennedy, D.C. *et al.* (2011) Cellular consequences of copper complexes used to catalyse bioorthogonal click reactions. *J. Am. Chem. Soc.* 133, 17993–18001
63 Uttamapinant, C. *et al.* (2012) Fast, cell-compatible click chemistry with copper-chelating azides for biomolecular labeling. *Angew. Chem. Int. Ed. Engl.* 51, 5852–5856
64 Astakhova, I.K. *et al.* (2013) Peptide-LNA oligonucleotide conjugates. *Org. Biomol. Chem.* 11, 4240–4249
65 Liu, T.-Y. *et al.* (2013) Self-adjuvanting polymer–peptide conjugates as therapeutic vaccine candidates against cervical cancer. *Biomacromolecules* 14, 2798–2806
66 Mamidyala, S.K. and Finn, M.G. (2010) *In situ* click chemistry: probing the binding landscapes of biological molecules. *Chem. Soc. Rev.* 39, 1252–1261
67 Sharpless, K.B. and Manetsch, R. (2006) *In situ* click chemistry: a powerful means for lead discovery. *Expert Opin. Drug Discov.* 1, 525–538
68 Lewis, W.G. *et al.* (2002) Click chemistry *in situ*: acetylcholinesterase as a reaction vessel for the selective assembly of a femtomolar inhibitor from an array of building blocks. *Angew. Chem.* 114, 1095–1099
69 Grimster, N.P. *et al.* (2012) Generation of candidate ligands for nicotinic acetylcholine receptors via *in situ* click chemistry with a soluble acetylcholine binding protein template. *J. Am. Chem. Soc.* 134, 6732–6740
70 Morris, G.M. *et al.* (2013) Automated docking with protein flexibility in the design of femtomolar 'click chemistry' inhibitors of acetylcholinesterase. *J. Chem. Inf. Model.* 53, 898–906
71 Ronco, C. *et al.* (2009) Synthesis and structure–activity relationship of Huprine derivatives as human acetylcholinesterase inhibitors. *Bioorg. Med. Chem.* 17, 4523–4536
72 Oueis, E. *et al.* (2014) Reaction site-driven regioselective synthesis of AChE inhibitors. *Org. Biomol. Chem.* 12, 156–161
73 Mohammadi-Khanaposhtani, M. *et al.* (2015) Potent acetylcholinesterase inhibitors: design, synthesis, biological evaluation, and docking study of acridone linked to 1,2,3-triazole derivatives. *Eur. J. Med. Chem.* 92, 799–806
74 Mondal, M. *et al.* (2016) Fragment-based drug design facilitated by protein-templated click chemistry: fragment linking and optimization of inhibitors of the aspartic protease endothiapepsin. *Chemistry* 22, 14826–14830
75 Haberland, M. *et al.* (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.* 10, 32–42
76 Suzuki, T. *et al.* (2012) Rapid discovery of highly potent and selective inhibitors of histone deacetylase 8 using click chemistry to generate candidate libraries. *J. Med. Chem.* 55, 9562–9575
77 Rakers, C. *et al.* (2015) Computational close up on protein–protein interactions: how to unravel the invisible using molecular dynamics simulations? *WIRES Computat. Mol. Sci.* 5, 345–359
78 Arkin, M.R. *et al.* (2014) Small-molecule inhibitors of protein–protein interactions: progressing toward the reality. *Chem. Biol.* 21, 1102–1114
79 Kulkarni, S.S. *et al.* (2011) Screening of protein–protein interaction modulators via sulfo-click kinetic target-guided synthesis. *ACS Chem. Biol.* 6, 724–732
80 Maki, T. *et al.* (2013) Chemical ligation of epoxide-containing fusicoccins and peptide fragments guided by 14-3-3 protein. *Mol. Biosyst.* 9, 940–943
81 Millward, S.W. *et al.* (2013) In situ click chemistry: from small molecule discovery to synthetic antibodies. *Integr. Biol. (Camb.)* 5, 87–95
82 Xu, J.L. and Davis, M.M. (2000) Diversity in the CDR3 region of V H is sufficient for most antibody specificities. *Immunity* 13, 37–45
83 Coppock, M.B. *et al.* (2014) Peptide-based protein capture agents with high affinity, selectivity, and stability as antibody replacements in biodetection assays. In *SPIE Sensing Technology+ Applications* (Cullum, B.M. and McLamore, E.S., eds), International Society for Optics and Photonics p. 910711
84 Maeda, H. (2010) Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjug. Chem.* 21, 797–802
85 Seo, J. *et al.* (2016) Polymeric materials for drug release system in drug eluting stents. *J. Pharm. Invest.* 46, 317–324
86 Golas, P.L. and Matyjaszewski, K. (2010) Marrying click chemistry with polymerization: expanding the scope of polymeric materials. *Chem. Soc. Rev.* 39, 1338–1354
87 Hoang, N.H. *et al.* (2016) Triblock copolymers for nano-sized drug delivery systems. *J. Pharm. Invest.* 47, 27
88 Tran, T.H. *et al.* (2016) Combined phototherapy in anti-cancer treatment: therapeutics design and perspectives. *J. Pharm. Invest.* 46, 505–517
89 Barner-Kowollik, C. *et al.* (2011) 'Clicking' polymers or just efficient linking: what is the difference? *Angew Chem. Int. Ed.* 50, 60–62
90 Talelli, M. *et al.* (2011) Synthesis and characterization of biodegradable and thermosensitive polymeric micelles with covalently bound doxorubicin-glucuronide prodrug via click chemistry. *Bioconjug. Chem.* 22, 2519–2530
91 Freichels, H. *et al.* (2011) 'Clip' and 'click' chemistries combination: toward easy PEGylation of degradable aliphatic polyesters. *Macromol. Rapid Commun.* 32, 616–621
92 Freichels, H.L.N. *et al.* (2012) Synthesis of poly (lactide-co-glycolide-co-ε-caprolactone)-graft-mannosylated poly (ethylene oxide) copolymers by combination of 'clip' and 'click' chemistries. *Biomacromolecules* 13, 760–768

93 Zhang, Z. and Fan, E. (2006) Solid phase synthesis of peptidotriazoles with multiple cycles of triazole formation. *Tetrahedron Lett.* 47, 665–669
94 Lundquist, J.J. and Toone, E.J. (2002) The cluster glycoside effect. *Chem. Rev.* 102, 555–578
95 Wang, X. *et al.* (2012) Comb-shaped glycopolymer/peptide bioconjugates by combination of RAFT polymerization and thiol-ene 'click' chemistry. *Macromol. Biosci.* 12, 1575–1582
96 Dan, K. *et al.* (2011) Aggregation and pH responsive disassembly of a new acid-labile surfactant synthesized by thiol-acrylate Michael addition reaction. *Langmuir* 27, 612–617
97 Iversen, F. *et al.* (2013) Optimized siRNA-PEG conjugates for extended blood circulation and reduced urine excretion in mice. *Theranostics* 3, 201–209
98 Ding, J. *et al.* (2013) Efficacious hepatoma-targeted nanomedicine self-assembled from galactopeptide and doxorubicin driven by two-stage physical interactions. *J. Control Release* 169, 193–203
99 Chan, D.P. *et al.* (2013) Double click: dual functionalized polymeric micelles with antibodies and peptides. *Bioconjug. Chem.* 24, 105–113
100 Oh, S.S. *et al.* (2014) Synthetic aptamer-polymer hybrid constructs for programmed drug delivery into specific target cells. *J. Am. Chem. Soc.* 136, 15010–15015
101 Iwasaki, Y. and Matsuno, H. (2011) Metabolic delivery of methacryloyl groups on living cells and cell surface modification via thiol-ene 'click' reaction. *Macromol. Biosci.* 11, 1478–1483
102 Ren, K. *et al.* (2015) Preparation of polymeric prodrug paclitaxel-poly(lactic acid)-b-polyisobutylene and its application in coatings of a drug eluting stent. *ACS Appl. Mater. Interfaces* 7, 11263–11271
103 Denis, I. *et al.* (2015) Histone deacetylase inhibitor-polymer conjugate nanoparticles for acid-responsive drug delivery. *Eur. J. Med. Chem.* 95, 369–376
104 Burts, A.O. *et al.* (2014) Brush-first and click: efficient synthesis of nanoparticles that degrade and release doxorubicin in response to light. *Photochem. Photobiol.* 90, 380–385
105 Sulistio, A. *et al.* (2011) Folic acid conjugated amino acid-based star polymers for active targeting of cancer cells. *Biomacromolecules* 12, 3469–3477
106 Gao, P. *et al.* (2016) Discovery of novel anti-HIV agents via Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC) click chemistry-based approach. *Expert Opin. Drug Discov.* 11, 857–871
107 Hapuarachchige, S. *et al.* (2014) Bioorthogonal, two-component delivery systems based on antibody and drug-loaded nanocarriers for enhanced internalization of nanotherapeutics. *Biomaterials* 35, 2346–2354
108 Pipkorn, R. *et al.* (2009) Inverse-electron-demand Diels-Alder reaction as a highly efficient chemoselective ligation procedure: synthesis and function of a BioShuttle for temozolomide transport into prostate cancer cells. *J. Peptide Sci.* 15, 235–241
109 Braun, K. *et al.* (2010) A cyclic-RGD-bioshuttle functionalized with TMZ by DARinv 'Click Chemistry' targeted to αvβ3 integrin for therapy. *Int. J. Med. Sci.* 7, 326
110 Devaraj, N.K. *et al.* (2012) Reactive polymer enables efficient *in vivo* bioorthogonal chemistry. *Proc. Natl. Acad. Sci. U. S. A.* 109, 4762–4767
111 Kakran, M. and Li, L. (2012) Carbon nanomaterials for drug delivery. *Key Eng. Mater.* 508, 76–80
112 Youn, Y.S. *et al.* (2017) Multifunctional nano-sized fullerenes for advanced tumor therapy. *J. Pharm. Invest.* 47, 1–10
113 Changwei, L. *et al.* (2013) The functionalization of graphene and graphene oxide via click chemistry. *Acta Chim. Sin.* 71, 1201–1224
114 Li, H. *et al.* (2005) Functionalization of single-walled carbon nanotubes with well-defined polystyrene by 'click' coupling. *J. Am. Chem. Soc.* 127, 14518–14524
115 Kumar, I. *et al.* (2008) Functionalization of single-walled carbon nanotubes with azides derived from amino acids using click chemistry. *J. Nanosci. Nanotechnol.* 8, 3351–3356
116 Iannazzo, D. *et al.* (2014) β-Cyclodextrin-grafted on multiwalled carbon nanotubes as versatile nanoplatform for entrapment of guanine-based drugs. *Colloids Surf. B Biointerfaces* 123, 264–270
117 Kawagoe, N. *et al.* (2011) Highly C6-selective and quantitative modification of cellulose: nucleoside-appended celluloses to solubilize single walled carbon nanotubes. *Cellulose* 18, 83–93
118 Lee, E. *et al.* (2012) Synthesis of single-walled carbon nanotube-incorporated polymer hydrogels via click chemistry. *Polym. Chem.* 3, 2451–2455
119 Choi, E.J. *et al.* (2015) Synthesis of electroconductive hydrogel films by an electro-controlled click reaction and their application to drug delivery systems. *Polym. Chem.* 6, 4473–4478
120 Bachl, J. *et al.* (2012) Hybrid bioactive hydrogels containing single-walled carbon nanotubes covalently integrated via strain-promoted azide-alkyne cycloaddition. *Nanosci. Nanotechnol. Asia* 2, 200–209
121 Steinmetz, N.F. *et al.* (2009) Buckyballs meet viral nanoparticles: candidates for biomedicine. *J. Am. Chem. Soc.* 131, 17093–17095
122 Nierengarten, I. and Nierengarten, J.F. (2015) The impact of copper-catalysed alkyne-azide 1,3-dipolar cycloaddition in fullerene chemistry. *Chem. Rec.* 15, 31–51
123 Munoz, A. *et al.* (2016) Synthesis of giant globular multivalent glycofullerenes as potent inhibitors in a model of Ebola virus infection. *Nat. Chem.* 8, 50–57
124 Magoulas, G.E. *et al.* (2015) Synthesis and evaluation of anticancer activity in cells of novel stoichiometric pegylated fullerene-doxorubicin conjugates. *Pharm. Res.* 32, 1676–1693
125 Pan, Y. *et al.* (2011) Water-soluble poly (N-isopropylacrylamide)–graphene sheets synthesized via click chemistry for drug delivery. *Adv. Funct. Mater.* 21, 2754–2763
126 Moore, E. *et al.* (2012) Clicking dendritic peptides onto single walled carbon nanotubes. *RSC Adv.* 2, 1289–1291
127 Wang, Z. *et al.* (2012) Polyvalent DNA-graphene nanosheets 'click' conjugates. *Nanoscale* 4, 394–399
128 Sarkar, K. *et al.* (2015) Dendron conjugation to graphene oxide using click chemistry for efficient gene delivery. *RSC Adv.* 5, 50196–50211
129 Singh, S. *et al.* (2015) Enhanced catalytic and antibacterial activities of silver nanoparticles immobilized on poly (N-vinyl pyrrolidone)-grafted graphene oxide. *RSC Adv.* 5, 81994–82004
130 Tu, Q. *et al.* (2016) Click synthesis of quaternized poly(dimethylaminoethyl methacrylate) functionalized graphene oxide with improved antibacterial and antifouling ability. *Colloids Surf. B Biointerfaces* 141, 196–205
131 Shi, L. *et al.* (2016) Modifying graphene oxide with short peptide via click chemistry for biomedical applications. *Appl. Mater. Today* 5, 111–117
132 Stern, B.R. *et al.* (2007) Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships. *J. Toxicol. Environ. Health B Crit. Rev.* 10, 157–222
133 Tisato, F. *et al.* (2010) Copper in diseases and treatments, and copper-based anticancer strategies. *Med. Res. Rev.* 30, 708–749
134 Wang, T. and Guo, Z. (2006) Copper in medicine: homeostasis, chelation therapy and antitumor drug design. *Curr. Med. Chem.* 13, 525–537
135 Hong, V. *et al.* (2010) Labeling live cells by copper-catalysed alkyne—azide click chemistry. *Bioconjug. Chem.* 21, 1912–1916
136 Macdonald, J.E. *et al.* (2007) Iron/iron oxide nanoparticle sequestration of catalytic metal impurities from aqueous media and organic reaction products. *Langmuir* 23, 9543–9545
137 Siemsen, P. *et al.* (2000) Acetylenic coupling: a powerful tool in molecular construction. *Angew. Chem. Int. Ed. Engl.* 39, 2632–2657
138 Massarotti, A. *et al.* (2014) ZINClick: a database of 16 million novel, patentable, and readily synthesizable 1,4-disubstituted triazoles. *J. Chem. Inf. Model.* 54, 396–406
139 Luong, N.D. *et al.* (2015) Functional graphene by thiol-ene click chemistry. *Chemistry* 21, 3183–3186