Functional groups in (bio)chemistry

SUMMARY: standard functional groups used in biochemistry for bioconjugation (by alphabetic order).

Reactive functional groups – for bioconjugations/labeling/activation — See NT-XLfctl

- Reactive to **amines (-NH2, -NH-)** & PEGYLu
  Succimide Esters (N-Hydroxy (NHS), Valerate (SVA), Succinate, Glutarate (SG),… | NPC (p-nitrophenyl carbonate) | IC (isocyanate) | Aldehyde (CHO)
  (poorly specific) : Iodo-&Bromo-acetyl (reacts with SH>NH2)
- Reactive to **carboxyls (-COOH)** & PEGYLu
  Hydrazide/EDC | Amine/EDC
- Reactive to **sulfhydryls (-SH)** & PEGYLu
  Maleimide | vinylsulfone (VS) | PyridylThio, OrthoPyridyl-diSulfide (OPSS) | Iodo-&Bromo-acetyl (reacts with SH>NH2)
- Reactive to **alcohols (-OH)** & PEGYLu
  Epoxide | NPC (p-nitrophenyl carbonate) | IC (IsoCyanante)
- **Clickable**-groups See NT-XLclic
  CuAAC & SPAAC (Azide & Alkyne, DBCO, BCN) | Tetrazine/Alkene ligation | Staudinger ligation (Phosphine) | Hydrazone chemistry (Aldehyde & HyNic) | AminoOxy/Aldehyde Ligation (Oxime Chemistry)
- Reactive to solid surfaces : **Gold**, **Glass** and Silicone,… & PEGYLu
  Silane
- Poorly reactive groups : Amine, carboxyl, Sylhydryls, hydroxyl,

Protective groups – for organic chemistry — See NT-XLprgr

- **Amino** protecting groups: reacts with Succinimides, …
  Primary Amines, Secondary Amines, Tertiary Amines, AminoOxy, T, T_2, Be, Ts
- **Carbonyl** protecting groups
  Carboxyl (COOH), Ketones (-CHO), 1,, 1,3-Dithianes et 1,3-Dithiolanes, *N*,*N*-Dimethylhydrazone
- **Carboxyl** Protecting group
  Methyl ester, *t*-Butyl ester, Benzyl ester, 2-Alkyl-1,3-oxazoline, *S*-*t*-Butyl ester
- **Hydroxyl** Protecting group
  Ols: MOM-OR, THP-OR, *t*-Butyl ether,Allyl ether , Bn-OR, TBDMS-OR, TBDPS-OR, Ac-OR, Pv-OR, Bz-OR
  1,2- and 1,3-diols: Acetonide, Benzylidene acetal

Functionnal groups introduction or Modification/Protection/Deprotection in aqueous media — See NT-XLprgr

- **Amine:** reacts with Succinimides, …
  Primary Amines, Secondary Amines, Tertiary Amines, AminoOxy, T, T_2, Be, Ts
- **Carboxyls**
- **Sulfhydryls**
- **Others groups**

Labeling groups

- **Fluorescent labels** (Conventionnal Coumarins/Fluoresceins/Rhodamines/Pyrenes/Others) ; FluoProbes, Cyanines, AFluor, others)
- **Enzymatic labels** (HRP, PA, GO, AchE,…)

Tags (non-chemical/affine binding groups)

- **Avidin/Biotin** family (Biotins, Strep-tag-tgacting, actiTag, SBP-tag), Biosourced tags, Peptide-based tags (Spot-tag, NE-tag, E-tag, Softag, TC tage, V5 tag Ty tag, Xpress tag)

Spacers

Protection&Blocking groups (block chemical reactions useful in conjugation/labelling applictions, removable or not)
See NT-XLprgr

Functionnal groups (chemically reactive groups & poorly reactive groups available for chemical reactions)

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• The **Succimide Ester** groups (-NHS, SVA,…) react readily with amines in aqueous solution at pH 7-10, making it on the most popular coupling method for conjugating and labeling proteins.
See more information XLNHS[NT]

• The **amino** group (-NH2) reacts readily with succinimidyl ester groups (in particular NHS), carboxylic groups (via carbodiimide mediation) and many other amine reactive functional groups either in aqueous buffer or organic solvents.

• The **Aldehyde** group reacts with hydrazides and amines at pH 5-7. The reaction with hydrazides is faster than with amines, making them useful for site-specific crosslinking. It aso react react spontaneously with amino groups to form Schiff base intermediates that can be stabilized by reduction with Sodium cyanoborohydride ($NaCNB_3$).

• The **Carboxyl** group (-COHH) can be conjugated using several conventional chemistries: via carbodiimide mediation to amines, with and many other amine reactive functional groups either in aqueous buffer or organic solvents.

• The **Epoxide** group reacts (PAS d'info encore/NTCrossl) non specifically…

• The **HaloAlkyls** groups react with sulfhydryls, and in lower extend with amines and even other groups.
Iodoacetamide more reacts slowly that iodoacetate. The reaction should be done in slightl molar excess of IA in a dark container to limit the generation of free iodine that may react with other aminoacids. The formed linkage is stable but slow cleavage of one of the amide linkages can occur by hydrolysis.

• The **HyNic** group (an aromatic hydrazine) reacts specifically with aldehydes at pH 5.0–6.0 to yield a stable hydrazone bond. The reaction can be speeded adding aniline catalyzer.

• The **Hydrazine** and **hydroxylamine** derivatives also have amine-like reactivity and, in some cases, can be coupled to water-soluble carbodiimide–activated carboxylic acid groups

• The **Hydrazide** group give a variety of reactions: they react with Carbonyls (aldehydes and ketones) much faster than amine does, making them useful for site-specific crosslinking.
Reaction with carboxyls better occur with activation by a carbodiimide.
Reactions with amines are nonetheless useful, but require mediators (NH_4BO_4, via Shiff'base formation)

• The **Isothiocyanate** group (R–NCS) forms thioureas upon reaction with amines.
The **isocyanate** group (-N = C = O) reacts readily with hydroxyls, forming a stable carbamate bond at pH 7-8.5. However, the formed bond (urethane) is not so stable than esters (cf NHS) or ether (cf Epoxy).

• The **Maleimide** group reacts with thiols readily at pH 6.5~7.5 to form a stable thioether bonds.

• The **Methanethiosulfonate** group reacts very rapidly and specifically with cysteine groups

• The **N-hydroxysuccinimydyl** group **(NHS)** reacts at pH 7.5-9 in aqueous phase on aliphatic primary ($–NH_2$) and secondary amines (=NH), optimally at neutral pH or higher. A peptidic bond (amide link -CO-NH-) is formed.

• The **NitroPhenyl Carbonate** group (**NPC)** can react with both amine and hydroxyl groups.

• The **Orthopyridyl disulfide** group (OPSS) reacts with thiol groups at pH 5.0~6.5 and generates a cleavable disulfide bond.

• The **pyridyl thiol (Pyridyl Disulfide** bridge **)** group reacts specifically at pH7-9 by exchange with sulfhydryls (free SH), leaving a pyridin-2-thione group that can be followed up: maximum absorption occurs at 343nm with an extinction coefficient of $8 . 10^3 M^{-1} cm^{-1}$ (Struchbury 1975).

• The **Thiol** group (SH, Sulfhydryl) is functional group that can be used by conventionnal chemistry. It notably reacts with maleimide, halogeno acetal (I, Br,…), Pyridyl thiols, Thiosulfinates (ThioSulfonates &VinylSulfone). Thiol also reacts to gold surfaces with high level of specificity.

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• The **VinylSulfone** group (VS) reacts with sulfhydryls in a similar manner than Maleimide, but unlike maleimides, are water soluble and are not proone to hydrolysis at neutral pH. Also, Michael addition to vinylsulfones does not generate stereoisomers. Caution may however be payed to the selectivity of the reaction because of possible unexpected reactivity with secondary targets, that depend on the protein structure (pka is modified locally): the ε-amino groups of lysine and to a lesser extent the imidazole ring of histidine side chain (i.e. at pH 7.7).

• The **Tosyl** group can react with amines, thiolates and alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.

Labeling groups

• **Fluorescence tags** are fluorescent moieties that allow direct detection and eventually indirect detection through the use of an affinity partner, typically an antibody specific to the fluorophore.
The most commonly used is **GFP and its variants**, that are proteins naturally fluorescent and used as reporter molecules co-synhetised in vitro or in vivo by cells that have be genetically modified. More advance tags are **folding-dependant reporters** ,e.g. the tag is fluorescent if completely folded, but colorless id not (the tag is portion of GFP, that assemblies upon addition of small fragment, revealing the color, similiarly to a substrate with en enzyme.
Many organic fluorochromes (incl.GFP, which is larger) can by used as as a fluorescent & epitope tag, when an anti-tag antibody is available. E.g. an anti-FITC allows FITC amplication, or at the opposite its quenching.

• **Enzyme tags** are not so popular, or used rather as reporter molecules in cell assays that as a tag.The enzyme is detected by its activity when adding its substrate. For example, luciferase and aquaeporin reporter system or based assays use both luciferin (or sub-type coelenterazine) substrates.

• **Biotin** is a small polar molecule that inds with extreme affinity to (strep)avidine protein. It is used to that point for labeling/detection purposes to create immunoreagents for assays (ABC systems). Serveral analos are available, with lower affinity for purification and pull-down assays.

Biotin | DesthioBiotin | …

Tags & labels

• **Tags** are more or less medium-sized molecules that can bind with high affinity and specifity to a partner molecule (a probe for the tag), generally a larger molecule. Most of tags are small peptides, that allows for their synthesis in biosystems.
Both the tag and the partner can be called ligands, or partner or bait and probe, and in some instances serve as a handle, receptor, catcher, sensor, label…:
The **ligand** term, which refer to the linking ability, is used typically for the smaller partner molecule thus the tag;
The **partner** term, which refer to the complementary role in a couple, is indistinctively for both;
The **bait** term, which refer to the unkwon molecule (or its linker molecule), it typically the tag (linked to biological target)
The **probe** term, which refer to a molecule that can generate a signal specific of a target molecule or condition, is larger partner that is labelled or immobilized.
The **label** term, which refer to a motif (a 'label') that is easy see/visualise, such as a dye/stain/fluorochrome/fluorophore, and even a not colored molecule such as an enzyme wich can produce a color our
The **handle** term, which refer to a molecule that can by handled in a controlled way by an other partner molecule, coorespond generally to the tag, its binding partner immobilized on a resin for purification. Ex polyHis tage with IMAC chelate.

• Many tags are inspired by binding compounds found in living cells : such **bio-originated tags** (and partners) include for exemple the biotin/(strept)avidin, proteinA./G/antibody, MBP/Maltose. Several modifications have been proposed to improve their performance, i.e. to increase or decrease the binding affinity, to increase the specificity and avoid binding in biological systems, to make the tage or its probe detectable or reversible. The bait and/or the probe are ofte of peptidic nature, but some a glycoside nature (MBP) or other organic types ().

• **Epitope tags** are typically short peptide sequences which are chosen because of their high-affinity to specific antibodies . Many are derived from viral genes. They include V5-tag, Myc-tag, HA-tag, Spot-tag and NE-tag and are particularly useful for western blotting, immunofluorescence and immunoprecipitation experiments, and eventually in antibody purification.

• **Fluorescent tags** : are not only tags (binding property) by also fluorescent for direct detection. Popular fluorescent labels can be used as tags (typically using an antibody specific toward the enzyme) are notably for larger compounds:
-when they are good immunogens, specific abs can be used, but this is not popular and generally affects fluorescence properties.
-fluorescent reporter proteins (gfp/yfp/rfp)

• **Enzyme tags**: enzymes are popular indirect labels (reporters) that can be used as tags (typically using an antibody specific toward the enzyme, or an affine partner -substrate or cofactor analog-)

Spacers

The spacer is structure between 2 functionnal groups, typically a molecular arm.

• An **Alkyl** spacer is flexible chain, a carbone backbone typically $(-CH_2)_n$- that is rather hydrophobic. Alkyl spacers are available in various lengths, butbecome too hydrophobic so for bioconjugation applications, there are short (C2, C4) or medium spacer (C6), eventually long ones (-LC- refer to Long Chain, typically C6-X-C6, where is can be noting (C12) or en ether (C) or an amide (-CO-N-)

• The **PEG** spacer is flexible and hydrophilic chain that usuall improves desired functionalities of the groups displayed at its ends. **PEGylation** can modify peptides and proteins and other materials, to create conjugates or to increase solubility and stability and reduce immunogenicity. It can also suppress the non-specific binding of charged molecules to the modified surfaces.

• **Cyclo hexane** ring, as in the classic SMCC, rigidy the thether between comjugated partners. In some applications this is helpful, but in a more general way, this reduc the mobility of partners dans so reduce their interactions, beside steric hindrance reasons.

Reactive functional groups

Following are functional groups that are convenient to use in biochemical reaction with aqueous media. The first sections cover majors reactions, followed by sections covering each individual representative functionnal group. Groups and reactions used with harsher conditions, in organic chemistry, are presented in next upper section.

>Amine-reactive functional groups

●Structure:

[] Amines are described as primary, secondary, or tertiary depending on the number of hydrogen available on the azote atom. Primary Amines are widely present naturally in proteins, but also in some natural forms of lipides, glucosides. They also can be introduced in biomolecules chemically, during synthesis (use of aminoallyl nucleotides), or genetically. Amine can finally be blocked chemically (with NHS-acetate), for site-directed coupling strategies. Amines behave as **bases**, and **nucleophiles**, primary amines being more reactive that secondary, or tertiary amines.

●Reactivity:

[] Amines can easily be coupled :

-to COOH groups via reductive amidation mediated by carbodiimides (see Carboxyls, and COOH/NH2 red.am.coupling)

-to CHO groups (see Aldehydes, and the glutaraldehyde-type NH2 to NH2 reticulation method).

-to any group/molecule via a reactive amine group of many kinds:

Main useful amine-reactive groups are acylating reagents, esters (NHS, ImidoEsters), not excluding other options notably aldehyde (for N-term peptide conjugation) and IsoThioCyanate or SulfonylChlorides (for labeling), but also (for organic chemistry) Epoxide, Acrylate, NPC, Tosyl,…:

More/detailled information : see +[XLNHS_]

.Succinimides esters (NHS, SCM, SS, SVA…) are surely the most popular and easy to use reactive group to modify amines in aqueous solutions, because they perform well in aqueous solution (in specificity and yield, despite competition with hydrolysis). Alternatively,

.ImidoEsters (PFP/**TFP**) are useful when a stronger reaction is needed (a)

.Aldehydes are useful especially for N-terminal modification. They give reductive amination with primary amines to produce secondary amines, in the presence of reducing agents such as sodium borohydride and sodium cyanoborohydride. The pH is important for reductive amination.

.Carboxyls can be coupled to amines once they are activated (i.e. by NHS).

.IsoThioCyanate may be useful for particular bioconjugations, and where popular for labeling biomolecules, but with lower specificity for amines. They react with amine to produce a stable thiourea linkage (less stable than with NHSesters)

.Sulfonyl Chloride may also be useful for particular bioconjugations, and where popular for labeling biomolecules.

.Epoxide perform useful nucleophilic additions, applicable to amines.

.Nitrophenyl Carbonate (NPC) Amine reacts with NPC functionalized PEG under proper conditions.

.**Acrylate** esters react by Michael addition with amine and acrylate . It is a relatively slow reaction.

Many other are less specific and useful in conjugation applications of biomolecules, including HalogenoAcetals, Acrylate, Tosyl, Sulfonyl Chloride, … despite for example the later (SC) have been popular in labeling applications.

Esters (NHS/Succinimides | ImidoEsters; Phenyl Esters)

Ester reagents (NHSuccinimide (NHSuc) | Other Sucinimides (SC, SCp, SPA, SVA, SS, SG) | Imido-Esters | Phenyl-Esters : STP, PFP)

Imido esters

See the TechnicalNotice NT-XLNHS *q*

Halogenated compounds useful for (bio)conjugations include:

-Halogenated Carbonyls: more active that COOH. Reactions less specific, i.e. to methylamine and ethanoyl chloride

-HalogenoAlkanes (-CH2-Br) : reactions to Amines

>Sulfhydryl-reactive functional group

.**Thiol (Sulfhydryl)** is a convenient group to target in proteins, but it also can be introduced chemically or by genetic engineering (via Cys aa). It forms with other thiols by oxidation a disulfide S-S bond (reversible), but more interestingly for conjugation and labeling applications, it reacts specificaaly with several reactive groups (below). As a result, it has been popularized in association with an amine-reaction for crosslinking biomolecules, known as the 'SMCC-type' or Maleimide/Succinimidyl conjugation method.

See below section 'Sulfhydryls / Thiols'

.**Maleimide**: reacts with thiols by Michael addition (on the C=C bond in the maleimic ring) and forms a physiological stable linkage. The best reaction condition is at pH 8. While it is still reactive to thiols even under acidic conditions pH 6-7, it is then not stable in water where it can undergo ring opening or addition of water across the double bond (hydrolyse).

See below section 'Maleimide'
See conjugation methods : 'SMCC-type' or Maleimide/Succinimidyl conjugation method [XL---]

.**VinylSulfone** (VS): thiols reacts by Michael addition with the C=C bond to form a physiological stable thioether linkage.
The reaction is in a similar manner than Maleimide, but unlike maleimides, without its potential hydrolysis. Also, Michael addition to vinylsulfones does not generate stereoisomers.
vinylsulfone reacts slowly with thiols to form a stable thioether linkage to the protein at slightly basic conditions (pH7–8) but will proceed faster if the pH is increased. Although VS derivatives (PEGs) is stable in aqueous solutions, I can react with lysine residues at elevated pH (unlike Maleimide that is more reactive to thiols even under acidic conditions pH 6-7 but it is not stable in water and can undergo ring opening or addition of water across the double bond).

See below section 'VinylSulfone'

.Pyridylthio/**OPSS**: (OrthoPyridiyl DiSulfide) OPSS reacts specifically with SH under both acidic and basic conditions (pH3-10 – but typically done at pH7-9) by exchange with sulfhydryls. The formed disulfide linkages are stable, except in reducing environment when the linkage is converted to thiols. This is taken to good account: Disulfide S-S bond formation can be reversed by reducing agents such as sodium borohydride and thioethanolamine.

See below section 'Pyridyl Disulfide '

.**Halides**: halides (chloride, bromide, iodide, tosylate and mesylate) react with free thiol. Haloalkyl reagents (Iodoacetate and notably **iodoacetamide**) are very reactive towards free thiols/sulfhydryls by nucleophilic substitution, creating a stable thioether linkage. The specificity is not so good as maleimide, as halide generally reacts also ± with amines.

See below section 'HalogenoAcetals'
Iodoacetamide more reacts slowly that iodoacetate(à vérifier). The reaction should be done in slightl molar excess of IA in a dark container to limit the generation of free iodine that may react with other aminoacids. The formed linkage is stable but slow cleavage of one of the amide linkages can occur by hydrolysis.

.**Thiosulfonate**

>Click Chemistries functional groups

Click chemistries are indirect conjugation and labeling using 2 partner functional groups that undergo a very specific reaction. They were introduced by an alkyne/Azide reaction, then popularized using cycloalkyne to avoir the used of a catalyzer, and finally click-like reactions includre Phosphine-Azide based (Staudinger) ligations, and Tetrazine-Alkene based ligations.

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Alkyne – Azide Ligation (for standard Click chemistry – CuAAC)

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See the TechnicalNotice NT-XLClic *q*

Introduction

Copper-chelating ligands that improve efficiency and biocompatibility of CuAAC :

- THPTA
- BTTAA
- BTTES
- BTTP

CycloOctynes: DBCO – Azide ligation (for Copper-free Click chemistry – SPAAC)

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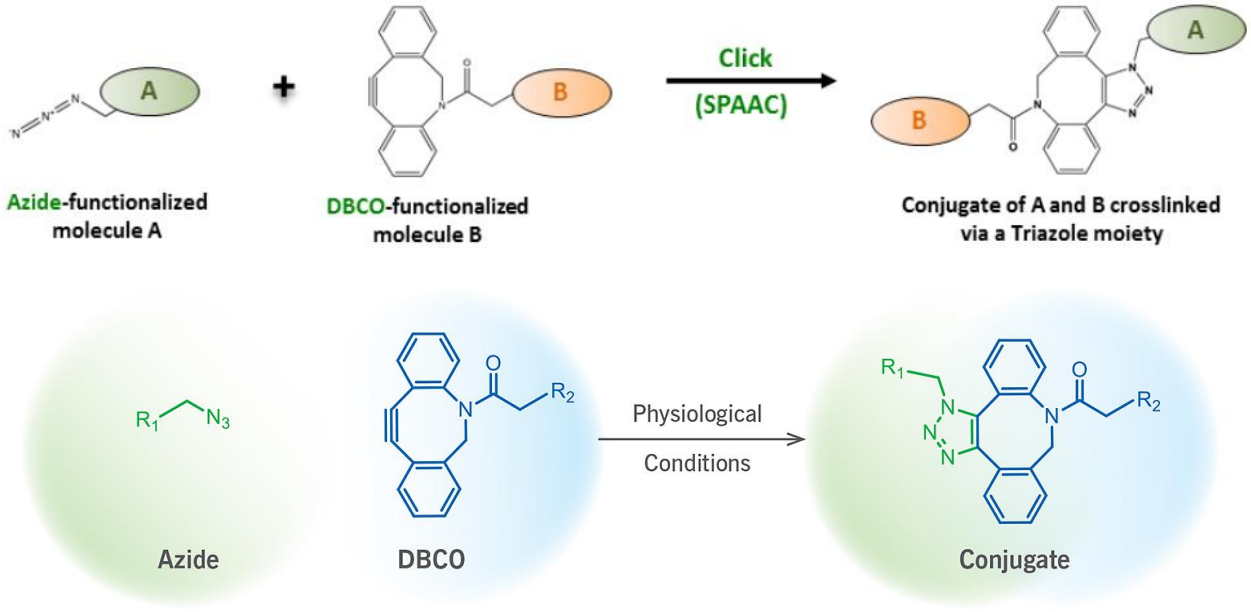
Features:
•Faster detection of small-sized Azides compared to CuAAC reactions (see 2.)
•Copper free and thus non-toxic
•No catalyst or accessory reagents and thus no extensive optimization of assay conditions required
•Suitable for dual-labeling approaches in combination with Tetrazine -trans-Cyclooctene Ligation

Principle:



(***Figure 1***). Schematic representation of a SPAAC ligation reaction.[CIK201904]

The strain promoted alkyne-azide cycloaddition, also termed as the Cu-free click reaction, is a bioorthogonal reaction utilizing a pair of reagents, cyclooctynes and azides that exclusively and efficiently react with each other while remain inert to naturally occurring functional groups such as amines (***Figure 1***). SPAAC enables labeling a wide variety of biomolecules without any auxiliary reagents in an aqueous and otherwise complex chemical environment through the formation of a stable triazole.
Among the large number of known cyclooctynes (DIFO, BCN, DIBAC, DIBO, ADIBO), the so-called **DBCO** (dibenzocyclooctynes, or Azadibenzylcyclooctyne ADIBO = DIBAC) compounds comprise a class of reagents that possesses reasonably fast kinetics and good stability in aqueous buffers with sufficient hydrophilicity[ref13,14]. Within physiological temperature and pH ranges, the DBCO group will not react with amines or hydroxyls that are naturally present in many biomolecules. Additionally, reaction of the DBCO group with the azide group is significantly faster than with sulfhydryl groups (–SH, thiol).

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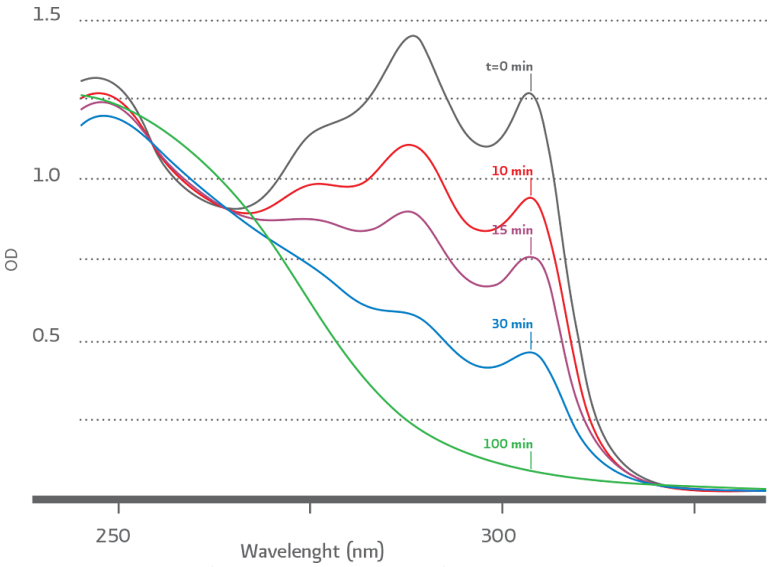
Unlike many other cyclooctynes, DBCO reagents possess an embedded chromophore that allows for the simple and non–destructive spectroscopic identification of DBCO – containing compounds. This chromophore can also be used for spectroscopic estimation of total incorporated DBCO molecules into a biopolymer.

Another important feature of DBCO compounds is that the **progress of SPAAC ligation can be followed in real time by simple UV – Vis spectroscopy**. As the "click reaction" progresses the signature an absorbance band at 310 nm disappears as illustrated ***Figure 1b***



(***Figure 1b***). Progress of SPAAC ligation reaction followed by UV–Vis spectroscopy.

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Limitation:
SPAAC has modest kinetics (e.g. 0.3 to 2.3 $M^{-1}s^{-1}$), so for low biomolecule concentrations (e.g. < 5 µM) one might see alternative method, such a Tetrazine/TCO Ligation.

See a list of DBCO reagents: DBCO (+ fluorescent labels: Cyanine, CF488/568/647, FAM, BrDIPY, …), (linkers: +mPEG, …) (crosslinkers: +Amine, Carboxyl Acid, NHSuc ester, Azide, Alkyne, AminoOxy, Hydrazide,…) (ligands: + Biotin/Desthiobiotin, Agarose, …)

Protocols
See the technical sheet

Phosphine-Azide (Staudinger) Ligation

The **Staudinger Azide/Phosphine ligation** use a aryl phosphine functional group to conjugate an azide functional group that react covalently to form an amide linkage. The molecules to be conjugated are preliminary derivatized with each partner molecule by suitable modidiers (i.e. NHS-Phosphine adn Maleimide-Azide).
See Phosphine functional group and reagents
See Azide functional group and reagents

Tetrazine-Alkene Ligation

See the TechnicalNotice NT-XLClic q

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Features:
•High-speed CLICK reaction that is ideally suited for in vivo cell labeling & low concentration applications
•Copper free and thus non-toxic
•No catalyst or accessory reagents and thus no extensive optimization of assay conditions required
•Suitable for dual-labeling approaches in combination with the strain-promoted Azide -DBCOreaction[17] .

Principle:

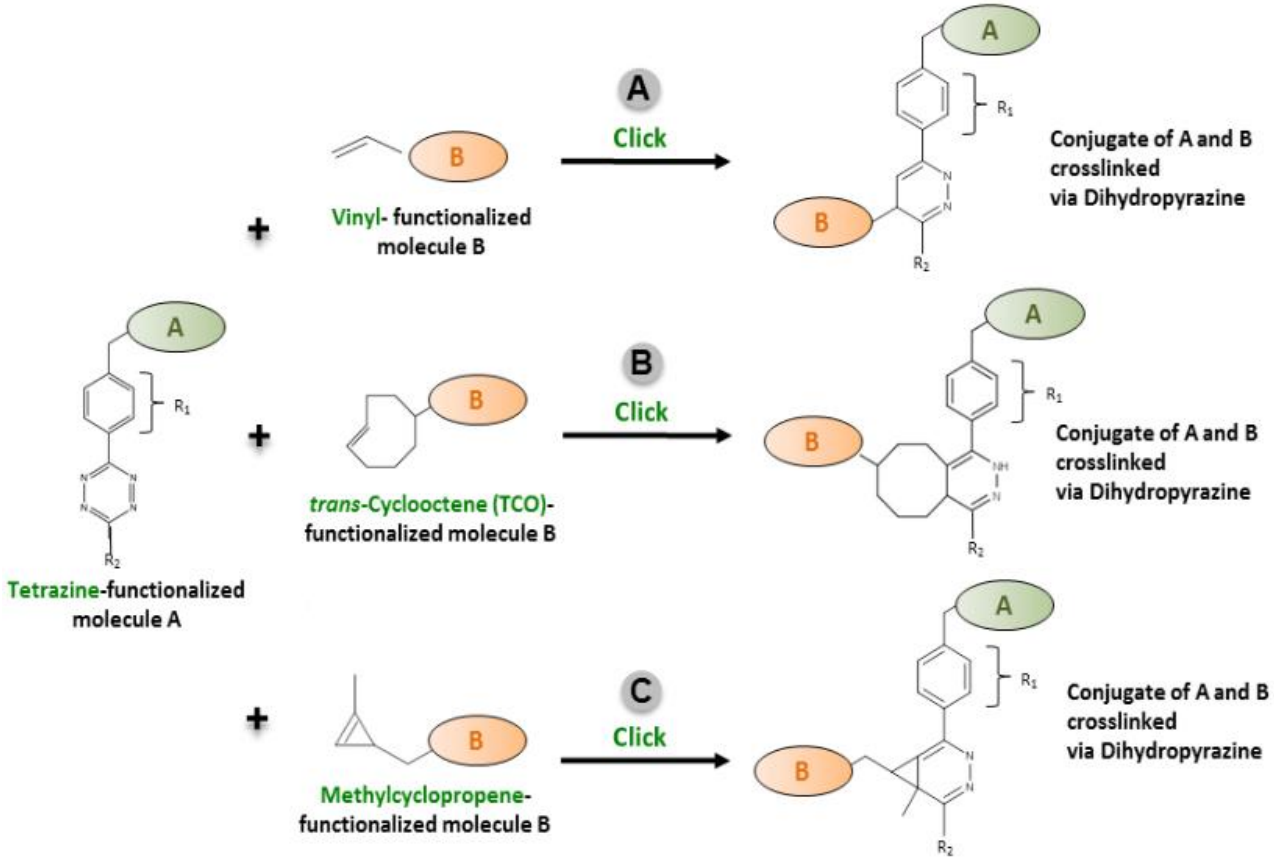


Figure 2: Principle of Tetrazine-Alkene Ligation.
A) Tetrazine - Vinyl Ligation.
B) Tetrazine - trans-Cyclooctene (TCO) Ligation.
C) Tetrazine –Methylcyclopropene Ligation. R1= Phenyl, R2= H or CH_3
A numberof structurally varied alkene and tetrazine derivatives have been developed that strongly differ in terms of reaction kinetics and stability. TCO has been selected (as strained alkene) since it possesses the highest reactivity towards tetrazine[18,19]. Methylcyclopropene (strained alkene)[21 - 24] and Vinyl[25] (terminal alkene) possess excellent substrate properties for enzymatic applications due to their small size.

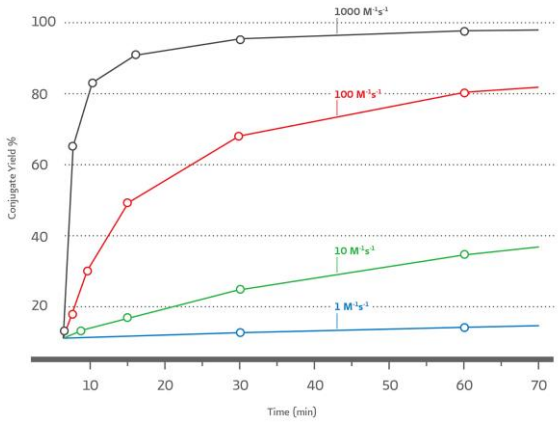
The Tetrazine - Alkene Ligation constitutes a non-toxic biomolecule labeling method of unparalleled speed that is ideally suited for in vivo cell labeling and low concentration applications. A Tetrazine -functionalized molecule A reacts with a terminal or strained Alkene - functionalized molecule B, via an inverse–demand Diels–Alder cycloaddition reaction, thereby forming a stable conjugate A-B via a Dihydropyrazine bond (*Fig. 4*).

The combination of ultrafast kinetics, selectivity, and long–term aqueous stability makes TCO–Tetrazines the ideal pair in low concentration applications such as protein–protein conjugations.

The reactivity of the tetrazine derivatives towards TCO is determined by the substituents in the 3 position (Fig. 4, R1) and 6 position (Fig. 4, R2). Two Tetrazine versions with different reactivities and stability characteristics have been selected that meet specific application requirements. **Tetrazine** (R1=phenyl,R2=H) reagents are the ideal choice if a rapid reaction kinetic is the key aspect, whereas **6-Methyl-Tetrazine**(R1=phenyl,R2=CH_3) reagents are ideally suited if an improved chemical stability is required [18].

TCO ligation is reaction of choice when low biomolecule concentrations (e.g. < 5 µM) render SPAAC poorly efficient due to modest kinetics (e.g. 0.3 to 2.3 $M^{-1}s^{-1}$), and where copper–catalyzed alkyne–azide cycloaddition click reaction might compromise system viability.

Reactivity can be defined by the second order rate constant for the bioorthogonal reactant pairs. The higher the 2nd order rate constant for product formation, the more efficient the conjugation at low reactant concentrations within reasonable time scales, at near neutral pH, and without having to use a large excess of either biomolecule. The relationship between2nd order rate constants ($M^{-1}s^{-1}$) for bioorthogonal reactants at 10 µM and the percent conjugate yield over time is illustrated in (*Figure 2b*).



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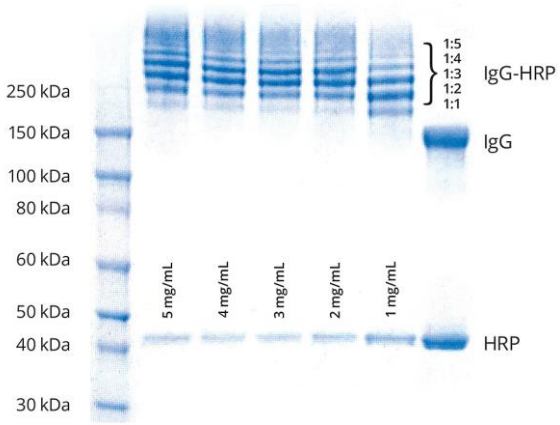
(***Figure 2b***). Simulation of 2nd order reactions at 10 µM reactants

Example Application

Five Goat IgG samples (100 µL at 5 mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL and 1 mg/mL) were labeled in BupH buffer (pH 7.5) using a 20–fold molar excess of Tetrazine–PEG5–NHS ester. Similarly, 0.1 mL HRP (500 µg) at 5.0 mg/mL in BupH buffer (pH 7.5) was labeled using a 20–fold molar excess TCO-PEG4-NHS ester for 60 min. After removal of excess reagents and determining each protein concentrations 3-fold excess of HRP–TCO was added to IgG–Tetrazine at 5 mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL and 1 mg/mL. After 60 minutes, an aliquot (1 µL) from each conjugation reaction was analyzed by SDS–PAGE.



(***Figure 2c***). Simulation of 2nd order reactions at 10 µM reactants

See Tetrazine functionnal group and reagents
See Alkene functionnal group and reagents

Hydrazone-chemistry functional groups (HyNic)

See below aldehyde group and Hydrazone group

>Oxime chemistry functional groups

'**Oxime ligation**' can conjugate AminoOxy reagetne to Carbonyls. Typically used to activate an CHO or Ketone (or even an an Amine or OH) in a CMO fonctional group.

'**CMO/Amine ligation**' cad couplage indirect~~Click like~~ by conventionnal chemistry between an amine (protein) and a COOH activated as CMO.

>Photoreactive functional groups

Photo-reactive reagents are chemically inert compounds that become reactive when exposed to ultraviolet or visible light. Historically, aryl azides (also called phenylazides) have been the most popular photo-reactive chemical group used in crosslinking and labeling reagents.
Diazirine reagents were more recently introduced.

Photoreactive cross-linkers react basically non specifically with free hydrogens upon illumination, but amine reactivity is usually predominant in presence of primary amines. Hence,
-they are used for site-directed or time-defined conjugations. For example, a ligand can be coupled to it's receptor inside cells during a controlled physiological condition.
-they are also used for specific conjugations of biomolecules that are difficult to handle with standard NHS/Mal methods.

The photoreactive group include:
-**ArylAzide** (such as in ABH, DTPA, HSAB) reacts non specifically.
-**HydroxyPhenylAzide** (N3/OH-Phe-CO-, such as in ASBA, ASIB, NHS-ASA) reacts mainly with amines upon UV 265-275nm photolysis
-**Nitrophenyl Azide** (N3/NO2-Phe-, such as in SANPAH, SAPB) reacts mainly with amines upon photolysis at 320nm-350nm
-**Phenyl glyoxal** (N3-Phe-CO-), such as in APG
All the phenyl based groups can be radiolabeled (iodinatable.)
-**Diazirin**-based reagents also react non specifically.

Remarkable photoreactivities include arginine specificity (APG), cleavability (BASED, DTPA). ASBA is also unique, allowing to conjugate carbonyls (though its amino group) to amines (throug its photoreactive group). Photoreactive and SH reactive crosslinkers such as APDP allows in situ and SH-oriented conjugations of Cys-containing molecules. It is thus useful to study many active sites (enzymes, channels, receptors), which contain cys residues.

>other: strongly reactive groups

-Acrylates:
Acrylate group can react with amines, thiolates and alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.
-Cyanates/IsoCyanates/Thiocyanates/**IsothioCyanates**:
Cyanate group can react with amines, thiolates and alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.
-Epoxides:
Epoxide group react with OH and Amines. Due to their strong reactivity, ther are usefull more particularly to modify surfaces, and also, under controled conditions, to modify biomolecules.
-Tosylates:
Tosyl group can react with Amines, Thiolates and Alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.

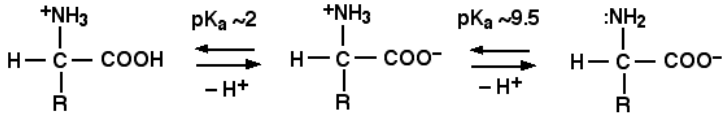
Functional groups for Biochemistry (alphabetic order)

.Acryl: see below Acrylate

.Amines (NH2)

●Structure: ● . -NH2 (primary A.) -NH- (secondary A.) =N- (tertiary A.) .
Amines are described as primary, secondary, or tertiary depending on the number of hydrogen available on the azote atom. Primary Amines are widely present naturally in proteins, but also in some natural forms of lipides, glucosides. They also can be introduced in biomolecules chemically, during synthesis (use of aminoallyl nucleotides), or genetically. Amine can finally be blocked chemically (with NHS-acetate), for site-directed coupling strategies.
Amines behave as **bases**, and **nucleophiles**, primary amines being more reactive that secondary, or tertiary amines.

predominant form at pH 1 (below the pK_a of both ionizable groups) | predominant form at pH 7 (dipolar ion, i.e. "zwitterion") | predominant form at pH 11 (above the pK_a of both ionizable groups)



●Reactivity:
[] The amino group (-NH2) reacts readily with succinimidyl ester groups (in particular NHS), carboxylic groups (via carbodiimide mediation) and many other amine reactive functional groups either in aqueous buffer or organic solvents.
Amines can be coupled :
-to COOH groups via reductive amidation mediated by carbodiimides (see COOH / NH2 reaction scheme)
-to CHO groups (see the glutaraldehyde NH2 to NH2 conjugation method).
-to reactive amine groups of many kinds:
Succinimidyl esters are surely the most popular and easy to use to modifiy amines in aqueous solutions, because they perform well in aqueous solution (despite competition with hydrolysis) by amine-specific reaction. Alternatively,
.**ImidoEster**, PFP/**TFP**, ... are useful when a stronger reaction is needed (a)
.**Aldehydes** are useful and is very good for N-terminal modification. They give reductive amination with primary amines to produce secondary amines, in the presence of reducing agents such as sodium borohydride and sodium cyanoborohydride. The pH is important for reductive amination.
.**Isothiocyanates** may be useful but with lower specificity

.**Epoxide**	Perform useful nucleophilic additions, applicable to amines.
.**Isothiocyanate**	React with amine to produce a stable thiourea linkage. No well specific (reacts also with …)
.**COOH**	Usually the acid needs to be activated, such as NHS ester.
.**NPC**	Amine reacts with NPC under proper conditions.
.**Acrylate** esters	React by Michael addition with amine and acrylate groups. It is a relatively slow reaction.

+ [XLAMIN]

●Protection of Amine groups : in amide function
See the technical notice NT-XLprgr +

.Aminooxy (AO)

●Structure: . -O-NH2 .
●Reactivity:
[] The **hydroxylamine** derivatives (aminooxy compounds – similarly to Hydrazines) have amine-like reactivity and, in some cases, can be coupled to water-soluble carbodiimide–activated carboxylic acid groups. They react with aldehydes and ketones to yield stable **oximes**.
See Oxime chemistry.

.Carbonyls (-COOH, -CHO, -CO-))

●Protection of Carbonyls : as carbamate form, and special others.

.Carboxyls (COOH)

●Structure: . -COOH .
●Reactivity:
[] The Carboxyl group (-COHH) can be conjugated using several conventional chemistries: via carbodiimide mediation to amines, with and many other amine reactive functional groups either in aqueous buffer or organic solvents.

COOH are poorly reactive (see Reactions of COOH), and widely present naturaly in proteins, but also in lipids, glucosides, nucleotides. They also may be introduced in biomolecules chemically, during synthesis, or genetically.
CHO are diversely present in biomolecules, notably in proteins, as the ?epsilon? terminal group of aa chain, but more importanly as COOH groups of certain (acidic) aminoacids (Glutamate, Asparate,...). They are also present in carbohydrates, forming the end of oxidized sugars and side groups of sialic acids. COOH are often present amidated

(Glutamine, Asparagine), or engaged in stable amide bonds (starting with the peptidic bonds). Whatever, COOH groups are abondant and a commun, target for conjugation after amines.

Carboxylic acids can be coupled chemically by different methods:
-**Amine** reagents are versatile intermediates for coupling to carboxylic acids such as in DNA and other biomolecules. The coupling involves a carbodiimide (i.e. EDAC, DCC, EDIC).
However, the procedure is successful only when amines are not present in the COOH-bearing compound, as for instance in the case of non-peptide drugs. In peptides and proteins the risk of cross-linking is difficult to avoid.
-**Hydrazide** reagents are useful[] for coupling carboxyls after their reduction in aldehyde (see Converting carboxyls in aldehyde, and Coupling to aldehydes). But Hydrazide can be coupled also to carboxyl group after their activation by EDAC at mild acidic pH, while (in proteins) the amino groups present in protein could remain inactive in this particular condition. The very low pK of the hydrazide group allows, in the presence of N-(-3-dimethylaminopropyl)-N'-ethylcarbodiimide hydro-chloride, its binding to carboxyl groups in acid media(pH 4.5-5), where all the protein amino groups are proto-nated and not reactive. This condition assures that intra-or inter-molecular protein cross linking between carboxylates and a PEG amine takes place.
-**Halides** (chloride, bromide, iodide, tosylate and mesylate) react with deprotonated carboxyl groups i.e. -COO- salt.
-Other methods are used in chemistry but usually do not suit biomolecule conjugations in physiological buffers because of too much strong reactivity, poor specificity, or harsh conditions.
+ [XLCARBX]

●Protection of Carbonyls : as carbamate form, and special others.

.Carbodiimides ()

●Structure: . -N=C=N- .
●Reactivity:
[] The cardodiimides are
-used **as a carboxyl activating agent for the coupling of primary amines** which is typically employed in the 4.0-6.0 pH range to yield amide bonds
-**used to activate phosphate groups**.
-**to couple a carboxylic acid to alcohol using DMAP as a catalyst**
Dialkylcarbodiimides are stable. Some diaryl derivatives tend to convert to dimers and polymers upon standing at room temperature, this mostly occurs with low melting point carbodiimides that are liquid at room temperature.[2] Solid diaryl carbodiimides are stable ,but can slowly undergo hydrolysis in the presence of water overtime.

See more information in technical sheet 52005A

.CyanoCyanate: see below Cyanates

.Aldehydes (CHO)

●Structure: : . -CHO .
[] CHO are diversely present in biomolecules [a], typically **in reducing oses** (contained in polysaccharides), rather rare in proteins and in lipids, or in relatively poorly reactive form in glycosides and nucleotides. They may be introduced in biomolecules chemically ([b] i.e. by oxidation from alcohols, that gives a classic method to couple glycosides with hydrazines), during synthesis, or genetically.
[a]Aldehydes and ketones are present in a number of low molecular weight molecules such as drugs, steroid hormones, reducing sugars and metabolic intermediates (e.g., pyruvate and -ketoglutarate). Except for polysaccharides containing free reducing sugars, biopolymers generally lack aldehyde and ketone groups. Even those aldehydes and ketones that are found in the open-ring form of simple carbohydrates are usually in equilibrium with the closed-ring form of the sugar.
[b]Techniques were developed to selectively introduce Aldehydes and ketones as functional groups into biomolecules, thus providing unique sites for chemical modification:
-reagents ManLev and ManLev tetraacetate
-oxidation of diols (by periodate, by permangagnate)
[] Aldhehydes are formed from cis-diol found notably in carbohydrates by specific oxidases such as galactose oxidase, or by mild oxidation with 10mM NaIO4 at RT in the dark (Chamow 1978):

R-CH(OH)-CH(OH)-R + NaIO4 → R-CHO

●Reactivity:
[] The most reactive reagents for forming stable conjugates with aldehydes and ketones are generically **Hydrazines** derivatives (including **hydrazides**, **semicarbazides** and **carbohydrazides),** as well as **Hydroxylamines** derivatives.

***Hydrazine** reagents can be coupled directly to aldehydes in mild conditions. While bonds formed by hydrazides usually need a stabilization by reduction, bonds formed with hydrazone chemistry do not (see Hydrazine/CHO method, i.e. dextran conjugation, conjugation/labeling antibodies through glycones).

Aldehydes and ketones are carbonyls [CO] without other reactive groups, and react:
-with hydrazides (-N-N3): The reaction yields an **hydrazone** bond. Arylated aldehydes (i.e.4FB) give hydrazine bonds, even with aromatic hydrazines (i.e. HyNic). As the reaction do not involves natural chemical groups (typically nor hydrazides nor aldehydes occur in biomaterials), the -CHO and –N-N3 groups are introduced in biomolecules prealably: see Hydrazine chemistry / HyNic.

*__Aminated__ reagents can be coupled directly to aldehydes in mild conditions.
-reaction of aldehydes **with amines** by **amidation**. The reaction (typicaly **Glutaraldehyde**) is very popular, although less rapid than with hydrazides, and yielding poorly stable conjugates.

-reaction of aldehydes **with amines** by **reductive amination**. The reaction is spontaneous giving a **Shiff's base** that poorly stable and should be stabilized **mild oxidation** (i.e. by $NaCNB_3$). The Schiff reaction sterically dependant, hence not typically not occurinig with ketones. Besides bioconjugations, it's colored by product allows colorimetric dosage of CHO groups..
-reaction of aldehydeswith **AminoOxy (-CO-NH)** ~~/ reaction avec les **aldéhydes** ou les **cétones** (carbonyls)~~, to form extrêmely stable **oximes** linkage(-CO-NH-)
+[XLALDE]

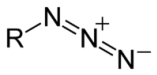
.Alkyne (CCH2)

●Structure: . -C≡CH . C_nH_{2n-2},
[] Alkyne (or acetylenic hydrocarbons) does not occur in commun biological samples, allowing biorthogonal reactions. However it can be found in some species (plants, fungi, bacteria, marine sponges, corals [12]) then often as bioactive compounds. Some drugs contain an alkyne group (and even ene-diynes).
●Reactivity:
[] Alkyne group allows diverse additions of Hydrogen, halides, thiols,… Most interestingly for bioconjugations, it allows cycloadditions with azides for specific and click type reaction that are biorthogonal:
Alkynes undergo diverse reactions. Most notable is the Diels–Alder reaction with 1,3-dienes to give 1,4-cyclohexadienes, extensively developed and electrophilic alkynes. Cycloadduct also occurs as alkyne trimerisation to give aromatic compounds and the [2+2+1]-cycloaddition of an alkyne, alkene and carbon monoxide in the Pauson–Khand reaction.
+ [XLALKY] [XLclic]
–

See the ClickChemistry reactions>TTCA

.Azide (N3)

●Structure: : . $-N_3$.



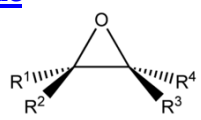
●Reactivity:
[] The **Azide** group can be used for a variety of reactions:
•Alkyl **Azides** are commonly used as a way to introduce an amine group, and also popular for their participation in the "click reaction" (with an alkyne group), and Staudinger ligation (with a phosphine group). They can participate to other kind of reactions as well.
.azide reacts with alkyne in aqueous solution catalyzed by copper or with constrained alkynes (i.e.DBCO reagents) without copper, giving popular click conjugations.
.azide reacts with phosphine, performing Click-like reaction known as the Staudinger ligation.
.azide reacts with hydrazides and amines at pH 5-7. The reaction with hydrazides is faster than with amines, making them useful for site-specific crosslinking. It also reacts spontaneously with amino groups to form Schiff base intermediates that can be stabilized by reduction with Sodium cyanoborohydride ($NaCNB_3$).
.azide reacts with carbonyls or ketones to form amines or amides. It can also be easily reduced into an amino group.

•**ArylAzides**: R is in above formula is a phenyl or an other aromatic moiety, which allow the compound to react, upon UV irradiation, with amines, freeH : see PhotoReactive groups.

+ [XLAZID] [XLclic]
–

NT-XLfctl

.Epoxide



●Structure: :

●Reactivity:

[] Expoxy react with OH and Amines. Due to their strong reactivity, ther are usefull more particularly to modify surfaces, and also, under controled conditions, to modify biomolecules.

Ring-opening reactions dominate the reactivity of epoxides. Epoxides undergo addition reactions with **Alcohols, water, amines, thiols** and many other reagents (and **water**, so in biochemical reaction there is a competiting hydrolysis).

Epoxide also oligo/polymerize readily, giving poleythers (PEO, PEG).

+ [XLEPOX]

.HaloAlkyls (A

●Structure: : . -OH .

●Reactivity:

HaloAlkyls groups react in moderatey specific way* with sulfhydryls by nucleophilic substitution, forming a stable thioether linkage. In most proteins, the site of reaction is at cysteine residues that either are intrinsically present or result from reduction of cystines. In addition, methionines can sometimes react with haloalkyl reagents. Other

*: nucleophilic groups in lower extend react with haloakykls, but this can be limited by the stocheometry of reagents making haloalkyls useful sulfhydryl reagents because of the stable formed link (compared with maleimide) and compatibility with azide groups.

Amongst haloalkyls, **iodoacetyls** then **bromoacetyls** are among the most frequently used reagents for thiol modification. When the specificity of reaction is found unsufficient, Maleimide or MTS reaction then preferred.

In organic chemistry, Haloalkyl can be used to react on amines.

Iodo-acetyl reaction with SH: The reaction with the free sulfhydryl proceeds by nucleophilic substitution of iodine with a thiol group, forming a stable thioether linkage. The reaction can occur with other groups, but the specificity of reaction with sulfhydryls can be driven using slight stoichiometric excess of iodoacetyl groups over the present number of free sulfhydryls, and by keeping a pH 7.5 and 8.5 (optimally at pH8.3) for the reaction.

Iodo-acetyl side reactions: If there is a gross excess of iodoacetyl group over the number of free sulfhydryls (or absence of free sulfhydryls), the iodoacetyl group can cross-react with amino acids. I.e. reaction occurs with Imidazoles at pH 6.9-7.0, for over a week [r]. Histidyl side chains and amino groups (unprotonated form) also react with iodoacetyl groups above pH 5 and pH 7, respectively [r].

Iodo-acetyl-NHS crosslinkers: The iodoacetyl group is much more stable to hydrolysis as compared to the ester, so the iodoacetyl reaction is usually performed in second step for conjugations in aqueous buffers. Reducing agents containing buffers should be precluded (i.e. DTT, mercaptoethylamine).

Iodo-acetyl-NHS crosslinkers:

HaloAcetyls stability/sensitivity: Iodoacetamides are well stable (more that maleimides), but in solutionthey undergo rapid **photodecomposition** to unreactive products. The iodoacetyl reactions should thus be carried out in the dark to limit the generation of free iodine, which has the potential for reacting with tyrosine, histidine, and tryptophan amino-acids [r].

>Reagents:

•**Iodoacetyl (IA)**[] | Crosslinkers: & **-Succinimide(NHS), -Maleimide, -COOH, -Amine,**
| Labels: & **-FluoProbes, FITC, Biotin, Resin**[], Agarose | **PEG**

•**Bromoacetyl** (BA, BrA)[]

.Hydroxyl (OH, Alcohol)

●Structure: : . -OH .

[] OH groups are ubiquitous in glycosides (alcohol or diols), but also present in nucleic acids (via the ribose sugar) and in proteins (via the Thr, Ser, Tyr aa, or glycones or other prosthetic groups). They are key fonctionality of the alcohols molecules.

●Reactivity:

NT-XLfctl
[] The **Hydroxyl** group (**OH)** is very poorly reactive in physiological conditions, allowing few direct specific reactions. In biological molecules, **viccinal hydroxyls of sugars** can however by targeted using some azides reagents.
There are few good options to conjugate hydroxyls in aqueous phase unless present alone (on materials to functionalise): hydroxyl can notably be modified using IsoThioCyanates (as with the few OH reactive like **PMPI** (MPITC) #88307B), or other with other non specific reactive groups (). There are more options in organic chemistry.
OH groups are added chemically to confer its chemically-inert and hydrophilic properties (including using the Pegylation approach)

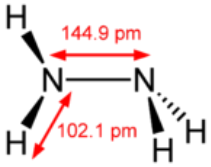
+ [XLOH--]
Hydroxyls allow few direct reactions, their modification fit to few options:
1/choose a direct chemistry (rare, and poorly specific of OH).
-Reaction of Hydroxyls / **IsothioCanate** (not well specific)
-Reaction of Hydroxyls / **Phosphoramidite** (=>phosphites esters):
see Phosphoramidite esterification of hydroxyls, performed in oligonucleotides synthesis.
2/choose **indirect chemistries**:

1-Use a hydroxyl-reactive cross-linker (PMI MaleimidoPhenyl-Isocyanate to activate OH)
2-Make it more reactive towards amines, so you can label with Aminated biomlc2, using **tosyl** reagents:
Tosyl Chloride + R1-CHO => R1-CCC; + R2-NH2 => R1-NH-R2
3-**Oxidize** hydroxyl to a ketone or aldehyde, then react with a bifunctional hydrazido cross-linker

3/choose to target other chemical group target than OH ()

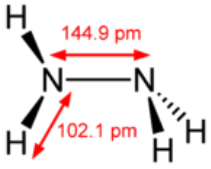
•Protection of Hydroxyl groups
See the technical notice NT-XLprgr +
+

.Hydrazide (-N-NH2)



●Structure: :
●Reactivity:
[] The **Hydrazide** group give a variety of reactions: they react with Carbonyls (aldehydes and ketones) much faster than amine does, making them useful for site-specific crosslinking.
Reaction with carboxyls better occur with activation by a carbodiimide.
Reactions with amines are nonetheless useful, but require mediators (NH_4BO_4, via Shiff'base formation)
+ [XLHYDR]

.Hydrazine



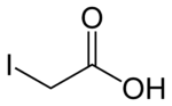
●Structure: :
Hydrazine derivatives (N-N bond), include **hydrazides**, **semicarbazides** and **carbohydrazides**.
[] The **HyNic** group (an aromatic hydrazine) reacts specifically with aldehydes at pH 5.0–6.0 to yield a stable hydrazone bond. The reaction can be speeded adding aniline catalyzer.

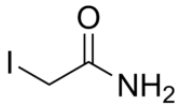
●Reactivity:
[] **Hydrazines** are useful reagents forming stable conjugates of aldehydes and ketones, but also have amine-like reactivity. They react typically with ketones to yield relatively stable **hydrazones**, and with aldehydes to yield **hydrazones** that are somewhat less stable, though they may be formed faster (their Shiff's base intermediate should be stabilized). **Hydrazones** share similar reactions.

See the TechnicalNotice 'Hydrazine'[XLfctl].
+

.HalogenoAcetals (-Cl, -Br, -I)

●Structure:

Iodoacetique Acid: 

IodoAcetamide : 

●Reactivity:

• **Iodoacetyl** group (-O-CO-CH_2-I) reacts rapidly with thiols by nucleophilic substitution at pH7.5-8.5 giving a thioether bond that is non cleavable. At lower or higher pH, with high ratios and long incubation times, undesired reaction may occur, for example with amines and histidines (Gurd 1967).

• **IodoAcetamide** (-N-CO-CH_2-I);) is an alkylating agent with similar reactivity.

Reactivity order: COOH > Acid Chlorides(-COCl) > Bromides (-COBr) > Iodides (-COI),

•Products examples:

Iodoacetyl-Biotin (FT-48198A) Fluoprobes®488 – IodoAcetamide FP-FH9780

+ [XLTHIO]

.ImidoEsters

●Structure: :

See 'Esters' for more information about related groups

(NHSuccinimide (NHSuc) | Other Sucinimides (SC, SCp, SPA, SVA, SS, SG) | Imido-Esters | Phenyl-Esters : STP, PFP)

●Reactivity:

Imidoesters (IE) react at pH8-10 with NH2-Lys to form stable amidine bond.

CNH-OCH3 + R-NH2 –(pH8-9)-> -CNH-NH-R + CH3OH

+ [XLIMES]

•Products and related documents:

DMA (dimethyl adipimidate) , DMP (dimethyl pimelimidate) , DMS (dimethyl suberimidate)

.Iso(Thio)Cyanate

●Structure: : (iso)cyanate : $[O{=}C{\equiv}N]^-$ IsoThioCyanate: $R{-}N{=}C{=}S$.

●Reactivity:

Cyanates (-O-C≡N <-> -N=C=O) are very reactive groups, hence not easy to use with biomolecules (uncontrolled reactions in specificity and extension).

The **isocyanate** group (-N = C = O) reacts readily with hydroxyls, forming a stable carbamate bond at pH 7-8.5. However, the formed bond (urethane) is not so stable than esters (cf NHS) or ether (cf Epoxy).

Finally, the **IsoThioCyanate** group (R–N=C=S) is more usefull in bioconjugations, being less reactive and more stable in water. Upon reaction with amines it forms thioureas, however the formed bond is less stable than that formed by Succinimide esters.

+ [XLITC_]

.Maleimide

●Structure: :

●Reactivity:

.**Maleimide**: reacts with thiols by Michael addition (on the C=C bond in the maleimic ring) and forms a physiological stable linkage. The best reaction condition is at pH 8. While it is still reactive to thiols even under acidic conditions pH 6-7, it is then not stable in water and can undergo ring opening or addition of water across the double bond Maleimide reacts to SH group in mild conditions.

+ [XLMAL_]

●Protection of Malemide group

NT-XLfctl
See the technical notice NT-XLprgr +
+

.Methanethiosulfonate

●Structure: :
●Reactivity:
The **Methanethiosulfonate** group reacts very rapidly and specifically with cysteine / Thiols
+ [XLMTS_]

.Nitrophenyl Carbonate (NPC)

●Structure: :
●Reactivity:
The **NitroPhenyl Carbonate** group (**NPC)** can react with both amine and hydroxyl groups.
+[XLNPC_]

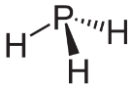
.Pyridyl Disulfide (Orthopyridyl disulfide group (OPSS) and pyridyl thiol)

●Structure: :
●Reactivity:
• The **Orthopyridyl disulfide** group (OPSS) reacts with thiol groups at pH 5.0~6.5 and generates a cleavable disulfide bond.

• The **pyridyl thiol (Pyridyl Disulfide** bridge **)** group reacts specifically at pH7-9 by exchange with sulfhydryls (free SH), leaving a pyridin-2-thione group that can be followed up: maximum absorption occurs at 343nm with an extinction coefficient of 8 . 10^3 M^{-1} cm^{-1} (Struchbury 1975).
+ [XLOPSS]

.Phosphine

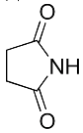
●Structure: . $-PH_3$.



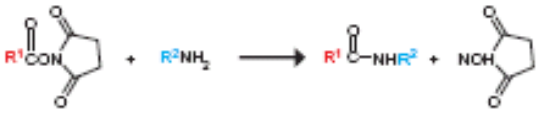
●Reactivity:
Phosphine-based chemistry allows specific and click type (bioorthogonal):
See **Staudinger Azide/Phosphine ligation** using a functionalized by aryl phosphines to conjugate an azide group in a covalent fashion forming an amides. The molecules to be conjugated are preliminary derivatized with each partner molecule by suitable modidiers.
+

.Succinimides (NHS & SG, SCM, SVA…)

[] The NHS group is the most popular Succinimide ester. See 'Esters' for more information about related groups
(NHSuccinimide (NHSuc) | Other Sucinimides (SC, SCp, SPA, SVA, SS, SG) | Imido-Esters | Phenyl-Esters : STP, PFP)
●Structure: R-CO-NR'-CO-R''
succinimidyl is a cyclic imide (secondary amide) :



●Reactivity:
• The **N-hydroxysuccinimydyl** group **(NHS)** reacts at pH 7.5-9 in aqueous phase on aliphatic primary ($–NH_2$) and secondary amines (=NH), optimally at neutral pH or higher. A peptidic bond (amide link -CO-NH-) is formed.



Succinimidyl ester Carboxamide

NHS functionality can be ***monitored by UV-Vis spectra at 260~280 nn***:
One limitation relies on its **susceptibility to hydrolysis**. Another limitation is the **weak solubility** of NHS, that as been adressed using Sulfo-derivative of NHS and PEGylated spacer derivates.

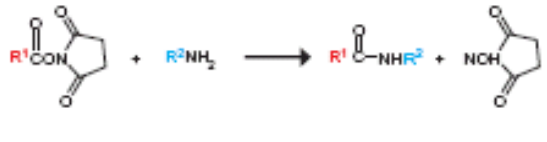
NHS is also useful to activate several group, i.e. carboxyl , Azide…

• Other **succinimydyl** esters are useful, which reactivity more or less differ for NHS **(SG, SCM, SVA,…)**.

•More/detailled information : 'NT-NHS reactivity'[XLNHS_]: [preferential reaction with N-terminal (alpha)-amines, other amine reactions for organic synthesis]

Succinimide ester (NHS, SE): acylation reaction with Amines

•The **N-hydroxysuccinimydyl (NHS)** group reacts at pH 7.5-9 in aqueous phase on aliphatic primary (–NH2) and secondary amines (=NH) (in fact on its deprotonated form), optimally at neutral pH or higher: amines present in proteins (Lys aminoacid) and in a lower proportion on ε-NH_2 located in terminal peptidic chains. A peptidic bond is formed, that confers many benefits compared to other chemistries (stability). The reaction however competes with hydrolysis, but this side reaction is usually slow below pH 8-9.



R-CO-O-Succ + R'-NH2 -(ph >7)-> R-CO-NH-R' + NHS

There are several differences in reactivity depending on the ester position (on the distance and spacer) :
see below other Succinimidyl esters: SVA, SC, SG, SS, SCM, SPA,…

•NHS has very low reactivity with aromatic amines, alcohols, phenols (including tyrosine) and histidine.
This drives the interest of NHS functional group for conjugation: NHS acts as a specific amine-reactive group, with efficient and mild reaction (while SulfoChroride, … are somewhat harsh), and stable link (with Isothiocyantaes, … are not so stable).

•One limitation relies on NHS susceptibility to **hydrolysis** that competes with amine reaction (concerns with the NHS reagent solubility, unsufficient concentration in the acylation reaction, hydrolysis with diluted proteins
This has been adressed using **sulfonated derivatives**. Attentioon should be payed however, when a water-soluble sulfoNHS derivative is dissolved in aqueous solultions as mother solution, without taking care of hydrolysis.
These drawbacks are usually limited, or acceptable, or can be compensated by increasing the ratio of NHS/molecule (or amines) in most applications.
An alternative method is to use **PEG/PEO containing NHS reagents** : this spacing motif not only confers hydrophilicity that allow fo easier dissolution, but the spacer lenght is adjustable and reduce steric hindrance between groups at both ends. Search NHSuccinide and PEG and NHSuc and PEO reagents (e.g. DBCOP-PEOx-NHSuc FT-DQP580)

Additionnaly, NHS functionality can be **monitored by UV-Vis spectra at 260~280 nn** when other reagents are not absorbing in this area. The OD should no change or poorly changing upon addition of 1 or 2 drops of alkaline solution

Other Succinimidyl esters: SVA, SC, SG, SS, SCM, SPA

Succinimidyl esters are available with a variety of spacers that modify more or less their reactivity:

Succinimide Ester structure (N-Succinimide ester)	Ester name (& Symbols)	Hydrolysis half-live (minutes) at pH 8, 25°C ()	(Interchim Symbol)
Ester			
-O-Succimide (-NHS)	**N-Hydroxy**-Succinimidyl ester (SE, NHS)	20.4 min	(NHSuc)
Esters of monoacids:			
-O-**COO**-Succimide	**Carbonat**e Succinimidyl ester (SC, CS)	20.4 min	(CSuc)
-O-**CH_2**-COO-Succimide	**CarboxyMethyl** Succinmidyl ester (SCM)	0.75 min	(CMSuc)
-O-**CH_2 CH_2**-COO-Succimide	**Propionate** Succinimidyl ester (SPA)	16.5 min	(PSuc)
-O-**$CH_2CH_2CH_2CH_2$**-COO-Succimide	**Valerate** Succinimidyl ester (SVA)	33.6 min (a)	(VSuc)
-O-**$CH_2CH_2CH_2CH_2CH_2$**-COO-Succ.			
Esters of diacids:			
-O-**CO**-**CH_2CH_2**-**COO**-Succimide	**Succinate** Succinimidyl ester	9.8 min	(SSuc)

NT-XLfctl

	(SS)		
-O-CO-$CH_2CH_2CH_2$-COO-Succimide	**Glutarate** Succinmidyl ester (SG, GS)	7.6 min	(GSuc)
With an amide link:	*: reactivité ??*		
-NH-CO-CH_2CH_2-COO-Succimide	**Succinamide** Succinimidyl ester (SAS)		(SASuc)
-NH-CO-$CH_2CH_2CH_2$-COO-Succim.	**Glutaramide** Succinimidyl ester (GAS)		(GASuc)

Protection of esters

See the technical notice NT-XLprgr +

+

.Sulfhydryls / Thiols

●Structure: : . -SH .

-Cysteine residues are present in most proteins, and involved in disulfide bridges (tertiary protein strucutre) or in bioactive sites (i.e. substrate site of proteases).
-cystein can be incorporated at desired position during the peptide synthesis step
-cystein car be incorporated at desired position by genetic engineering in recombinant proteins
-Sulfhydryls can be introduced chemically on proteins with SATA type reagent.
-Sulfhydryls can be generated for disulfide bonds of proteins; I.e. reducing an immunoglobulin under mild conditions can create SH in the hinge region available for labeling, or create Ab fragments bearing SH groups.

●Reactivity : [XLfctl] [XL]

[] The **Thiol** group (SH, Sulfhydryl) reacts readily with maleimide, halogeno acetals (I, Br,… : haloacetamides), Thiosulfinates (ThioSulfonates &VinylSulfone). It also binds by exchange to another thiol group to form a disulfide bridge and to Pyridyl thiols.
Hence, Sulfhydryls are coupled easily and specifically (even in the presence of amine groups.1,2) using these reagents at near-neutral (physiological) pH.
-Maleimide reacts with SH group in mild physiological conditions (optimal at pH 8), forming a stable linkage.
-Vinylsulfone reacts with SH group at high pH, forming a stable linkage.
-PyridlThiol /OPSS: (OrthoPyridiyl DiSulfide) OPSS reacts specifically with SH under both acidic and basic conditions (pH3-10 – but typically done at pH7-9), forming a stable thioether linkage.
-Thiol reacts to SH groups on cysteine side chains under mild reaction conditions.
-Other reactions include HaloAlkyl (less stable formed linkage), ThioSulfonates (i.e. MTS reagents, more useful for receptors/channels/enzymes studies),…
+ [XLTHIO]

●Stability:

[] Thiolated PEGs are manufactured and will stay in reducted form if you stock them in right condition. As disulfide bond will come out if you stock it for a long time, we suggest to use thiol-reagents in 6 months from the manufacture date.

●Products : Thiol reagents:

mPEG-X(Thiol) (FT-AXEST1); Thiol-PEG-COOH (FT-WT9771); Thiol-PEG-Azide (FT-1I6661); Thiol-PEG-Hydroxy (FT-T9781)

●Protection of Thiol groups

Thiols can be protected form oxidation See the technical notice NT-XLprgr +

+

.Sulfonyl Chloride (SC)

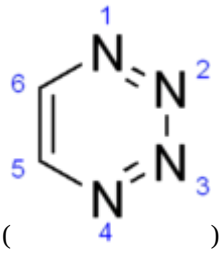
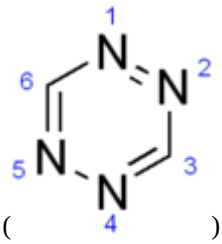
[] **Sulfonyl Chloride** (R-SO2-Cl) derivative is a highly reactive toward amines. Even aromatic amines can be modified. Their use for amine conjugation is limited by poor stability at the required pH 8.5-9. SC group reacts also readily with alcohols to form sulfonic esters.

NT-XLfctl
Methane Sulfonyl Chloride is a versatile, reactive chemical, which offers a mesyl group (CH3S02-) for substitution in a number of reactions. Methane Sulfonyl Chloride, an acid chloride, undergoes reactions, which typify this class of compounds, such as replacement of hydroxyl, amino and active alpha hydrogens, hydrolysis, etc.

Sulfonyl chlorides form very stable sulfonamides that can survive complete protein hydrolysis, but since they are more difficult to work with, we do not recommend these for most routine conjugations with proteins.

•Products : TFP, SDP, and succinimidyl esters are preferred.

.Tetrazine

●Structure: *Structure* of 1,2,3,4-tetrazine () and 1,2,4,5-tetrazine ().

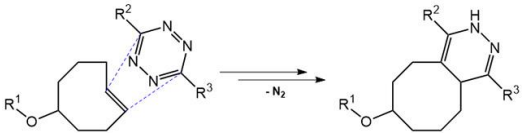
●Reactivity:
[] Tetrazine group reacts with TransCycloOctenes by cycloaddition of Diels-Alder, forming a dihydropyridazine stable bound. It occurs in aquous medium with a very rapid kinetic (l>800M-1s-1: superior to other click reactions and conventional ones). Conjugates are ready within 30mins. This made it popuilar for very low concentration of compounds to be coupled. This reaction is perfectly specific, bioorthogonal, and does not need catalyzer.
See ClickChemistry reactions>TTCA

See the technical notice 'ClickReactions[XLclic].

Tetrazine ligation principle

[]

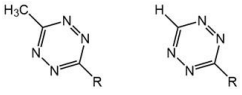
Tetrazine ligation offers a new copper-free, rapid and fully bioorthogonal type of click chemistry. Mechanistically, this reaction proceeds *via an* inverse electron-demand Diels-Alder cycloaddition reaction between a *trans*-cyclooctene (TCO) and a tetrazine, followed by a retro-Diels-Alder reaction under elimination of N_2.



This method excels at very low concentrations (e.g. in biological systems) due to the extremely rapid second order reaction kinetics (between approx. 800 $M^{-1}s^{-1}$ and 30000 $M^{-1}s^{-1}$). Moreover, the tetrazine-TCO ligation can be performed in aqueous media and is specific, so has been applied in live cell imaging. These properties make tetrazine click chemistry the method of choice for labeling or crosslinking biomolecules in living cells.

Stability vs. even Faster Reaction Kinetics:

●Methyl-substituted tetrazines exhibit a high stability even when dissolved in aqueous media, while still offering faster reaction kinetics with TCO derivatives than any other bioorthogonal reaction pairs (approx. 1000 M-1 s-1). Moreover, they tolerate a wide array of reaction conditions. This makes them the prime choice for applications like protein labeling.
●Hydrogen-substituted tetrazines, on the other hand, show lower stability and less tolerance to harsh reaction conditions, but offer extremely fast reaction kinetics (up to 30000 M-1 s-1) for applications like in vivo imaging.
There are two main types of tetrazines that are widely applied: 6-Methyl-substituted tetrazines and 6-hydrogen-substituted tetrazines.

 6-Methyl-substituted tetrazine (**6-Me Tetrazines** - left) and 6-hydrogen-substituted tetrazine (**6-H Tetrazines**)

NT-XLfctl

Choice of Spacer: Alkyl or PEG?

Tetrazines equipped with alkyl spacers are suitable for reactions in organic solvents. For experiments in aqueous media, however, PEG spacers are usually the best choice. Moreover, tetrazines equipped with PEG-spacers are ideal for the functionalization of proteins, since they reduce the aggregation of labeled polypeptides.

References

Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging; N. K. Devaraj, R. Weissleder and S. A. Hilderbrand; Bioconjugate Chemistry 2008; 19: 2297-2299. doi:10.1021/bc8004446

Synthesis and Evaluation of a Series of 1,2,4,5-Tetrazines for Bioorthogonal Conjugation; M. R. Karver, R. Weissleder and S. A. Hilderbrand; Bioconjugate Chemistry 2011; 22: 2263-2270. doi:10.1021/bc200295y

Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides; C. W. Tornøe, C. Christensen and M. Meldal; The Journal of Organic Chemistry 2002; 67: 3057-3064. doi:10.1021/jo011148j

A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes; V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless; Angewandte Chemie International Edition 2002; 41: 2596-2599. doi:10.1002/1521-3773(20020715)41:143.0.co;2-4

Tetrazine Reagents from Interchim.com

•Uptima+Tetrazine:
Sulfo-6-Methyl-Tetrazine-DBCO #MRU280 (FT a faire!!! actualmt Jena!!!)
Tetrazine-mPEG (5-10-20-30KDa) #1N691
•FluoProbes+Tetrazine:
BrDIPY FL – Tetrazine #AWHFD0
(FT-WXS720): Cy – Tetrazines : Cy3/5/5.5/7 and DiSulfo, TriSulfo versions
•All Tetrazine reagents: TCO, Tetrazine (+ fluorescent labels: Cyanine, CF488/568/647, FAM, BrDIPY, …), (linkers: +mPEG, …) (crosslinkers: +Amine, Carboxyl Acid, NHSuc ester, Azide, Alkyne, AminoOxy, Hydrazide,…) (ligands: + Biotin/Desthiobiotin, Agarose, …)

.Thiol: cf Sulfhydryl

.ThioCyanate: see below **Cyanates**

.Tosyl: see below **Tosylates**

.VinylSulfone

●Structure: :
●Reactivity:
[] The **VinylSulfone** group (VS) reacts in a similar manner than Maleimide, but at high pH and unlike maleimides, without its potential hydrolysis. The reaction occurs by Michael addition with the C=C bond to form a physiological stable thioether linkage.
Also, Michael addition to vinylsulfones does not generate stereoisomers. Caution may however be payed to the selectivity of the reaction because of possible unexpected reactivity with secondary targets, that depend on the protein structure (pka is modified locally): the ε-amino groups of lysine and to a lesser extent the imidazole ring of histidine side chain (i.e. at pH 7.7).
Vinylsulfone reacts slowly with thiols to form a stable thioether linkage to the protein at slightly basic conditions (pH7–8) but will proceed faster if the pH is increased. Although VS derivatives (PEGs) is stable in aqueous solutions, I can react with lysine residues at elevated pH (unlike Maleimide that is more reactive to thiols even under acidic conditions pH 6-7 but it is not stable in water and can undergo ring opening or addition of water across the double bond).

See the technical notice 'VinylSulfone'[XLVISU].

>other fonctionnals groups (strong/poorly selective – for organic chemistry)

.Acrylate

●Structure: Acryl :
●Reactivity:
[] The **Acrylate** group can react with amines, thiolates and alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.
[XLACRYL].

.Cyanate

●Structure: Cyano :
●Reactivity:
[] The Cyanate group can react with amines, thiolates and alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.
[XLCYANI]. See also IsoThioCyanate (more usefull for bioconjugations)

.Tosylates

●Structure: :
●Reactivity:
[] The **Tosyl** group can react with amines, thiolates and alkoxides to form more stable carbon-N, -S or -O bonds, respectively. It readily reacts with amine groups at a pH of from 8.0 to 9.5.
+ [XLTOSY].

Functional Groups for organic chemistry

Functionnal groups for Modification/Protection/Deprotection in aqueous media

More technical information and ordering information

Ask Interbiotech@interchim.com for any question about these protective agents.