Click Chemistry – insights to help you choose when and how to use click chemistry for innovative applications

Content

1. Why using click chemistry? 1
2. Click chemistry in different applications 3
2.1 How click chemistry can ameliorate cell proliferation detection 4
2.2 Current methods, procedures and limitations of short nucleic acid modification 5
2.3 Click aptamers – nucleic acid antibodies 5
2.4 Pole position in bioconjugation for RNA conjugates & mRNA drug development 6
2.5 Click chemistry in DNA and RNA sequencing, sample preparation & system reagents 7
2.6 Innovative technology and products 7
3. Summary – are you now prepared for your click chemistry experiments? 8

1. Why using click chemistry?

Even after almost two decades, since the initial concept of click chemistry was developed by Sharpless, Kolb and Finn in 2001[1], creative new applications demonstrate the unique power and modularity of these type of reactions. The idea was to join small modular compounds by a few very good reactions to generate chemical diversity instead of using a plethora of reactions that would need extensive optimization for each transformation. The copper-catalyzed version of the Huisgen cycloaddition (CuAAC) between a terminal alkyne and an azide is arguably one of the most popular click reactions.[2,3] The reaction proceeds in the presence of many functional groups in good to excellent yields in aqueous solvent (mixtures) and has resulted in more than 1000 research articles based on the reaction within the first decade after its discovery.[4] The strength of the click technology becomes crucial when it is performed in complex mixtures, e.g. for the detection of cell proliferation. Therefore, key applications comprise molecular cell biology, next generation sequencing (NGS), polymerase chain reaction (PCR), molecular diagnostics (MDX) and nucleic acid drug development, in situ hybridization (ISH), and many more. But even today new applications and technologies based on the CuAAC principle are being developed.

What renders CuAAC superior in many applications compared to the state-of-the-art or alternative click reactions, like e.g. copper-free click reactions? And how can innovative applications benefit from this expertise?

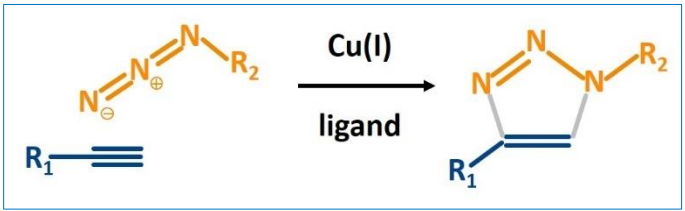


Figure 1: CuAAC -efficient formation of a single product

Click-chemistry: One "simple" reaction – multiple options

Due to its widespread application, the copper(I)-catalyzed reaction between a terminal alkyne and an azide (CuAAC) has become "the classic click reaction". The reaction proceeds in the presence of many functional groups in good to excellent yields in aqueous solvent (mixtures) and results in a single stable triazole (1,4-regioisomer only). Apart from the efficiency and the modularity of the reaction, several other properties render the CuAAC superior to many other click reactions. The required functional groups can be introduced by rather simple chemical transformations, they have good long-term stability and they have a relatively low steric demand. The latter property is one major reason for acceptance of click-ready building blocks by many enzymes, like e.g. polymerases, glycosyl transferases and many others. Moreover, since neither azides nor terminal alkynes are generally present in natural systems, the background signals from side reactions are eliminated.

Some advancement in click protocols have been key to the development of click based applications. Most protocols use Cu(I) chelating ligands, like TBTA, THPTA or BTTAA. These ligands serve a dual role: They protect the biomolecule from free copper in solution that could generate oxidative species, but at the same time they increase the reaction kinetic. Thus, they enable to perform reactions at lower copper salt concentrations, decrease the incubation time or allow reactions at lower reactant concentrations.

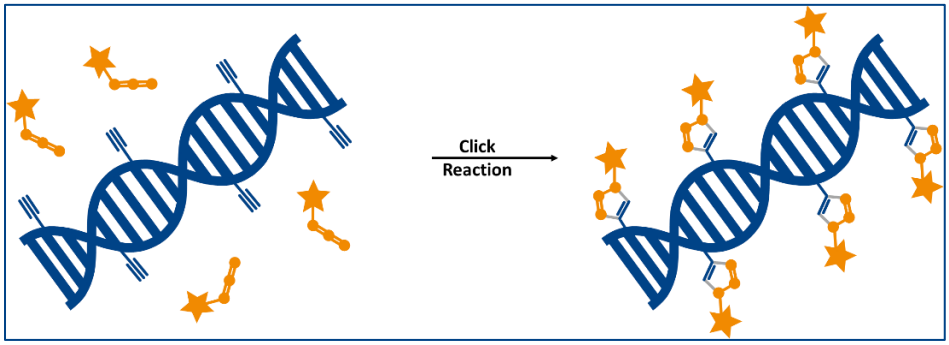


Figure 2: Click Chemistry-based nucleic acid labeling works for RNA & DNA modification with high bioorthogonality and superior versatility (and very broad spectrum of functional moieties can be made "click-ready").

Despite the simple outcome of the reaction, the formation of a covalent bond between two reaction partners, the detailed catalytic cycle most likely involves two Cu(I) species and the mechanism is matter of an ongoing debate.

Copper catalyzed click chemistry has gained high interest by researchers and most recently by pharmaceutical industry, expending its wide application fields continuously. As the reaction is only depending on the azide and alkyne partners and precursors are easily made available, theoretically there is no limitation in the functional moiety to be attached. At the moment we offer various carbohydrates, dyes, fatty acids, peptides, vitamins, small molecules, nucleotides and oligonucleotides with click-ready functional groups. By continuously developing new click applications (see some of them presented below), we are convinced that the importance of this enabling technology will even increase.

2. Click chemistry in different applications

Fueled by scientific creativity numerous applications have been developed based on the "simple" click chemistry, which range from bioconjugation of DNA[5], RNA[6], proteins[7] and carbohydrates[8] to sophisticated detection applications. At baseclick we mostly focus on DNA and RNA based applications that are enabled by click chemistry.

Figure 3: One Click – multiple applications.

2.1 How click chemistry can ameliorate cell proliferation detection

Cell proliferation is of fundamental importance for all living organisms, as it is the basis for growth and regeneration. Before cells can divide, the genetic information in form of the DNA needs to be duplicated. The ability to replicate is an important property of a cell and allows evaluation of several processes which can influence the fitness and viability of a cell, e.g. toxicity of the substances added to the cell medium. This can be a desired effect when drug candidates against cancer cell lines are screened.

For decades tritium labeled thymidine (^{3}H thymidine) was fed to cells and allowed sensitive detection of cell proliferation by detecting radioactive emissions in the cells that had incorporated the nucleoside into their *de novo* synthesized DNA. Despite the sensitivity of this cell proliferation method, the radioactive hazard during handling and disposal has prompted scientists worldwide to look for a non-radioactive replacement.

The BrdU assay and the EdU assay are both non-radioactive alternatives for the ^{3}H thymidine assay in cell proliferation direct analysis. In both cases the method relies on metabolic activation of a thymidine analogue and incorporation into DNA. However, detection of BrdU and EdU labeled DNA differs considerably. While the BrdU assay has a fluorophore-labeled antibody for detection, which forms a non-covalent interaction with BrdU containing DNA, EdU containing DNA is detected by covalent attachment of fluorophore azides.

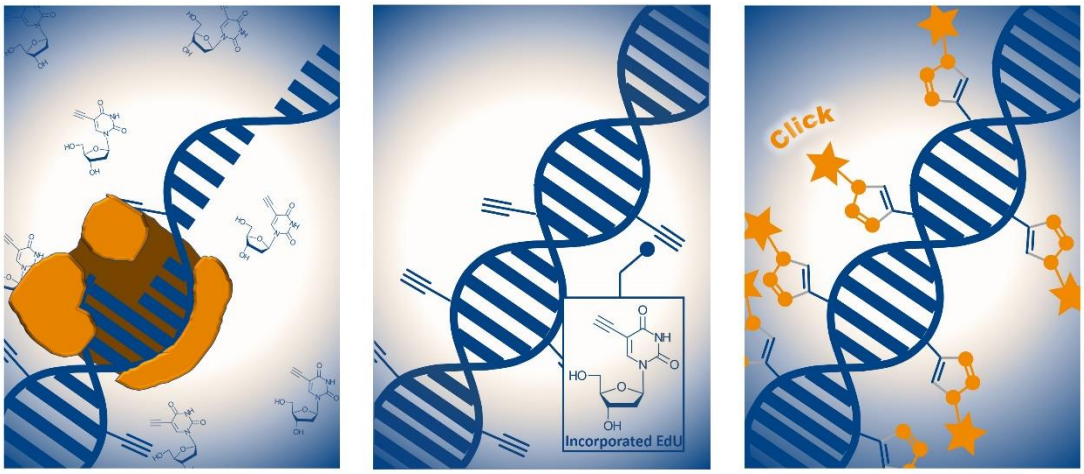


Figure 4: Schematic representation of click chemistry-based EdU cell proliferation assays. A) Incubation of cells with EdU. B) EdU is incorporated during active DNA synthesis. C) Detection of cell proliferation via click chemistry, wherein the use of a range of different fluorescent dyes is possible.

Taking advantage of the easiness or the click reaction, the EdU based proliferation test has already gained wide acceptance. It is important to note that for the EdU assay, both functional groups undergoing the detection reaction, alkynes and azides, do not occur in natural cell systems and do not react without catalytic reagents. Thus, no undesirable further cell compartments or components are falsely marked using this procedure (onlike the BrdU technique). This results in the high selectivity, sensitivity and

reproducibility. Furthermore, the technology allows for an almost unlimited selection of different and easy to synthetize fluorescent dyes and offers maximum flexibility for the choice of the detection mode. But not only the "detection color" is selectable, but also the readout equipment. Cell proliferation can be detected by fluorescence microscopy, flow cytometry, and high throughput screening. Currently, commercial assays exist for the application of the assay in these three analytical procedures, and four standard dyes (FITC, Cy3, Texas Red, Cy5 and their analogs) are offered worldwide.

Easy protocol, fast procedure and reliable results are just some characteristics of the EdU assays. For a detailed description on the methods and a comparative overview, we would like to draw your attention to the extra white paper we have prepared for you. It is also available in our webshop (section Learn / Cell Proliferation)

2.2 Current methods, procedures and limitations of short nucleic acid modification

Currently, short modified nucleic acids (with a length of 100 nt) are prepared using respective building blocks in solid phase synthesis. These could be either already e.g. dye-labeled phosphoramidites or functional groups ready for post-synthetic coupling with dye molecules. Anyhow the use of dye-building blocks in the oligonucleotide synthesis can be cumbersome, due to the relative harsh conditions for synthesis and deprotection. Therefore, post-synthetic labeling e.g. *via* amine/NHS-ester, thiol/iodoacetamide or maleimide chemistry is one preferred option to introduce labels into oligonucleotides. This allows introduction of dye molecules that are incompatible with solid-phase synthesis, but these methods suffer from low efficiency and limited scalability and thus require large excess of dye.

Since the discovery of click chemistry an ever-increasing number of oligo houses and customers have recognized the great advantages of this technique. Therefore, it is a state-of-the-art labeling method, not only when it comes to complex and multiple labeling of nucleic acids with sensitive labels but also for PNAs and LNAs. Most importantly marker azides used for click functionalization are stable to hydrolysis, which allows storage in solution (in contrast to sensitive NHS esters and maleimides).

2.3 Click aptamers – nucleic acid antibodies

Antibodies help our body to repel pathogens and have become valuable therapeutics against diseases like e.g. cancer. Aptamers are DNA or RNA oligonucleotides which possess specific binding activities similar to antibodies. Since aptamers are smaller, are evolved *in vitro* and can be prepared by synthetic methods, they have some interesting advantages compared to antibodies from mammalian cell culture production. For example, it was demonstrated that aptamers have superior detection properties compared to antibodies, when they are applied in cellular imaging.[9]

Scientists and companies worldwide are currently developing methods for efficient production and testing of aptamers. In contrast to antibodies, many of the handling steps can be automated and thus save resources (including animal models). As DNA and RNA are mostly composed of four building blocks, the

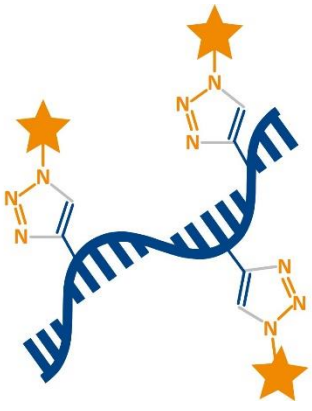
chemical diversity of the resulting polymers is decreased compared to the 20 (21) amino acids in proteins. This has drastically limited the number and properties of the targets that aptamers can normally bind.

The invention of the so-called click-SELEX procedure has at least partly overcome this bottleneck for the aptamer technology. In click-SELEX an alkyne-modified nucleotide is used for enzymatic incorporation during PCR and the resulting oligonucleotide is subsequently clicked with functionalized azides in order to increase the chemical diversity of the nucleic acid.[10] Due to a relatively small sterical demand of the alkyne-modified nucleotide, 5-ethynyl deoxyuridine, polymerase acceptance is almost as good as for a natural nucleotide. Moreover, the post-enzymatic introduction of chemical functions *via* click enables straightforward preparation of modified oligonucleotides with a diversity that can hardly be achieved by any other method. Thereby the success rate to find a good target binder is increased and the physico-chemical properties of the oligonucleotides can be modulated.

2.4 Pole position in bioconjugation for RNA conjugates & mRNA drug development

In recent years RNA therapeutics are becoming a novel class of drugs which should enable transient gene therapies against diseases that have no cure so far.[9] The first FDA approved siRNA drug, Patisiran, from Alnylam marks an important cornerstone for all RNA therapeutics technologies. Key to this landmark achievement was conjugation of the siRNA with N-acetylgalactosamine for receptor-mediated uptake, which enables targeted delivery of the oligonucleotide drug. Due to the small size of the siRNA molecule, production by chemical synthesis was possible and enabled a medicinal chemistry approach for lead structure optimization.

As mRNA therapeutics are at the advent of becoming drugs, they face even greater challenges compared to such siRNAs. Due to their much larger size, chemical preparation is not an option and therefore mRNA is produced *via in vitro* transcription from a DNA template. So far, chemical modification of the resulting mRNA is mostly limited to the nucleotides that are accepted by the RNA polymerase during transcription. Therefore, targeted delivery of mRNA is much more complex and is achieved by modifying transfection agents instead of the mRNA itself.



Other current solutions to these challenges are based on optimized sequence design, and the incorporation of modified nucleotides during mRNA production. Due to the complexity of the molecules, chemical modifications and the preparation of conjugates, e.g. a tissue-specific uptake of mRNA, are still mostly unknown.

Click chemistry offers the ideal conditions to chemically modify mRNAs easy and effective. Incorporation of clickable groups, enables "decoration" of the mRNA with a multitude of reporter molecules, stabilization reagents or even target-specific units is possible. This could be the breakthrough for a widespread usage of RNA in therapy. We are convinced that click chemistry could become an important option to overcome the remaining challenges in mRNA drug development.

2.5 Click chemistry in DNA and RNA sequencing, sample preparation & system reagents

Sequencing technologies for nucleic acids have evolved at a tremendous pace and are considered as a key technology for diagnostics and might represent the analytical basis for personalized medicine. Despite enormous technological improvements, there is an ongoing need to increase efficiency, throughput and thus cost. This is achieved by decreasing the size of individual sequencing reactions and by massive parallel setups.

Already today, some methods sequence a single DNA molecule in real-time. In order to achieve this, reagents which are required for the sequencing reaction must meet strict specifications. For some of these reagents, this is only feasible when using click chemistry as it combines high yields in complex mixtures with mild reaction conditions and can be used to simply create chemical diversity.

Real-time sequencing is based on a principle in which the sequence of bases is determined during the enzymatic incorporation of ultra-labeled nucleotides. Therefore, the signals of nucleotide incorporation must be clearly distinguishable from the background and the nucleotides must meet the highest quality standards.

Our partners have already experienced that click chemistry is the favorite for their preparation of highly complex four different fluorophore-labeled nucleotides in NGS to achieve the modularity, the high efficiency and the high yield.

Moreover, chip surface density can be increased aiming for augmented sequencing efficiency, higher throughput and drastically decreased costs as the click reaction of click ready surfaces with clickable adapters is almost quantitative and straightforward.

Furthermore, baseclick's "click ligation" of oligonucleotides in NGS library preparation has the power to be a more efficient alternative to enzymatic ligation circumventing e.g. problems related to bias generation. In order to exemplify the advantages of the "click ligation" in this area, we show convenient incorporation of azide modified nucleotides by several enzymes as well as broad acceptance of the obtained triazole linkage by several polymerases. Encouraged by these findings we are developing new protocols, tools and kits in this application field.

2.6 Innovative technology and products

As exemplary shown above the click chemistry technology turns conventional nucleic acids into augmented molecules with "click" anchors. These are well within established workflows, but now ready for the addition of a myriad of useful functional moieties with a simple "click" reaction.

The basic concept allows numerous adaptions to create efficiency disruptions in nucleic acid sample preparation, in labeling and in medicinal chemistry for nucleic acids (DNA, RNA, LNA, others).

Our high-end nucleic acid modification and bioconjugation platform opens the door to accelerated innovation of superior tools, automated read-outs, kits & reagents.

New and value-added applications are developed continuously (see image above - One Click - multiple applications) and is mostly limited by researchers' imagination.

3. Summary – are you now prepared for your click chemistry experiments?

We hope you have got a few new insights into the current click chemistry methods out there. If you have further questions on our kits and their procedures and / or other products, then please to not hesitate to check our FAQs and descriptions online or get in touch directly with us. We are looking forward to helping you getting the best studies.

Selected literature

1. Kolb, H. C., Finn, M. G. & Sharpless, K. B. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chemie - Int. Ed.* **40**, 2004–2021 (2001).
2. Rostovtsev, V. V., Green, L. G., Fokin, V. V. & Sharpless, K. B. A stepwise huisgen cycloaddition process: Copper(I)-catalyzed regioselective 'ligation' of azides and terminal alkynes. *Angew. Chemie - Int. Ed.* **41**, 2596–2599 (2002).
3. Tornøe, C. W., Christensen, C. & Meldal, M. Peptidotriazoles on solid phase:-triazoles by regiospecific copper (I)-catalyzed 1, 3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **67**, 3057–64 (2002).
4. Liang, L. & Astruc, D. The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) "click" reaction and its applications. An overview. *Coord. Chem. Rev.* **255**, 2933–2945 (2011).
5. Gierlich, J., Burley, G. a., Gramlich, P. M. E., Hammond, D. M. & Carell, T. Click chemistry as a reliable method for the high-density functionalisation of alkyne-modified oligodeoxyribonucleotides. *Org. Lett.* **8**, 3639–3642 (2006).
6. Schulz, D. & Rentmeister, A. Current approaches for RNA labeling in vitro and in cells based on click reactions. *ChemBioChem* **15**, 2342–2347 (2014).
7. Ullrich, M. *et al.* Bio-orthogonal labeling as a tool to visualize and identify newly synthesized proteins in Caenorhabditis elegans. *Nat. Protoc.* **9**, 2237–2255 (2014).
8. Zhang, Y. *et al.* Carbohydrate-protein interactions by 'clicked' carbohydrate self-assembled monolayers. *Anal. Chem.* **78**, 2001–2008 (2006).
9. Strauss, S. *et al.* Modified aptamers enable quantitative sub-10-nm cellular DNA-PAINT imaging. *Nat. Methods* **15**, (2018).
10. Pfeiffer, F. *et al.* Identification and characterization of nucleobase- modified aptamers by click-SELEX. **13**, 1153–1180 (2018).