The growing applications of click chemistry

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Click chemistry, the subject of this *tutorial review*, is a modular synthetic approach towards the assembly of new molecular entities. This powerful strategy relies mainly upon the construction of carbon–heteroatom bonds using spring-loaded reactants. Its growing number of applications are found in nearly all areas of modern chemistry from drug discovery to materials science. The copper(I)-catalysed 1,2,3-triazole forming reaction between azides and terminal alkynes has become the gold standard of click chemistry due to its reliability, specificity and biocompatibility.

Introduction

In 2001, Kolb, Finn and Sharpless published a landmark review describing a new strategy for organic chemistry, or as the authors also put it "the reinvigoration of an old style of organic synthesis". The name click chemistry (CC) was coined to describe this 'guiding principle'—a principle born to meet the demands of modern day chemistry and in particular, the demands of drug discovery.[1]

Since the foundations of CC were laid, there has been an explosive growth in publications describing a wealth of applications of this practical and sensible chemical approach. This review is intended to highlight key areas where CC has had significant impact, and is thus separated into the three most relevant categories: (i) bioconjugation, (ii) materials science, and (iii) drug discovery. It would be impossible in an article of this size to provide a complete account of the dramatic impact that CC has had in recent years in a diverse range of applications, and readers are directed towards more detailed and subject specific reviews.[2–5]

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The click chemistry philosophy

Examination of the molecules created by nature (the quintessential chemist), reveals an overall preference for carbon–heteroatom bonds over carbon–carbon bonds; for example, nucleic acids, proteins and polysaccharides are condensation polymers of subunits linked through carbon–heteroatom bonds. This strategy of making large oligomers from relatively simple building blocks can be described as nature's way of performing combinatorial chemistry with remarkable modularity and diversity. All proteins are created from 20 building blocks that are joined *via* reversible heteroatom links. Following nature's lead, and limiting the search for new substances to those which can be generated by joining small units together through heteroatom links, Sharpless *et al.* defined CC.[1]

Click chemistry serves as a powerful strategy in the quest for function, and can be summarised neatly in one sentence: "*all searches must be restricted to molecules that are easy to make*".[1]

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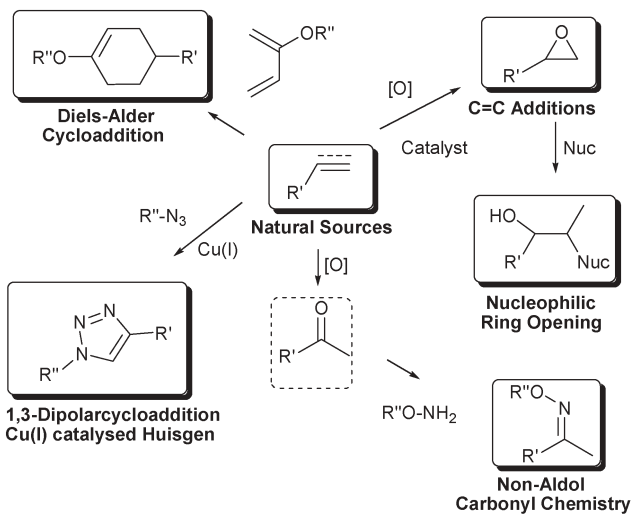
Since chemists lack nature's ability to perfectly control reversible carbonyl based chemistries, the focus of CC rests exclusively on highly energetic 'spring loaded' reactants.

These reactions are governed by kinetic control and are highly reliable and selective processes. A set of stringent criteria that a process must meet to be useful in the context of CC has been defined by Sharpless *et al.*,[1] as reactions that: "*are modular, wide in scope, high yielding, create only inoffensive by-products (that can be removed without chromatography), are stereospecific, simple to perform and that require benign or easily removed solvent*". Ideally, starting materials and reagents for 'click' reactions should be readily available, and it is a convenient coincidence that unsaturated-hydrocarbon based organic synthesis is currently at the heart of this powerful approach, since these materials are readily available from nature or can be obtained by steam cracking of alkanes in the petrochemical industry.

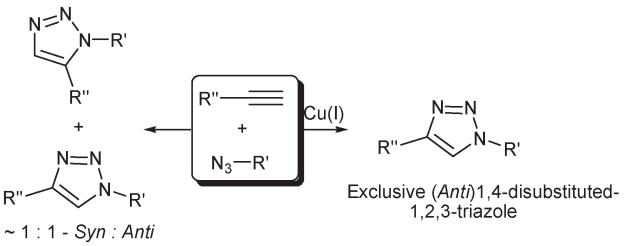
Although meeting the requirements of a 'click' reaction is a tall order, several processes have been identified which step up to the mark (Scheme 1): nucleophilic ring opening reactions: epoxides, aziridines, aziridinium ions *etc.*; non-aldol carbonyl chemistry: formation of ureas, oximes and hydrazones *etc.*; additions to carbon–carbon multiple bonds: especially oxidative addition, and Michael additions of Nu–H reactants; and cycloaddition reactions: especially 1,3-dipolar cycloaddition reactions, but also the Diels–Alder reaction.

The cream of the crop

Of all the reactions which achieve 'click status', the Huisgen 1,3-dipolar cycloaddition of alkynes and azides to yield 1,2,3-triazoles is undoubtedly the premier example of a click reaction. The ease of synthesis of the alkyne and azide functionalities, coupled with their kinetic stability and tolerance to a wide variety of functional groups and reaction conditions, make these complementary coupling partners particularly attractive. However, it was the recent discovery of the dramatic rate acceleration of the azide–alkyne coupling event[6,7] under copper(I) catalysis and the beneficial effects of



Scheme 1 A selection of reactions which match the Click Chemistry criteria.



Scheme 2 The Cu(I) catalysed Huisgen 'click reaction' results in exclusive formation of the 1,4-triazole, whilst the thermally induced Huisgen cycloaddition usually results in an approximately 1 : 1 mixture of 1,4- and 1,5-triazole stereoisomers.

water that have placed this reaction at the 'center stage' of CC (Scheme 2). This new reaction process requires no protecting groups, and proceeds with almost complete conversion and selectivity for the 1,4-disubstituted 1,2,3-triazole (*anti*-1,2,3-triazole). No purification is generally required. This 'near perfect' reaction has become synonymous with CC, and is often referred to as 'The Click Reaction'. This powerful bond forming process has proven extremely versatile, and has driven the concept of CC from an ideal to a reality. A detailed review describing the intricate mechanistic aspects of this remarkable reaction has recently been published.[4]

In order to introduce the non-specialist to this exciting field of research, this introductory review will highlight recent examples of the application of CC, with particular emphasis on the Cu(I) catalysed Huisgen reaction.

Application of click chemistry in bioconjugation

Bioconjugation encompasses a broad arena of science at the interface between molecular biology and chemistry. Bioconjugation techniques generally involve the covalent attachment of synthetic labels to a biomolecular framework. Examples include the modification of proteins and nucleic acids by incorporation of fluorophores, ligands, chelates, radioisotopes and affinity tags; or modifications such as fusing two or more proteins together or linking a complex carbohydrate with a peptide. The power of bioconjugation extends to the labelling of biomolecules *in vivo*.

Currently, only a handful of reactions have proven useful in this context. Generally, these 'fusion-type' reactions have two complementary components which are orthogonal to the functionality present in biological systems. Examples include carbonyl based formation of thiazolidines, oximes and hydrazones, which also fall under the umbrella of CC. The Diels–Alder reaction and Staudinger ligation have also been recognised as important.

Click chemistry is the latest strategy called upon in the development of state of the art exponents of bioconjugation. As with other applications of CC, and specifically the Cu(I) catalysed Huisgen reaction discussed in this review, the ease with which azides and alkynes are introduced into organic compounds, their bioorthogonal properties and tolerance to a wide range of solvents (including water) make them ideal partners for bioconjugation purposes. Azide functionality is particularly well suited, as it is absent in all known natural

compounds, and despite a high intrinsic reactivity, azides allow selective ligation with a limited number of reaction partners.

The applicability of CC towards bioconjugation was first hinted at in the work of Meldal *et al.*[6] in their seminal publication as the joint discoverers of the Cu(I) catalysed variant of the Huisgen cycloaddition reaction. Their study coincidentally led to the first peptidotriazoles by utilisation of solid phase synthesis techniques, and the 'click reaction'. From their work it was noted that the reaction conditions (*N*-ethyldiisopropylamine and CuI at RT), were "mild and fully compatible with Fmoc- and Boc- peptide chemistry" and that "free amino groups, carboxylic acids, thioglycosides, and Fmoc, ^{t}Bu, trityl, Boc, and Pmc groups were found to be completely stable under the reaction conditions".

Following the discovery and development of the 'click reaction' in water, the potential for facile introduction of varying functionality into the biomolecular environment has been realised. As a result, numerous biomolecules including DNA, peptides, proteins, oligosaccharides and glycoconjugates have been labelled with various appendages. Many of these new molecular entities have proven extremely useful in the study of biological systems.

High density functionalisation of modified DNA was carried out by Carell *et al.*[8] wherein the 'click reaction' was used to post-synthetically decorate alkyne modified DNA. The DNA was synthesised by standard means using phosphoramidite chemistry, but with the incorporation of the modified uridine nucleosides **1** and **2** (Fig. 1).

Azides **3–5** (Fig. 1) were chosen for conjugation, since they represented potentially useful labels. Azido-sugar **3** is a semiprotected aldehyde used for selective Ag staining, whereas coumarin **4** fluoresces only after triazole formation, and **5** is a fluorescein azide which is used in a variety of biophysical applications. The DNA was successfully labelled using the 'click reaction' with the added ingredient

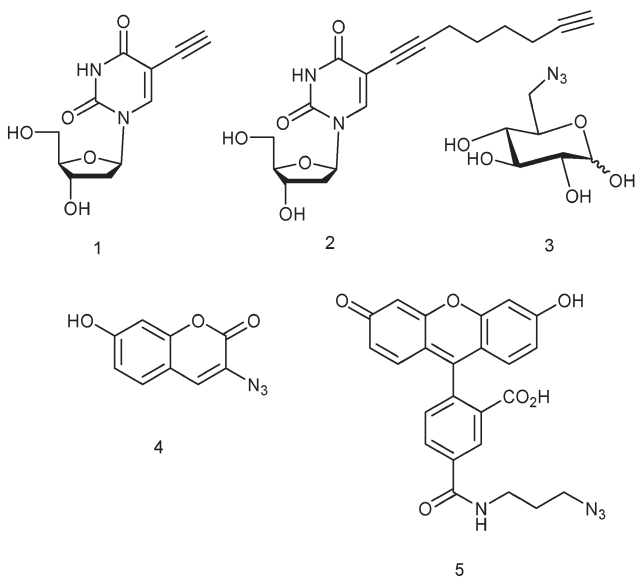


Fig. 1 Alkyne modified uridine nucleosides (**1** and **2**) and azide labels (**3–5**) used in the high-density functionalisation of alkyne modified DNA.

tris(benzyltriazolylmethyl)amine, a ligand which stabilises the Cu(I) oxidation state.[9] In the absence of the stabilising ligand DNA was fragmented, whereas upon stabilisation of the Cu(I) oxidation state, conversion to the coupled triazole product was achieved with no apparent degradation. This result was in line with previous observations that the addition of the Cu(I) stabilising ligand protects biomolecules from unwanted aqueous Cu(I) mediated chemistry. Of the two labelling nucleosides **1** and **2**, the former often resulted in partial conversion, whereas **2** resulted in fully labelled DNA strands. These studies demonstrated that highly reliable and complete high-density functionalisation of oligodeoxyribonucleotides is made possible by applying CC principles.

CC has also been shown to be a useful tool in the ligation and decoration of peptides and peptoid structures. Eichler *et al.*[10] performed ligation of peptides to form assembled (ligation of two protein fragments) as well as scaffolded (peptide fragments ligated on to a multivalent peptide scaffold) peptides. This technique has proven to be compatible with several types of peptide functionalities. For example, three peptidic azides were sequentially 'clicked' onto a cyclic peptide. This was achieved by incorporating orthogonal protection at three points on the oligomer, followed by selective deprotection and decoration with alkyne functionality and finally the 'click reaction' itself, to give the scaffolded peptide depicted in Fig. 2. This strategy offers great potential in the development of combinatorial libraries of assembled and scaffolded proteins.

The 'click reaction' has been applied in the not too far removed arena of peptidomimetic oligomers. Biomolecular-like oligomers consisting of *N*-substituted glycine monomer units are known as peptoids. These and other peptidomimetics have a range of interesting properties, and have been shown to form a range of secondary structures, as well as displaying some potentially useful biological activities. Such peptoid structures presented an ideal application for CC as a means of ligation and chemical conjugation. Work carried out by the Kirshenbaum group presents two variations for decorating peptidomimetic scaffolds. The first enabled access to oligomers, derivatised with a repeating azide–alkyne motif following post-translational CC modifications.[11] A peptide oligomer, modified with an alkyl–azido or propargylalkyl functionality at certain regular points along the chain, was synthesised.

Following conventional solid phase synthesis, the azide–alkyne bearing peptide chain was treated with a complementary coupling fragment, then cleaved from the support, thus

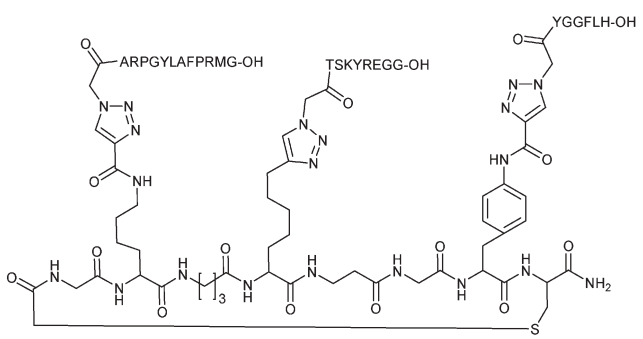
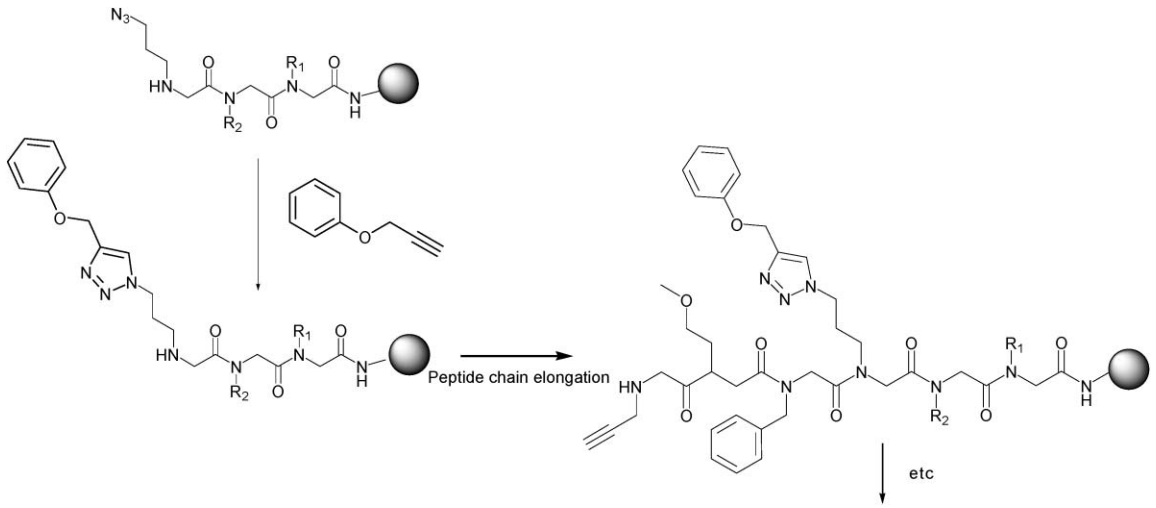


Fig. 2 Scaffolded peptide functionalised *via* click chemistry.



Scheme 3 Sequential synthesis of highly functionalised peptoid oligomers containing multiple functionalities.

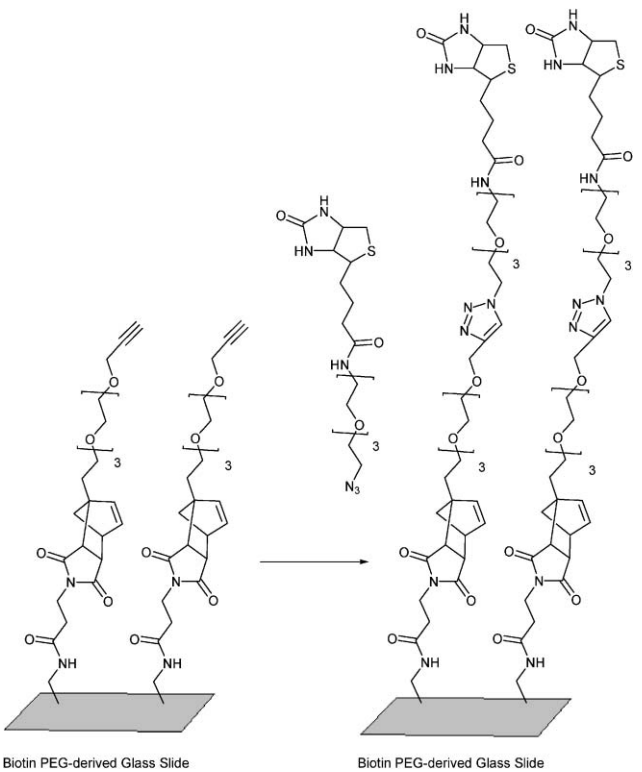
arriving at the decorated oligomer. The group subsequently developed a complementary synthesis, enabling the preparation of peptoid oligomers which contained multiple functionalities. This was achieved by reacting the azido/alkynyl bearing peptoid fragment *via* the 'click reaction' before each round of peptoid chain elongation, thus presenting only one 'naked' azide/alkyne functionality in the peptoid molecule at any point (Scheme 3).[12]

The application of CC in the straightforward derivatisation of biomolecules and pseudo-biomolecules provided the foundations for more challenging tasks in the field of bioconjugation.

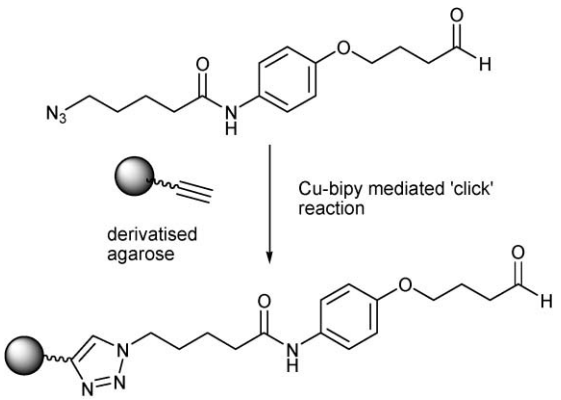
One particularly novel example by Chaikof *et al.* involved the immobilisation of carbohydrates and proteins onto solid surfaces.[13] Successful immobilisation of biomolecules with preserved activity onto solid surfaces is an increasingly useful technique with many potential applications such as the development of microarrays, microbeads and biosensor chips. There are few reliable methods applicable to the immobilisation of biomolecules onto solid surfaces, including adsorption, direct covalent attachment, or non-covalent interaction between a polypeptide and an appropriately derivatised surface. Limiting factors have been found to be reduction in biomolecular activity through denaturation, random orientation of the biomolecules on the surface, and deleterious reactions at or near the active domain. Recently, work has been carried out which incorporates the triazole formed *via* CC as a linker between a glass slide derivatised with alkyne bearing substituents. The slide was then treated with a selection of azide derived substrates, with varied recognition properties (biotin, lactose and truncated thrombomodulin). Each of the three labels was shown to successfully bind the appropriate fluorescein (FITC)-labelled protein partners (streptavidin for the biotinylated surface, lectin for lactose labelled, and a protein that specifically binds an *N*-terminal peptide sequence found on the thrombomodulin protein (Scheme 4).

These results demonstrated that the 'click reaction' is ideally suited for immobilisation of carbohydrates and proteins, without the production of unwanted side products, and that this protocol could be used to immobilise a wide range of substances onto solid surfaces.

In the above example, a Diels–Alder (DA) reaction between a cyclopentadiene derived linker molecule and a maleimide-derivatised solid surface was employed to immobilise the alkyne functionality onto the glass slide. This reaction was also of key importance to the success of the work. Although the Cu(I) catalysed Huisgen reaction between azides and terminal alkynes (click reaction) has received much of the CC limelight, it is important to recognise the contribution and significance of



Scheme 4 Immobilisation of biotinylated PEG onto solid surface using click chemistry.



Scheme 5 Click chemistry in the development of agarose based affinity chromatography agents.

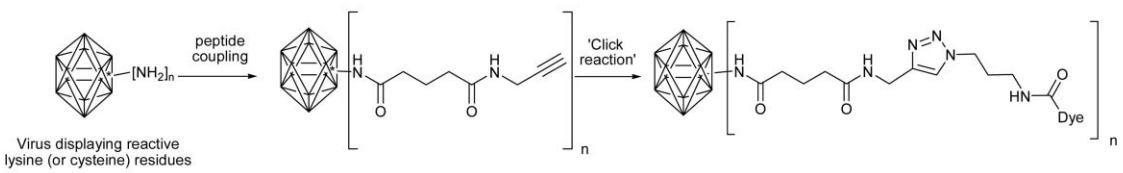
other reactions which meet 'click' status. Unlike the main body of 'click' reactions which generally form carbon–heteroatom linkages, the classic DA reaction is a carbon–carbon bond forming process. Nevertheless, there is much application in the arena of bioconjugation where the reliability and chemoselectivity of the DA reaction have been advantageous, particularly with the discovery of a general rate enhancement of this [4 + 2] cycloaddition process in water. The DA reaction may therefore described as a 'click' reaction itself,[1] as it offers modularity, is wide in scope, and produces no by-products. An example of a more intricate use of the DA reaction in bioconjugation was demonstrated by Grandas *et al.*[14] In their work, the [4 + 2] cycloaddition between a diene-modified oligonucleotide (synthesised using traditional phosphoramidite chemistry) and a maleimide-derivatised peptide (synthesised using standard solid phase chemistry) was used in the synthesis of peptide–oligonucleotide conjugates in aqueous media.

Affinity chromatography exploits various pairing interactions that exist between certain biological molecules (for example the exclusive interactions between antibodies and antigens, carbohydrates and lectins, and enzymes and inhibitors). These interactions are used to analyse or purify mixtures of biomolecules. One member of the interacting species is covalently immobilised onto an insoluble support, and then upon passing a solution of biomolecules down the column, the desired species from the mixture is bound to its interacting partner on the solid support and thus isolated.

Traditionally, the support was prepared by the reaction of the appropriate interacting species with beaded agarose bearing reactive groups such as amine, thiol, carboxylic acid, aldehyde or hydroxyl. However, complications can arise due to the lack of selectivity inherent in the aforementioned functionalities, which may result in ligands being bound in such a way to be detrimental to its affinity function (when more than one of the attachment units is present in the ligand), or when other functional groups on the ligand compete with the immobilisation chemistry. Yet again CC represented an attractive solution. Finn *et. al.*[15] demonstrated that agarose beads could be readily functionalised with azide and terminal alkyne groups, and could then prove to be successful in various affinity based applications. Azide and alkyne functionalised beads were synthesised using standard amide chemistry upon carboxy-link agarose containing free reactive amine groups. Upon arrival at the reactive resin, the feasibility of functionalisation using CC was demonstrated by the successful irreversible attachment of dye or fluorescent molecules, and observation of the maintained optical characteristics of the beads after washing with DMF. The biomolecular binding properties were then tested by using a biotinylated alkyne derivative which was covalently attached to azide derived agarose beads. The resulting agent was the used to successfully bind avadin HABA (HABA = 2-(4′-hydroxyphenyl)azobenzoic acid) complex, determined by the observation of a persistent red colour in the beads. The power of this particular click chemistry approach to immobilise a polypeptide bearing various functionalities, such as carboxylic acid, thiol, primary amine and alcohol functionalities was also achieved. Successful affinity chromatography was demonstrated using an aldehyde based affinity agent, useful in purification of certain antibodies by virtue of reversible imine formation. Protection/deprotection steps would have been a necessity to attach such an aldehyde unit to agarose using standard amide/ether bond formation, whereas click chemistry was used in one step to attach the molecule (Scheme 5) to the functionalised agarose, and this agent was then successfully used in the appropriate antibody purification.

One particularly striking application of CC was reported by Finn *et al.*,[16] in their studies on the conjugation of fluorescein dye molecules onto the cowpea mosaic virus. The virus itself resembles a cage-like molecule, formed from 60 identical copies of a two-protein asymmetric unit, which surrounds the genetic information in the core. The virus particle presents functionality on its exterior surface, including the free amines found in lysine, and thiols found in cysteine residues. It was at these points that the virus particle was decorated with azide and alkyne functionality, *via* peptide coupling (Scheme 6) and thio-ether formation.

In initial studies, fluorescein was conjugated to three different functionalised virus particles, *via* the 'click' reaction. Some important conclusions were made from this work; firstly the use of ascorbate or *p*-hydroquinone reductants led to substantial disassembly of the virus capsid, and secondly,



Scheme 6 Functionalisation of cowpea mosaic virus particle.

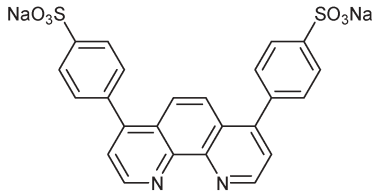


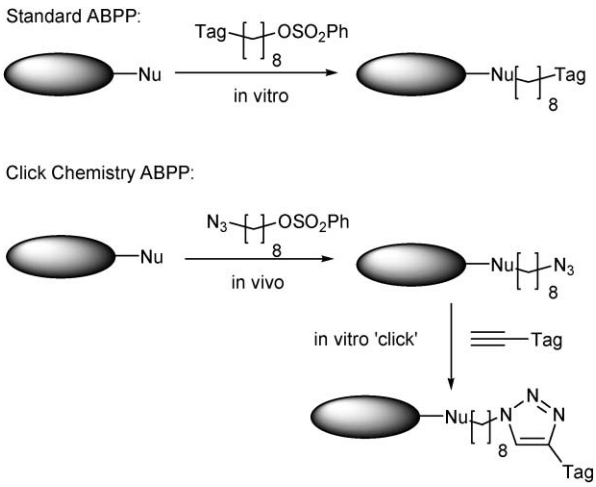
Fig. 3 Water soluble sulfonated bathophenanthroline ligand.

triazole formation in the presence of Cu(II) led to virus decomposition, despite the virus being stable to Cu(II) alone. Again, it was found that addition of the tris(benzyltriazolylmethyl)amine ligand could protect the virus from Cu–triazole induced disassembly.

However, in a further study by Finn *et al.*,[17] the procedure was developed to overcome the need for large excesses of expensive substrate molecules necessary in order to allow the reaction catalysed by the tris(benzyltriazolylmethyl)amine ligand (itself not very soluble in water) to proceed at a useful rate, and to alleviate the possibility of protein damage arising from insufficient amounts of ligand being available in solution. A new water-soluble sulfonated bathophenanthroline ligand (Fig. 3) was employed. When compared to the tris(benzyltriazolylmethyl)amine ligand under otherwise identical conditions, a much lower concentration of labelling substrate was required. Using the modified procedure, various appendages were conjugated to the virus particles *via* the 'click' reaction, including complex sugars, peptides, poly(ethylene oxide) polymers, and the iron carrier protein transferrin. Coupled products from the modified procedure included cases that were previously resistant to azide—alkyne coupling using the conventional ligand tris(triazolyl)amine. This work paved the way for further developments in the field of *in vitro* and also *in vivo* CC.

Bioconjugation has proved particularly fruitful in the field of activity based protein profiling (ABPP). This technique involves the engineering of molecules which comprise active site-directed probes with broad target selectivity. The probes are employed to help determine the functional state of enzymes in complex proteomes. These site directed probes generally contain two key elements: a reactive group for binding and/or covalently labelling the active sites of a particular enzyme class, and a reporter tag, *e.g.* biotin and/or a fluorophore, for detection and isolation of probe-labelled enzymes. Generally, the isolation of these probes is preceded by homogenisation of the cell tissue in question. As a consequence of this homogenisation, there is potential loss of information relating to the differences in protein activity in the relevant physiological setting (disruption of cell tissue may cause differences in concentrations of substances affecting protein activity). One potential solution would be to carry out the ABPP *in vivo*. The ABPP probes are however typically quite large due to their bulky reporter tags, which limits their cellular uptake and distribution.

Click chemistry offers a solution to the *in vivo* labelling problem. Cravatt and co-workers have demonstrated that the bioorthogonal azide and alkyne functionalities involved in the 'click reaction' can be passively incorporated into an enzyme binding substrate for use in ABPP.[18,19] These functionalities

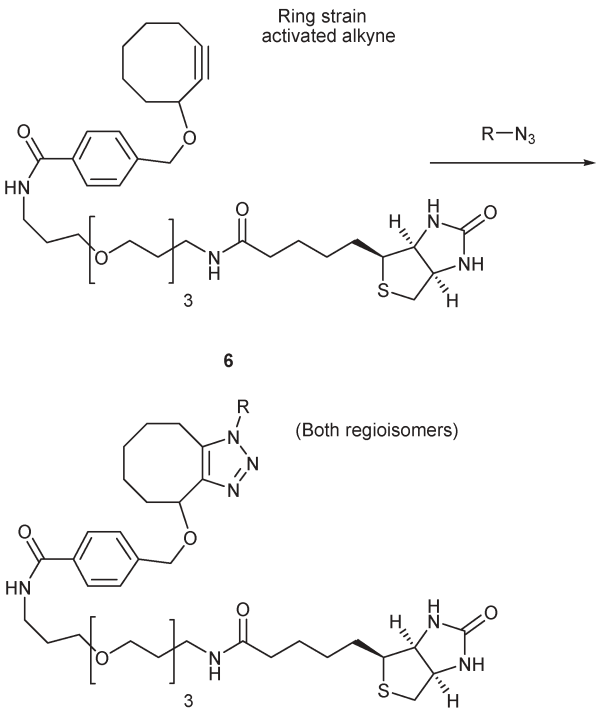


Scheme 7 Comparison of standard and 'click' activity-based protein profiling.

can form an inert handle which can be 'clicked' with bulkier reporter tags [*i.e.* only the relatively small azide and alkyne activity based probes are administered *in vivo*, followed by *in vitro* analysis, using CC to attach the reporter tags before analysis] (Scheme 7). The procedure was developed and tested to evaluate whether it could be used in the identification of targets of enzyme inhibitors *in vivo*, and secondly to test if homogenisation did in fact affect enzyme activity profiles. Mice were used to test whether the CC–ABPP method could identify changes in enzyme activity *in vivo* due to the actions of chemical inhibitors, such as disulfiram, which acts to inhibit the aldehyde dehydrogenase (ALDH-1). After treatment with an alkyne reactive fragment, the mice were sacrificed. Disulfiram treated mice displayed a 2.6-fold reduction in ALDH-1 labelling intensity compared to vehicle-treated animals. It was also shown by the CC-ABPP method, that homogenisation did indeed affect enzyme activity profiles.

The enzyme activities of living cancer cells were tested *in vivo* using the CC–ABPP method, and the results of these tests showed that some enzymes were labelled more strongly *in vivo* compared to *in vitro*, suggesting that homogenisation results in the activation of some proteins. These developments are enabling the visualisation and tracking of complex biopolymers. However, the 'click reaction' is still only performed after sacrifice and lysation of the animal, due to the toxic nature of the copper catalysis.

Ideal bioconjugation reactions can be carried out without affecting living tissues, and do not require ancillary catalysts (namely copper which has been found to exhibit considerable cell toxicity) and ligands such as tris(2-carboxyethyl)phosphine (TCEP) and tris(benzyltriazolylmethyl)amine. The classic uncatalysed Huisgen reaction has been developed towards becoming this 'perfect bioconjugation reaction' by Bertozzi *et al.*[20,21] The group employed a reactive 'spring-loaded' cyclooctyne alkyne fragment activated by ring strain (Scheme 8). In this manner the activation energy for the cycloaddition process is lowered, enabling this powerful cycloaddition reaction to be carried out at lower temperatures and without need for catalysis. The group initially employed this modified reaction



Scheme 8 Octoalkyne fragment utilised in strain promoted Huisgen reaction.

towards *in vitro* labelling of biomolecules. Azide-functionalised GlyCAM-Ig (formed from expression of the recombinant glycoprotein GlyCAM-Ig in Chinese hamster cells (CHO) in the presence of peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz), leading to incorporation of *N*-azidoacetylsialic acid (SiaNAz) into its glycans) was incubated with the cyclooctyne modified biotin containing fragment **6** overnight. Biotinylation was observed for GlyCAM-Ig modified with SiaNAz, whereas native GlyCAM-Ig lacking azides showed no background labelling. A similar reaction was performed using biotin functionalised with a terminal alkyne. This experiment showed no glycoprotein labelling in the absence of copper, whereas with the addition of $CuSO_4$, TCEP, and a triazolyl ligand it resulted in facile labelling of the azido modified glycoprotein.

Taking this powerful methodology to the next level, Bertozzi *et al.* performed *in vivo* labelling experiments with live Jurkat cells.[20] After incubation with 25 μM Ac4-ManNAz for 3 days, the cells displayed SiaNAz residues in their surface glycoproteins. The 'functionalised' cells were then treated with the alkyne probe **6**, followed by staining with FITCavidin. Consequently, the cells displayed a dose-dependent increase in fluorescence upon treatment with the cyclooctyne probe.

Application of click chemistry in materials science

Dendrimers are large regularly branched synthetic molecules which continue to receive much attention due to their unique properties and applications in medicinal and materials chemistry.

After more than 25 years of dendrimer synthesis, problems continue to arise due to the difficulties of purification, and lengthy chromatographic separations of impure products. These problems have somewhat been addressed by the fidelity of the Cu(I) catalysed 'click reaction'. The first example of a CC based dendrimer was synthesised by Fokin *et al.*[22] utilising a convergent pathway. Individual 'branches' were first synthesised by sequential 'click reactions' onto a bis-alkynyl scaffold, which comprised a labile Cl group, amenable to substitution with azide anion at a later stage. This first generation dendrimer was 'clicked' onto another bis-alkynyl core to yield the second generation dendrimer *etc.*, resulting in a number of products including the dendrimer depicted in Fig. 4.

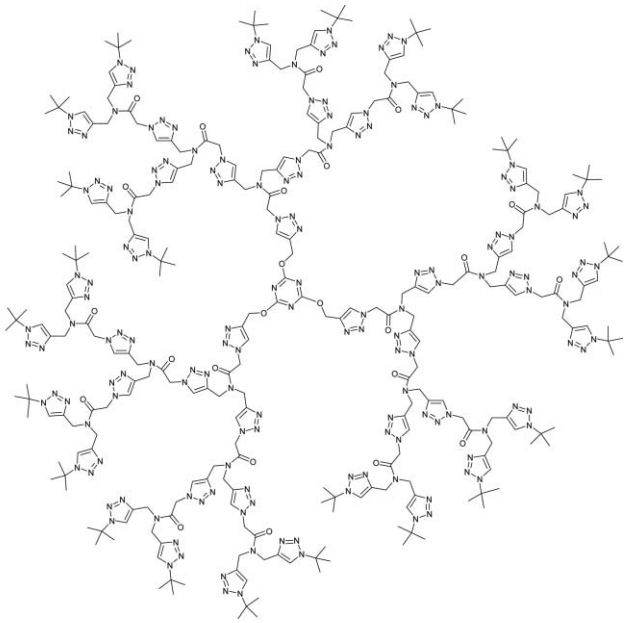


Fig. 4 Fokin *et al.* dendrimer synthesis using click chemistry.

This synthesis required only stoichiometric quantities of reagents, and the reactions were mostly driven to completion, greatly simplifying purification at each stage. Dendrons propagated to the 3rd and 4th generation were synthesised with a variety of monomers and chain-end azides, and all second-generation and some third-generation dendrimers were isolated directly as pure solids.

Click chemistry has been further adopted in the synthesis of bivalent dendrimers, containing both mannose binding and coumarin fluorescent units.[23] These have been shown to be highly efficient, recognition/detection agents for the inhibition of hemagglutination.

The fundamental cornerstones of CC—high efficiency, fidelity and ease of work-up—make it an ideal system for polymer synthesis. Less efficient transformations generally suffer from incomplete reactions as a result of the steric inaccessibility of the reaction site in a polymer structure. Using the 'click reaction', Haddleton *et al.*[24] achieved the synthesis of neoglycopolymers (sugar derived polymers). These compounds are receiving much attention due to their potential medical applications and favourable interactions with protein receptors (known as the 'glycoside cluster effect'). Alkyne derived

polymer backbones of both homo- and co-polymer types were prepared, followed by derivatisation with complementary azido-sugar residues, fused together using the Cu(I) catalysed Huisgen reaction, to give model glycopolymers in close to 100% yield. Interestingly, a novel 'co-click' strategy was developed utilising a mixture of two azide bearing sugar moieties (α-mannoside (**8**) and β-galactoside (**9**)) and an alkyne derived homopolymer chain (**7**) to furnish a polymer which contained both α-mannoside and β-galactoside side chains, linked to the polymer backbone *via* a triazole linker (Scheme 9). NMR analysis was used to confirm that the molar ratio of the two incorporated sugar moieties in the polymers was essentially the same as the initial ratio in the 'co-click' reaction.

The CC functionalisation of macromolecular structures provides the basis for more complicated processes in the arena of polymer synthesis and functionalisation. Grayson *et al.*[25] investigated the use of CC in the development of novel polymer architectures, namely the synthesis of macrocyclic polymer structures. Linear polystyrene precursors functionalised with azide and alkyne groups at their termini were prepared. Continuous addition of these linear precursors to a solution containing the Cu(I) catalyst in the form of CuBr and bipyridine prevented competing condensation reactions, and the cyclised polymer was isolated by extraction and precipitation. Both azide formation and cycloaddition steps were nearly quantitative, preventing the need for time consuming purification steps, thus living up to the expectation of CC.

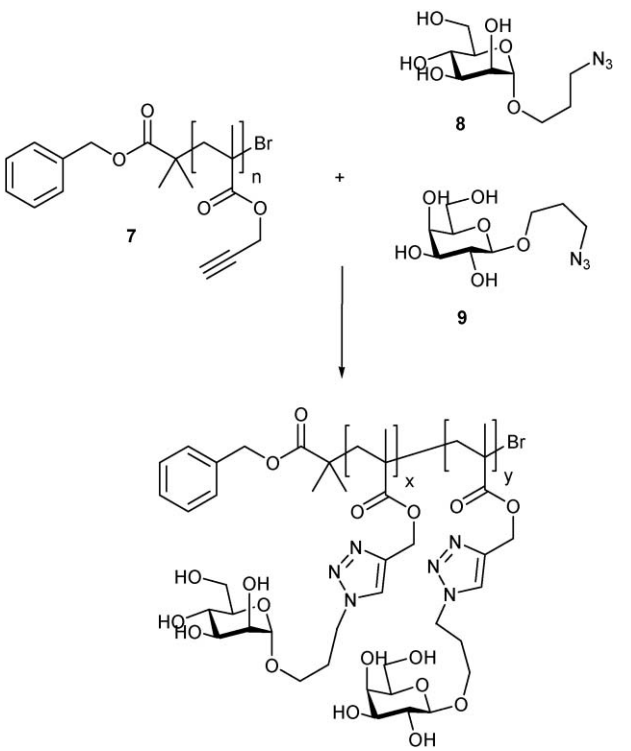
Dendronised linear polymers have many interesting applications, including catalysis, drug delivery and nanoscale electronics. Click chemistry was first applied to this field of science by Fréchet *et al.*[26] In this initial study, dendronised polymers were synthesised by 'clicking' known dendrimer azides of different generations on to a poly(vinylacetylene) scaffold. Again, results for 1st and 2nd generation dendrimers highlighted the efficacy of the 'click reaction', with quantitative yields being observed. It was only upon attempting reaction with the 4th generation dendrimer azide that the product was unobtainable. Due to this fact, it was hypothesised that the 'click reaction' was kinetically controlled, *i.e.* that copper acetylide formation was rapid, but accessibility of azide was the rate determining step. It was supposed that in the aqueous environment, the azide functionality of the 4th generation dendrimer would be shielded by hydrophobic interaction with the dendron branches.

This work was complemented by Monteiro *et al.*[27] with the synthesis of 3-miktoarm star polymers and 1st generation mikto dendritic copolymers using CC. Miktoarm star polymers contain arms with different chemical compositions. They have applications in drug delivery, diagnostic assays, nanopatterned structures and photonics. The group first synthesised a series of well defined miktoarm stars using the 'click reaction', and polystyrene (PSTY), poly(*tert*-butyl acrylate) (P^tBA), poly(acrylic acid) (PAA), poly(methyl acrylate) (PMA) polymers. Azide derivatives of these polymers were synthesised, then 'clicked' onto a tripropargylamine core (Scheme 10). The first polymer was reacted with an excess of tripropargylamine to give the 1st arm, then the second polymer was added in two equivalents to the mono-arm product to give the tri-substituted product in good yields.

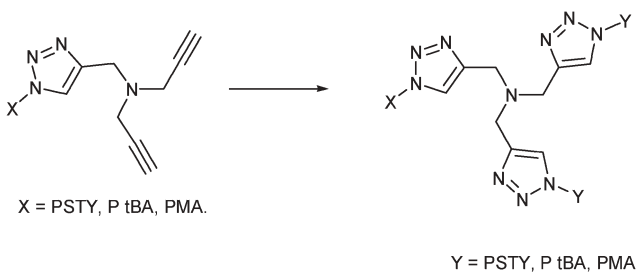
A modified version of this protocol was also used to synthesise 1st generation polymeric dendrimers, which contained 2 pairs of polymeric arms separated by a polymeric chain.

In the field of materials chemistry, CC is being applied in more 'off the wall' applications.

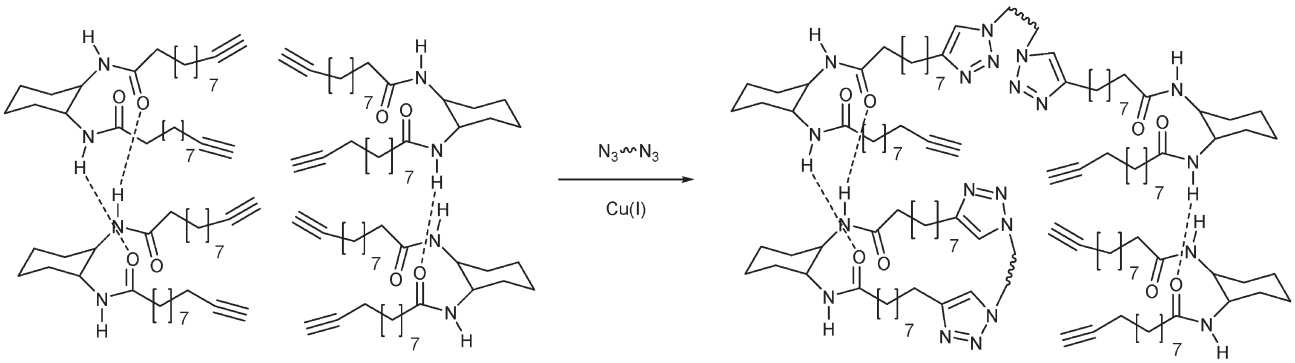
Organogels are substances which undergo molecular self-assembly. Their aggregation into fibrous networks is driven by multiple weak interactions such as dipole–dipole, van der Waals, and hydrogen bonding. They are useful substances, and have found numerous biomedical applications ranging from drug delivery to tissue engineering scaffolds. Until the advent of CC, cross-linking of organogels, which serves to enhance gel thermostability, has traditionally relied upon uncontrolled reactions, like free radical chemistry. Numerous groups have since employed the 'click reaction' in the cross linking of hydrogels. One such example is in the stabilisation of low molecular weight organogelators based on the undecynylamide of *trans*-1,2-diaminocyclohexane, synthesised by Finn *et al.*



Scheme 9 Synthesis of neoglycopolymers using alkyne derived polymer and azido derived sugars in a 'co-click' reaction.



Scheme 10 General synthesis of 3-miktoarm star polymers using the Cu(I) catalysed 'click reaction'.



Scheme 11 Cross linking of organogelators using azide cross-linkers.

(Scheme 11).[28] These materials comprised alkynyl functionality which was subsequently cross-linked with a variety of bis-azide cross linkers.

Click chemistry has been applied to the very fashionable field of nanotechnology. Single walled carbon nanotubes (SWNTs) have potential applications in molecular electronics, sensors, field emission devices, and components in high-performance composites. The insolubility of these structures presents problems when it comes to solution phase processing. Sidewall modification with polymeric structures has been shown to improve solubility, and functionalisation with polymers allows control over the final properties of the nanotube–polymer conjugate. Adronov *et al.* used CC to successfully functionalise single-walled carbon nanotubes with polystyrene (Fig. 5).[29] The 'click reaction' enabled high density functionalisation, by incorporation of a small alkyne bearing reactive species on to the surface of the nanotube, followed by coupling of these fragments to terminal azide bearing polystyrene chains. The group were able to functionalise the carbon nanotube to such a density that the composite material consisted of about 45% polymer. The materials synthesised exhibited high solubility in a range of organic solvents.

Microcontact printing is a surface engineering technique used to pattern self-assembled monolayers (SAMs) as etch resists or chemical templates on gold and silicon oxide substrates. Chemical synthesis can also be carried out utilising microcontact printing in which case the nanoscale confinement between stamp and substrate increases reaction rate. This technique has potential applicability in the development of biological arrays by the localised immobilisation of biomolecules onto surfaces. Reactions which have been carried out previously by this method include the printing of amines onto reactive anhydride SAMs, peptide bond synthesis by printing *N*-protected amino acids onto an amine SAM and also the formation of imines by microcontact printing of amines onto aldehyde SAMs. Recently CC, and particularly the Huisgen cycloaddition reaction has shown promise as an alternative means of this type of chemical functionalisation.[30] The cycloaddition reaction was carried out successfully between azido-terminated SAMs on silicon oxide and 1-octadecyne without the need for Cu(I) catalysis. This was possible because of the high local concentration of reagents between the surface and the stamp, which drive the reaction to completion within minutes without need for catalyst. The utility of the process was further demonstrated by the printing of fluorescent alkyne LRA (lissamine rhodamine with a terminal acetylene unit) onto the azido modified SAM. The well defined printed areas were then visualised using fluorescence microscopy.

Inspired by the potential use of CC in the synthesis of dendrimers based on polyvalent scaffolds, Finn *et al.* developed a new class of triazole containing crosslinked polymers employing di-, tri-, and tetravalent azides and alkynes capable of adhesion to metal surfaces.[31] Encouraged by the precedent for metallic copper to be a convenient source of Cu(I) for catalysis of the azide–alkyne cycloaddition (from comproportionation of Cu(II), from oxidation of metal surfaces, and Cu(0)), as well as the known affinity of 1,2,4- and 1,2,3-triazoles for metal surfaces, it was proposed that polymeric 1,2,3-triazoles would demonstrate adhesive properties. Indeed it was demonstrated that spreading a mixture of a variety of different polyvalent azide and alkyne building blocks in the minimum amount of solvent on the surface of the two metal plates led to adhesion. Many of the samples tested led to strong bonds to copper, and mixtures involving tripropargyl amine and the trialkyne **10** (Fig. 6) significantly outperformed commercial glues.

The copper surface serves as a source of Cu(I) ions for the formation of the copper acetylide at the metal surface as well as in the developing organic matrix responsible for fusing the

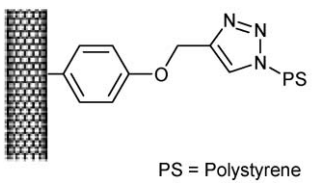


Fig. 5 Functionalisation of carbon nano-tubes using the Cu(I) catalysed 'click reaction'.

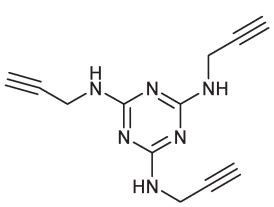


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Fig. 6 Trialkyne used in the synthesis of adhesive polymers.

two surfaces together. Other interactions with the metal surface (as well as multiple triazole–metal interactions between the growing polymer and the metal surface) include those from the dangling alkynes in σ- or π-based interactions. It was also considered that the extraction of catalytic Cu(I) ions from the metal could 'etch' the surface, leading to an ill-defined metal–organic boundary at the site of interaction, and further increase adhesion. Various factors affecting the efficacy of the glues derived from differing monomeric mixtures, catalysts and sample loading were noted, and the best adhesives were found to be those formed from mixtures of monomers with increased numbers of 'arms', relating to the amount of cross-linking which could take place. It was also discovered that amine containing monomers were beneficial as they assisted in the production of Cu–acetylide intermediates. As polytriazoles bind to metals of different kinds, the method was also tested using zinc metal instead of copper. The zinc surfaces were not adhered to each other by mixtures of monomers alone (as is the case for copper), but when a Cu(I) source was added to the mixture the load bearing capacities of the adhesives were comparable to those of commercial glues, and in the range of those tested on copper. The Cu(I) source used was either Cu(I) salts, or Cu(II) salts with Zn metal as the reducing agent to generate/preserve the active Cu(I) state.

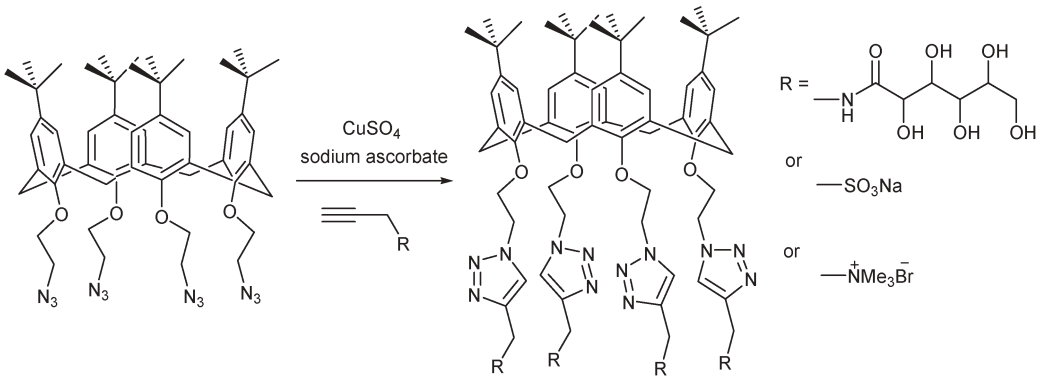
Supramolecular refers to the area of chemistry which focuses on the noncovalent bonding interactions of molecules. Calixarenes have become useful building blocks in supramolecular chemistry. More specifically, water soluble calixarenes with their well formed hydrophobic cavities make it possible to study molecular recognition in water. Introduction of these water soluble groups is often hampered by the poor functional group compatibility of the reactions involved (*e.g.* sulfonation). In order to overcome such problems, Ryu and Zhao employed a CC approach towards the synthesis of water soluble calixarenes.[32] These compounds were synthesised *via* two complementary routes. The first involved preparation of polyvalent alkynyl decorated calixarenes that were then treated with water soluble azides using the Cu(I) catalysed Huisgen reaction. The second route involved polyvalent azido-decorated calixarenes, which were treated with a range of water soluble alkynes (Scheme 12). These reactions were high yielding, and it was found that coupling between azidocalixarenes and water soluble alkynes gave much better yields than those between alkynylcalixarenes and water soluble azides, because of possible side reactions between the alkynes in close proximity on the alkynylcalixarene substrate.

Click chemistry has also found application in the synthesis of rotaxanes. These complex structures have become readily accessible due to advances in template based approaches, and until recently, stoichiometric amounts of templating catalyst were required. Click chemistry has been applied in a substoichiometric metal-templating pathway to produce mechanically interlocked architectures. In the work carried out by Leigh *et al.*[33] a Cu(I) atom co-ordinated to the macrocycle acts as both a catalyst and a templating agent. The two fragments, one azide and one alkyne, are then ligated *via* co-ordination with the Cu(I) ion such that the two fragments are threaded through the initial macrocycle as depicted (Scheme 13).

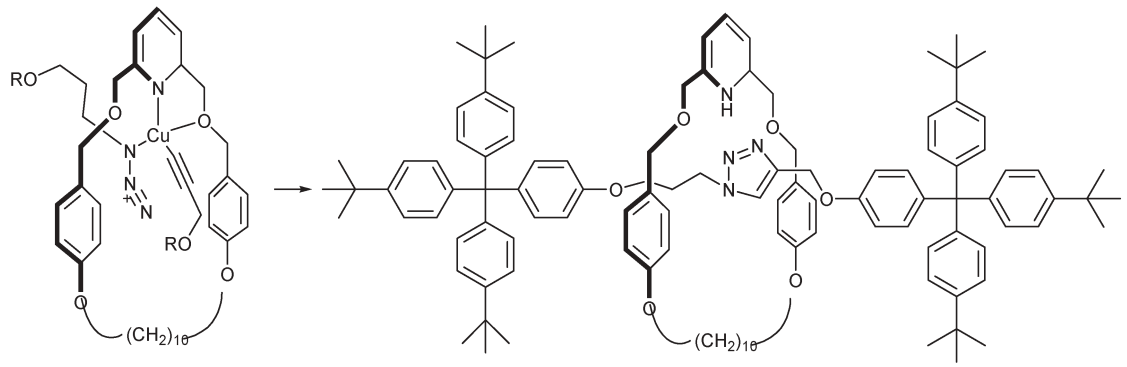
Another, loosely related example of CC appearing in this section is in the popular field of catalysis. The CC power to produce large numbers of diverse organic compounds simply and efficiently has been exploited in the synthesis of triazole based ligands for transition metal catalysts. The main impetus for the design of the ligands was that they offered a means to attain great ligand variation, and thus to tune the catalytic properties of the system such as activity, selectivity and stability, all of which are dictated by the electronic properties of the ligands co-ordinated to the metal. Two classes of compound are described here. Firstly compounds of the generic structure **11** in Fig. 7.

These ligands known as 'ClickPhos', are readily prepared,[34] and their palladium complexes have provided excellent yields in the amination and Suzuki–Miyaura coupling reactions of unactivated aryl chlorides.

The second class of compounds, named 'Clickphine', are novel catalysts of the P,N-type ligand family (Scheme 14).[35] These catalysts are useful in the regioselective Pd-catalysed allylic alkylation reaction. Furthermore, this particular type of solid supported triazole based catalyst can be recycled. Thus the 'click reaction' was performed using both a previously reported azide decorated carbosilane dendrimer, as well as polystyrene supported azide. The formation of the appropriate solid phase metal complexes was confirmed by NMR, and the reactivity of the polymer supported catalyst was shown to only decrease slightly upon recycling.



Scheme 12 Preparation of water soluble calixarenes.



Scheme 13 Copper templated synthesis of a rotaxane compound.

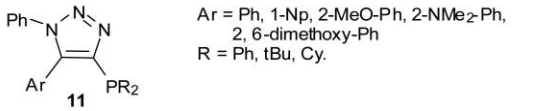
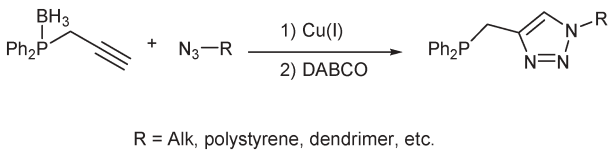


Fig. 7 Generic structure of 'ClickPhos' ligands.



Scheme 14 Synthesis of 'Clickphine' type ligands.

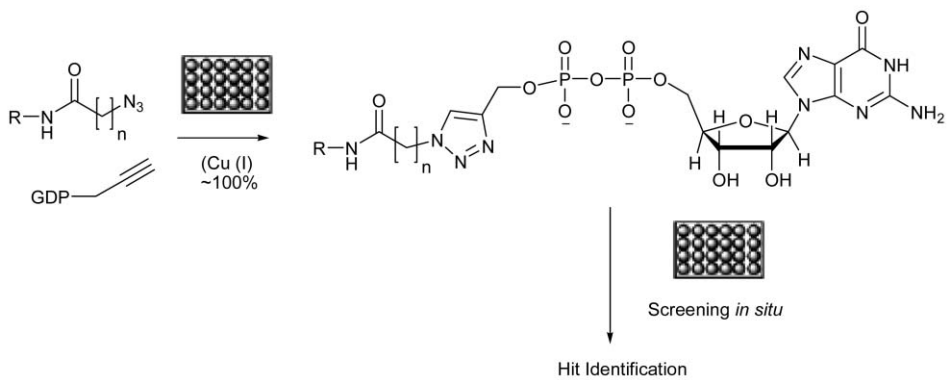
Application of click chemistry in drug discovery

In the demanding world of medicinal chemistry, CC is impacting right at the core, with significant development of novel approaches to screening compound libraries. The reliability of the 'click reaction', means that compounds can be screened directly from the reaction mixture. This was demonstrated, by Wong *et al.*[36] in a paper wherein the Cu(I) catalysed Huisgen reaction was utilised in the development of high throughput methodology which led to the discovery of a novel and selective Inhibitor of Human r-1,3-Fucosyltransferase (Fuc-T). The enzyme is responsible for the catalysis of the final glycosylation step in the biosynthesis and expression of many important saccharides. Previous strategies employed to identify inhibitors of Fuc-T generally relied upon the design of acceptor, donor and transition state analogues. Difficulties arise, however, due to the complexity of the transition state composition, which consists of sugar donor, acceptor, divalent metal, and nucleotide. It was known the majority of the binding energy of Fuc-T for its substrate resulted from a guanosine diphosphate β-L-fucose (GDP-fucose) moiety (which is responsible for the transfer of the L-fucose to the corresponding glycoconjugate acceptor), and the hydrophobic pocket adjacent to the binding site of the acceptor molecule, which enhances the affinity of the acceptor molecule by 70-fold.

A range of azide fragments (85) were synthesised, which comprised hydrophobic residues and alkyl chain linkers of varying length. These were then coupled with an alkyne GDP core in a 96-well microtiter plate, *via* the 'click reaction', and the crude product formed was screened directly (Scheme 15). Three hits were observed from the screens. Scale-up, purification and re-testing of these compounds revealed that the most active agent had an inhibitory constant K_i (comp) of 62 nM, making it the first known nanomolar inhibitor of Fuc-T.

Recently, we have demonstrated another example of the use of CC in lead discovery, but more importantly, we have demonstrated further possibilities for the triazole functionality. In this study, the 1,4-triazole group not only functioned as a linker, but as a key part of the pharmacophore.[37]

The G-quadruplex is a tertiary structure of DNA which has become an illustrious target in the field of cancer therapy. It has been shown that stabilisation of G-quadruplex structures by small molecule ligands can cause inhibition of the enzyme



Scheme 15 Triazole synthesis in microtiter plate for screening *in situ*.

telomerase which is responsible for the 'immortality' of cancer cells. Although there are many molecules which have demonstrated the ability to bind strongly to the G-quadruplex, few have apparent selectivity for the G-quadruplex as opposed to other tertiary structures of DNA. However, to date there are no telomerase targeting cancer therapies available. We aimed to remedy this situation by addressing the rate of discovery of new target ligand structures by applying CC and a fragment based approach. *En route* to this goal, we investigated whether the triazole functionality could actually form part of a planar pharmacophore, which is normally a prerequisite for G-quadruplex ligands. Several ligands were prepared and shown to stabilise human telomeric G-quadruplex structures, and consequently inhibit the function of telomerase (Fig. 8). Moreover, these ligands were found to be highly selective towards G-quadruplex structures even in the presence of large excesses of duplex DNA. Thus CC, enabled the rapid and efficient synthesis of a new class of telomerase inhibitors.

In the last decade, novel means of lead discovery approaches have been investigated in which the biological target is actively involved in the synthesis of its own inhibitory compound. These fragment-based approaches, also termed target-guided synthesis (TGS), show great promise in lead discovery applications by combining the synthesis and screening of libraries of low-molecular-weight compounds in a single step. Of all the TGS methods, the kinetically controlled variant is the least well known, but it has potential to emerge as a reliable lead discovery method.

Irreversible TGS discovery relies on low binding affinities of fragment molecules, or building blocks with complementary reactive sites, which, when mixed in the presence of a biological target molecule, combine irreversibly to (ideally) form inhibitors of the target, which are preferentially selected due to the highest affinity. Recently, several elegant and thorough experiments have demonstrated the efficacy of the 1,3-Huisgen cycloaddition towards this application, and this has been the topic of recent review by Sharpless and Manetsch.[5] The particular properties of this reaction which make it ideally suited for the discovery of lead compounds through *in situ* target directed synthesis are: (1) the reaction is extremely thermodynamically favourable, but kinetically slow at room temperatures. However, in TGS, kinetic barriers are overcome by the templating of the two reactive species, which puts the reactive functionalities in close proximities, thus allowing the selection of high affinity pairings (only when both fragments are bound to the substrate can reaction occur); (2) the reaction does not involve any third party participants, such as catalysts or other reagents. The reaction proceeds cleanly without the formation of by-products (which otherwise may interfere to an unknown extent with delicate tertiary structures of the biomolecules of interest, and alter the 3D structure of the binding site); (3) bioorthogonality, *i.e.* both azide and alkyne functionalities are inert in the presence of biomolecules, and can survive in biological conditions. Click chemistry has been successfully employed to a selection of biological targets, with the discovery of improved inhibitors produced by more conventional strategies.

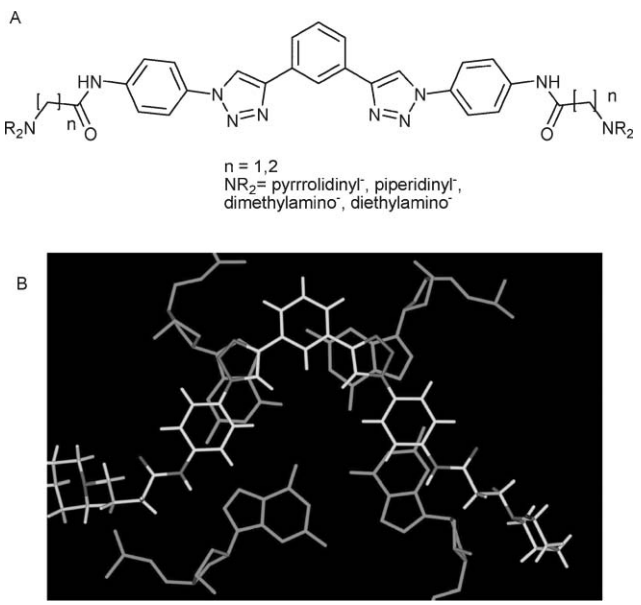
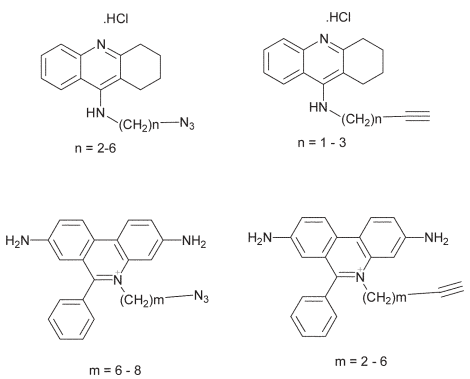


Fig. 8 (A) A generic structure of the five contiguous ring telomerase inhibiting G-quadruplex ligand, synthesised using the Cu(I) catalysed Huisgen reaction; (B) a qualitative molecular model showing the overlap of the ligand with a G-tetrad.

For example Sharpless *et al.*[38] recently described the application of acetylcholinesterase, an enzyme which is involved in neurotransmitter hydrolysis in the central and peripheral nervous systems, in the application of TGS. This enzyme was chosen since much structural data were available (including the structure of the active site), small molecule ligands for both parts of the active site were known and inhibitors that span both parts of the active site are known. It was envisaged that a triazole link made between two of these small molecule inhibitors appropriately decorated with complementary alkyne and azide functionalities could potentially form *in situ*, to produce a new 'bivalent' inhibitor of the enzyme. Sixteen building blocks were synthesised allowing for 98 potential inhibitors, 34 possible regioisomeric pairs (*syn* (1,5) and *anti* (1,4) triazoles) formed from mixed tacrine–phenthridinium adducts, and 15 regioisomeric pairs formed from only tacrine–tacrine triazoles (Scheme 16).

Previous work had shown that an uncatalysed reaction between a tacrine azide and a phenthridinium alkyne proceeded at a negligible rate, and that the uncatalysed reaction



Scheme 16 Alkyne and azide, tacarine and phenthridium fragments utilised *in situ* in the presence of acetylcholinesterase.

would take approximately 40 years to reach 80% completion. It was reasoned that if a product was formed in detectable amounts in the presence of enzyme, then it would suggest that the reaction was templated by the enzyme. In the 49 reactions, one tacarine azide and phenthridinium alkyne combination produced detectable product. Non- specific enzyme interactions were ruled out by blocking the active site, thus preventing product formation. The tacrine–phenthridinium 1,3-triazole product formed in the study is the most potent non-covalent AChE inhibitor known to date. After this initial foray into the application of CC to irreversible TGS, and successful proof of principle, Kolb *et. al.* produced a follow up paper,[39] describing improved sensitivity of the analytical method, by employing LC/MS. The increase in sensitivity allowed for the reduction in reaction times from 6 days to 6 hours, and smaller amounts of product adduct could be detected, further enhancing the efficiency of the *in situ* lead discovery protocol. Armed with this new method, the group revisited their tacrine and phenylphenanthridinium library. As a result of the new screen, three new hit compounds were identified, which displayed good binding affinity to AcHE, though not as great as the original hit. In these compounds, the triazole acts as a pharmacophore, due to observed hydrogen bonding, and π-stacking interactions between the triazole and neighbouring amino acid residues from the crystal structure.

The group have also developed this protocol one step further by demonstrating that the CC *in situ* screen could be used not only to assemble binary adducts from a complementary azide–alkyne pair, but could pick the most potent combination from a mixture of fragments. A single tacrine azide anchor was incubated with four alkyne phenanthridinium building blocks. This experiment resulted in the formation of the expected product (the potent inhibitor), and encouragingly, when the combinational screen was expanded to contain the entire library of fragments, the mixed *in situ* screen led only to the expected product formation. The experiment displays the potential for CC in the wider arena of drug discovery, due to the ability of the triazole linker functionality to act as a pharmacophore.

This brand of *in situ* CC has been developed further with other enzyme targets, namely carbonic anhydrase (CA),[40] and HIV-1 protease.[41] These two systems were studied in much the same way as the AcHE system but with some important differences that widen the scope of *in situ* lead discovery. Firstly, in the CA system, only the alkyne fragment, an acetylenic benzenesulfonamide (selected based upon previous research which had highlighted the inhibitory nature of 4-carboxybenzenesulfonamide derived compounds towards CA) showed any binding affinity for the target, thus acting as an anchor for the azide fragments. 24 azide functionalised compounds which were selected to cover six different structural themes, weighted towards α-azido carboxamides, analogues of the known peptidic carboxybenzenesulfonamide derived inhibitors. 12 out of the possible 24 azide–acetylene combinations displayed *in situ* hits, 11 of which did not display hits in the corresponding control experiments. This result broadens the scope of *in situ* CC even further, by demonstrating that an inhibitor with increased binding affinity can be formed from a fragment which shows moderate binding (in this case, the alkyne fragment) coupled with a fragment that shows no binding (the azide fragments).

In the case of HIV-1 protease, only weakly binding fragments were available. However, the *in situ* experiment generated a potent inhibitor of this important target from two complementary azide and alkyne fragments. The reaction was confirmed to be catalysed by the enzyme active site with the usual control experiments, thus demonstrating that fragments with no inherent affinity could 'click' together *in situ* to produce a potent inhibitor.

Interestingly, the *in situ* CC approach has been incorporated into a microfluidic chip device, which could perform in parallel 32 *in situ* reactions with reduced consumption of target proteins and reagents, as well as reduced reaction times.[42] By selecting the known bCAII system described previously, Tseng *et al.* demonstrated that the new microfluid format gave the same results as control reactions carried out in 96-well plates.

Conclusion and outlook

In the short period since click chemistry was conceived, it has had a dramatic and diverse impact in many areas of modern chemistry. Research and development in this field are still increasing exponentially, and this review is intended to provide an introductory overview of CC to demonstrate the diversity of applications of this chemical strategy.

The versatility of CC and particularly the Cu(I) catalysed Huisgen cycloaddition (or 'click reaction') seem endless, yet we are still in the early developmental stages of this concept driven research. With the discovery and invention of new chemical transformations which meet 'click' status, the future looks bright for CC.

Acknowledgements

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