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Cleavable linkers in chemical biology

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ABSTRACT

Interest in cleavable linkers is growing due to the rapid development and expansion of chemical biology. The chemical constrains imposed by the biological conditions cause significant challenges for organic chemists. In this review we will present an overview of the cleavable linkers used in chemical biology classified according to their cleavage conditions by enzymes, nucleophilic/basic reagents, reducing agents, photo-irradiation, electrophilic/acidic reagents, organometallic and metal reagents, oxidizing reagents.

1. Introduction

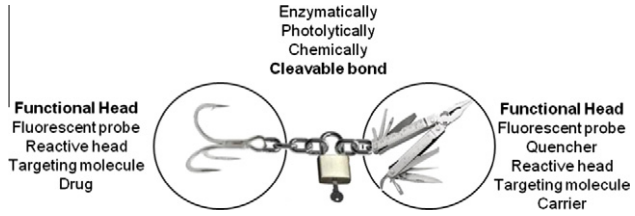
The ability to break chemical bonds that link two molecular entities can be an effective tool, and consequently many cleavable linkers with this capability have been developed. Applications of cleavable linkers have been characterized for organic synthesis and more recently chemical biology.

Progress in combinatorial chemistry, solid phase synthesis (SPS) and micro-chip chemistry in the early 1990s renewed the interest in cleavable linkers.[1–4] These linkers connect the organic substrate to the solid support. They are required to be stable during the synthesis steps and are eventually cleaved to release the product. The choice of linker is crucial during the planning of the synthesis strategy. An array of linkers have been developed and optimized for specific small molecule synthetic strategies; these were optimized to be resistant to a diverse range of conditions involved in synthesis, while reactive towards other chemistries. Generally, these linkers are cleaved under harsh chemical conditions. The linker strategies in solid-phase organic synthesis have been extensively reviewed.[5]

The recent rise of chemical biology has lead to the demand for cleavable linkers that are compatible with the biological molecules and systems. Chemists started to reassess potential functional groups and cleavage reactions that could be used in biological chemistry, since the harsh conditions required by organic synthesis linkers are unsuitable. The new types of cleavable linkers have to satisfy various challenging constraints, such as mild cleavage conditions, usage of bio-orthogonal reagents, high yield at low concentrations, and the ease of eliminating excess of reagents and by-products. Additional technical requirements will depend on the specific application of the linker, for example often the objective of proteomic experiments is to isolate and identify labelled proteins, which are expressed at low levels, and to determine where the label is incorporated in the protein.[6–9] Consequently, the ideal cleavable affinity purification linkers enable the quantitative and specific release of proteins in low abundance from solid supports, without generating by-products detrimental to subsequent analysis, such as mass spectrometry (MS). The linkers must be resistant to the labelling and purification steps, but are also required to be labile in mild conditions, so not to degrade the labelled proteins.

In this review, we define a linker as a molecule with two functional heads joined together through a cleavable bond (Scheme 1). The functional heads serve to interact with or to manipulate the biological target; they can be reactive groups for protein cross-linking, ligation or for click chemistry; fluorescent probes for diagnostic tools, proteomic analysis or cell imaging; tags for MS analysis or purification; targeting molecules for functional proteomics or activity based probe profiling (ABPP).

The flexibility in the design of the cleavable linkers has led to their application in many different biological disciplines including drug development, proteomics, imaging and DNA sequencing.



Scheme 1. General representation of a cleavable probe.

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1.1. Drug development

Many drugs often cause unwanted, dose-limiting, and debilitating side effects because they are untargeted, toxic compounds that act on the normal and disease cells alike. Prodrugs and drug delivery systems have been developed to try to reduce amount of off-target effects. Prodrugs are defined as chemicals which have little or no pharmacological activity undergoing biotransformation to a therapeutically active metabolite. The objective of prodrugs is to improve the pharmacokinetic and pharmacodynamic profile of the molecule.[139] Prodrugs generally consist of two elements, the active agent and a promoiety "carrier", which are joined together by a linker and are frequently activated by enzymatic hydrolysis or chemical conditions specific to the diseased tissue.

Drug delivery systems aim to administer an active drug to achieve a therapeutic effect and to improve the product's efficacy and safety, compared to the drug alone. Many different drug delivery systems have been developed and can be prepared from soluble and insoluble, or biodegradable natural and synthetic polymers, microcapsules, cells, cell ghosts, lipoproteins, liposomes, and micelles. All of the different carriers must be able to carry, target and release the drugs within the required timeframe, and at the desired location. Cleavable linkers used for drug delivery should be sensitive to enzymatic hydrolysis or to chemical conditions.[140,141]

1.2. Proteomics

Cross-linking agents aim to form a covalent bond between two spatially adjacent residues within one or two polypeptide chains. To identify protein binding partners, the cross linking agents need to be able to detect and stabilize transient interactions. The cross-linking agents frequently form covalent links between lysine or cysteine residues in the proteins. Alternatively, the cross-linking agent can be photoreactive. Cross-linking cleavable linkers can be used to distinguish between inter- and intra-protein interactions of receptors, signalling cascades, and the structure of multi-protein complexes.[142]

Affinity purification is used to obtain an uncontaminated sample of a protein or to determine a physical interaction between a protein and interacting partners. The cleavable affinity purification probe can be used to conjugate the ligand to the solid support and it is cleaved to liberate the proteins under mild conditions. The ligand can be an inhibitor, a suicide substrate, a small molecule substrate (for ABPP), a metal ion chelate, or a drug. The identification of the proteins can be determined through MS, and the purified proteins could be used in functional studies.[143,144]

1.3. Imaging

Medical imaging is used for instance to diagnose or examine diseases and to study anatomy and physiology. The detection and staging of tumors currently relies on computed tomography, which has the disadvantages of being expensive and using radiation; while magnetic resonance imaging (MRI) scans are cheaper, they are limited by the sensitivity of gadolinium chelates, or iron. Currently, 25% of the MRI examinations use contrast agents to increase the clarity of the soft tissue being visualized. Recently developed MRI scan contrast agents contain cleavable linkers, which enable the detection of enzymes involved in malignancy and metastasis.[18,26]

Molecular imaging can be used to characterize biological processes or conditions in vivo, using animal and cellular models. Most molecular imaging probes use inducible fluorescence to monitor biological reactions. Some rely on a cleavable linker to separate the fluorophore from either a quencher or another fluorescent dye. The cleavable bonds can be responsive to specific conditions such as enzyme activity, or the presence of thiol compounds.

1.4. DNA sequencing

Recently developed DNA sequencing by synthesis has reduced the cost and increased the speed of sequencing. The development of this technology has been possible due to the conjugation of nucleotides bases to a fluorescent probe via a cleavable linker. These linkers can be cleaved via exposure to light, palladium, or by enzymes. In DNA sequencing by synthesis, a DNA polymerase incorporates a single fluorescently modified nucleotide, complementary to the template base, and this stops the sequencing reaction. The remaining unincorporated nucleotides are washed away. The identity of the incorporated nucleotide is recorded and then the fluorescent dye is removed by a cleavage reaction. The next incorporation step is started after washing. DNA sequencing is used in a wide range of research sectors including comparative genomics and evolution, forensics, epidemiology, and applied medicine for diagnostics and therapeutics.[145,146]

Table 1
Examples of different cleavable groups and their applications

Cleavage conditions	Cleavable group	Applications
Enzymes	TEV,[10] trypsin,[11,12] thrombin,[13] cathepsin B,[14] cathespin D,[15] cathepsin K,[16] caspase 1,[17] matrix metalloproteinase[18–20] sequences, phosphodiester,[21,22] phospholipid,[23] ester,[24] β-galactose[25,26]	Protein purification, imaging enzyme activity and tumor, drug delivery, DNA sequencing, metabolite enrichment
Nucleophilic/ basic reagents	Dialkyl dialkoxysilane,[27–29] cyanoethyl group,[30,31] sulfone,[32] ethylene glycolyl disuccinate,[33,34] 2-*N*-acyl nitrobenzenesulfonamide,[35] α-thiophenylester,[36] unsaturated vinyl sulfide,[37,38] sulfonamide after activation,[39–41] malondialdehyde (MDA)-indole derivative,[42] levulinoyl ester,[43] hydrazone,[44] acylhydrazone,[45] alkyl thioester[46–48]	Protein modification and purification, structural biology, imaging, synthesis of oligonucleotides
Reducing reagents	Disulfide bridges,[49–64] azo compounds[65–79]	Protein modification and purification, structural biology, tumor-targeting, imaging, visualization of PEG shedding, drug delivery
Photo-irradiation	2-Nitrobenzyl derivatives,[80–90] phenacyl ester,[91,92] 8-quinolinyl benzenesulfonate,[93] coumarin,[94,95] phosphotriester,[96] bis-arylhydrazone,[97] bimane bi-thiopropionic acid derivative[98]	Protein purification, imaging protein activity, structural biology, drug delivery, DNA sequencing, metabolite enrichment
Electrophilic/ acidic reagents	Paramethoxybenzyl derivative,[99,100] *tert*-butylcarbamate analogue,[101] dialkyl or diaryl dialkoxysilane,[102] orthoester,[103,104] acetal,[103,105] acontityl,[106,107] hydrazone,[55,108–110] β-thiopropionate,[111–113] phosphoramidate,[114] imine,[115,116] trityl,[117] vinyl ether,[118] polyketal,[119,120] alkyl 2-(diphenylphosphino)benzoate derivatives[121]	Protein purification, structural biology, drug delivery
Organometallic and metal catalyst	Allyl ester,[122,123] 8-hydroxyquinoline ester,[124] picolinate ester[125]	DNA sequencing
Oxidizing reagents	Vicinal diols,[126–136] selenium compounds[137,138]	Structural biology

In this review we will present an overview of cleavable linkers classified according to their cleavage conditions, along with a selection of their biological applications (Table 1).

2. Enzymatically cleavable linkers

In chemical biology, among the six different enzyme classes (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases), hydrolases are widely used to induce chemical-bond cleavage using water as the nucleophile. Many different types of bonds (ester, peptide, glycoside, etc.) are able to be cleaved but each one requires a specific enzyme (esterase, protease, glycosidase, etc.). This enzyme–substrate specificity distinguishes enzymes from other chemical catalysts and enables enzymes to be used as a cleavage reagent. Proteases (also called peptidases) are enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds.

Hydrolytic activity is restricted when one amino acid or a specific sequence of amino acids is recognized by the enzyme. The longer the amino acid sequence required for the enzyme to recognize the cleavage site, the more specific the cleavage. Compared to commonly used proteases, such as pepsin, trypsin or chymotrypsin, virus proteases have more stringent sequence specificities.[147] Speers et al. designed a tobacco etch virus protease (TEV)-sensitive probe, using the ENLYFQG sequence as the cleavable linker, a biotin group for affinity purification and an azide for a click reaction.[10] The probe was designed to be used in tandem orthogonal proteolysis (TOP) experiments. Following proteome labelling with an alkynyl ABPP probe, the cleavable biotin probe was attached to proteins by a click reaction. The proteins were captured on a solid support, purified from the cell lysate and digested by trypsin. The MS analysis of the tryptic peptides can be used to identify the protein. The proteins remaining on the solid support can be subsequently cleaved with TEV and analyzed by MS to identify the probe's labelling site. In this study, cleavage times were overnight and 12 h for trypsin and TEV proteases respectively. Based on this trypsin cleavable linker approach, the same group developed a protease cleavable probe strategy to chemically tag and enrich specific sets of metabolites based on shared functional-group composition.[11]

Since proteases have key roles in many diseases, protease-responsive linkers are widely used in drug release systems[13] or in diagnostic tools.[18,19,148] MRI scan contrast agents can detect specific types of malignant and metastatic cancer. Activatable cell penetrating peptides (ACPPs) are polycationic cell penetrating peptides conjugated to a polyanionic inhibitory domain which minimizes the peptides' adsorption and cellular uptake. The release of the inhibitory domain by proteolytic cleavage leads to the uptake of the ACPP by surrounding cells. ACPPs labeled with Cy5 or gadolinium(III) can be 2 and 9, which are overexpressed in a wide variety of tumors.[20] This can be used for disease progression monitoring or for fluorescence guided surgery. The use of fluorescence imaging to visualize the tumor during surgery improved survival in murine models of cancer.

Proteases are not the only enzymes that can be exploited in chemical biology; other enzyme-sensitive linkers have been developed such as esterases and β-galactosidases. FRET-based probes were designed to image esterase activities. The insertion of phospholipid and phosphodiester groups between two fluorescent dyes or a dye/quencher pair allows the detection of phospholipase and phosphodiesterase cleavage activity, respectively.[21–23]

According to our definition the linker's cleavable group needs to be modified on two positions; for enzymatically cleavable linkers this requirement can affect enzyme–substrate recognition. For example, β-galactosidase can only tolerate the modification of its substrate, β-galactopyranoside, on the anomeric carbon. Komatsu et al. used quinone methide chemistry to overcome this problem and developed a FRET-based probe by conjugating a trifunctionnal 4-hydroxymandelic acid to β-galactopyranoside on the phenol position, and adding two fluorescent dyes on the mandelate linkage, with a carbamate as the leaving group (Scheme 2).[25] In the presence of β-galactosidase, the sugar unit is removed to form quinone methide. The species created reacts with a nucleophile and releases acceptor of fluorescence to restore fluorescence of donor.

3. Nucleophile/base sensitive linkers

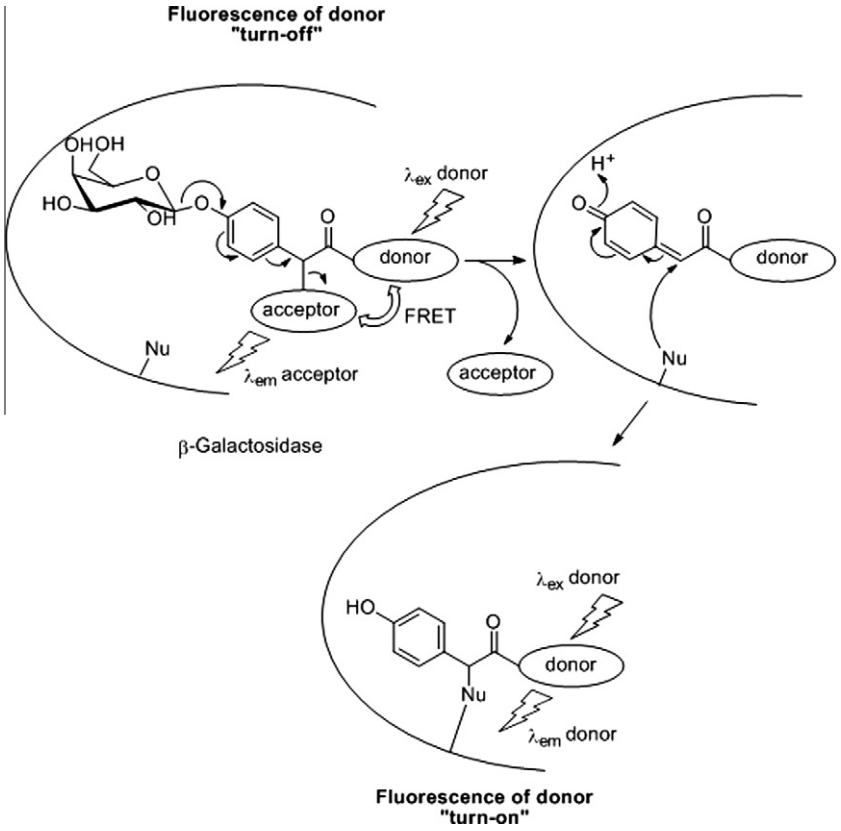
3.1. Halogen nucleophiles

Fluoridolyzable linkers are widely used in organic chemistry as silicon-based protecting groups for alcohols. The high thermodynamic affinity of fluorine for silicon allows their removal in orthogonal and mild conditions using a fluorine source. In this reaction a fluoride ion reacts with silicon as nucleophilic species and the cleavage conditions depend on the steric hindrance of the silicon's alkyl group. Since amino acid residues or DNA bases are not covalently altered by fluoride ions, fluoridolyzable linkers appear to be a suitable candidate for bioorthogonal and biocompatible cleavage conditions. Lin et al. were the first to describe silicon cleavage in physiological conditions.[27] The authors prepared a probe that contained a biotin group for affinity purification, a dialkoxy silane function as the cleavable group and a chloroacetyl ester to target the cysteine-protease papain. Once formed, the biotinylated protein was purified by affinity chromatography using potassium fluoride as eluent. It was shown that elution was temperature dependent, and 0.5 M potassium fluoride was required for a complete cleavage at 4 °C in less than 6 h. Since this work, this cleavage methodology has been used for oligonucleotide purification rather than in the proteomics field.[28,29]

Fluoride ions can also trigger bond cleavage due to their basic properties. Knapp et al. designed a fluoride cleavable linker based on a 2-cyanoethyl function that is widely used as a protecting group in oligonucleotide synthesis.[30] In basic conditions, the labile acidic vicinal proton of the nitrile function is removed to induce the molecule dissociation by β-elimination. Associated with a reporter molecule (like azide or NHS group), this linker was used to reversibly label oligonucleotides. However, currently cleavage is limited to nonaqueous systems (TBAF 1 M in THF) and this linker is only used in organic synthesis.

3.2. Oxygen nucleophiles

According to our literature review, two different oxygen nucleophile sensitive linkers were used with reactiveness toward hydroxyl ions. The first generation was developed for protein cross-linking and is based on a sulfone linker, which could be cleaved at pH 11.6 at 37 °C for 2 h.[32] The second generation of linkers were the ester groups cleaved by saponification. The reactivity of the ester to cleavage can be enhanced by the use of electron-withdrawing groups. In chemical biology, ester-based cleavable compounds were initially used for protein purification[149] and in structural biology. The structure of protein complexes is determined by cross-linking the proteins and MS and MS/MS analysis; Petrotchenko et al. described a bi-reactive sulfo-NHS ester cross-linking reagent with a deuterated ethylene glycolyl disuccinate as the cleavable group.[33] After cross-linking and enzyme digestion, cross-linked peptides were incubated in a 1 M ammonium hydroxide cleavage solution for either 2 h or overnight at 25 °C. The cleavage cocktail was directly analyzed by MS and the interacting subunits of the HIV-reverse transcriptase complex were identified. For this approach, chemical cleavable cross-linkers were used to identify the interpeptide and intrapeptide interactions within the HIV-reverse transcriptase complex.



Scheme 2. FRET-based cleavable probe sensitive to β-galactosidase activity.

3.3. Thiols nucleophiles

In secondary amine synthesis or solid phase synthesis, nitrobenzenesulfonamides are known to be cleaved with a thiol nucleophile, like β-mercaptoethanol.[150,151] Based on this reaction, Yokoshima et al. designed a cleavable photoaffinity probe which enables the release of proteins in a solution of 3% mercaptoethanol at pH 9.2 at 20 °C for 4 h.[35]

Long et al. developed a FRET based ratiometric thiol probe inspired by native chemical ligation strategies and the well-known reactions between the *N*-terminal cysteine's nucleophilic thiol group and the electrophilic carbon of α-thioesters.[36,152] The probe has a thiophenylester group as a cleavable linker, between a Bodipy molecule and rhodamine dye. Without a thiol reactive species, these dyes are closely coupled together in a restricted spatial environment and rhodamine quenches Bodipy's fluorescence via a FRET mechanism. In presence of free cysteines, the probe is turned-on: *trans*-thioesterification and intramolecular rearrangement of the probe affords a non-fluorescent rhodamine spirolactam compound, and a free Bodipy. This reaction can also be used in living cells to detect intracellular thiols, such as cysteine and glutathione, which play an important roles in redox related biological processes. A variety of human diseases with abnormal levels of biological thiols are associated with slow growth, liver damage, skin lesions, Alzheimer's diseases, cardiovascular diseases and coronary heart disease.[38]

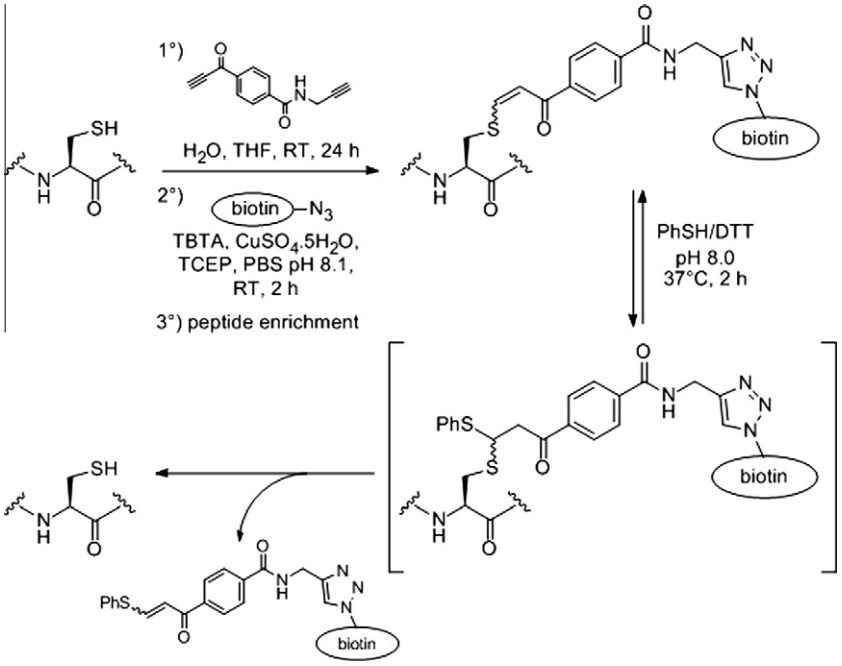
Cysteines can be modified by electron-deficient alkynes to form a vinyl sulfide linkage. Interestingly, depending on the structure of the alkynes used, the vinyl sulfide bond can be either stable or sensitive to thiol nucleophiles and DTT; however, they are consistently insensitive to TCEP. Shiu et al. reported that the modification of cysteines by terminal alkynones can result in a vinyl sulfide linkage, which can be cleaved by thiophenol, cysteine or β-mercaptoethanol via an addition/elimination mechanism (Scheme 3). Using this approach, it was possible to label peptides with biotin for purification from a solid support (71% yield). For this study, the cleavage cocktail was an aqueous solution of thiophenol/DTT at pH 8 at 37 °C for 2 h.[37] The same sensitive linker was also used to image thiols in cells using a quencher/dye pair.[38]

3.4. Safety-catch linkers sensitive to nucleophiles upon activation

Safety-catch linkers allow greater control over the timing of the bond breakage, because the linker will remain stable until it is activated for cleavage by a chemical modification. This principle was first demonstrated by the acylsulfonamide linker in SPS.[153,154] The main advantage of these molecules is their stability towards both basic and strongly nucleophilic reaction conditions. The linkers have to be activated via N-alkylation before they can be cleaved. The highly reactive *N*-acylsulfonamide species created provides the reactive linkage structure for cleavage by various nucleophiles. Park et al. were the first to describe the use of this two-step cleavable linker in proteomics.[40] They developed a probe that contains a sulfonamide linker surrounded by three functional groups: a biotin group for affinity purification, a ligand for galactose-specific lectins and a diazirine-based-photoprobe. After photo-cross-linking and washing the solid support; the linker was activated with 10 mM of iodoacetonitrile (pH 9 for 1 h), and the addition of 20 mM ammonium hydroxide (1 h) cleaved the linker and released 76% of the proteins. Alternatively, during N-alkylation of the acysulfonamide linker, iodoacetic acids salts in acidic medium (pH 5 for 10 min) can be used for instead of the iodoacetonitrile.[41] These milder reaction conditions can minimize protein degradation.

3.5. Nitrogen nucleophiles

Displacement reactions involving a specific nitrogen species as a nucleophile can occur in mild cleavable conditions. These



Scheme 3. Chemoselective cysteine modification by electron-deficient alkynes and cleavage of the formed vinyl sulfide linkage by thiophenol.

reactions can be classified into two groups; cleavage by aminolysis or exchange reaction.

For aminolysis cleavage, examples include the cleavage of a malondialdehyde (MDA) indole derivative by either pyrrolidine or hydrazine,[42] and the cleavage of an ester linker by hydroxylamine[34] or hydrazine.[43] In organic synthesis, a levulinoyl ester, an acid stable protecting group, can be removed either by sodium borohydride, sulfite anion donors, or by hydrazinolysis.[155] The presence of the γ-keto ester group in levulinoyl esters allows the reaction with hydrazine to occur in mild conditions, generating the corresponding alcohol and dihydropyridazinone compounds. Geurink et al. fine-tuned the levulinoyl ester structure to overcome base lability.[43] An optimized affinity probe was designed to contain a cleavable levulinoyl ester linked to a biotin tag and a selective proteasome inhibitor. After capture and pull-down, proteins were quantitatively released from the solid support using 100 mM hydrazine solution at 38 °C at pH 7.5 for 15 h.

Acylhydrazones[44] and hydrazones[45,156] can be used as cleavable linkers through transimination in a mildly acidic medium. An amine catalyst (e.g., aniline, *p*-anisidine or hydroxylamine) accelerates hydrolysis and enables the effective transition between stable and dynamic states, which is required for cleavage and exchange. This approach has the advantage of enabling the replacement of one functional group with another, when the linker is cleaved; this provides additional possibilities for protein labelling. For example, authors reported purification proteins by biotin–avidin capture followed by exchange of the biotin tag for a fluorescent dye or an isotope-coded affinity tag (ICAT) for further analysis. To identify new binding partners of anticoagulant protein S, Dirksen et al. modified protein S with a purification probe which contained a bisarylhydrazone cleavage site and a biotin tag (Scheme 4).[44] Proteins complexes were eluted using a mixture of 100 mM hydroxylamine and 100 mM aniline at pH 6.0 at room temperature for overnight. Since the use of strongly nucleophilic amines could induce protein denaturation, Dirksen et al. proposed an alternative strategy to cleave the hydrazone bond using an electrophilic reagent, like an aldehyde. However, this strategy also requires aniline as a catalyst for transimination, because the aldehyde cannot directly react with the hydrazone linkage. Although this method reduced the proteins yield, it released aldehyde-functionalized proteins, which provided a new group for chemical modification.

4. Reduction sensitive linkers

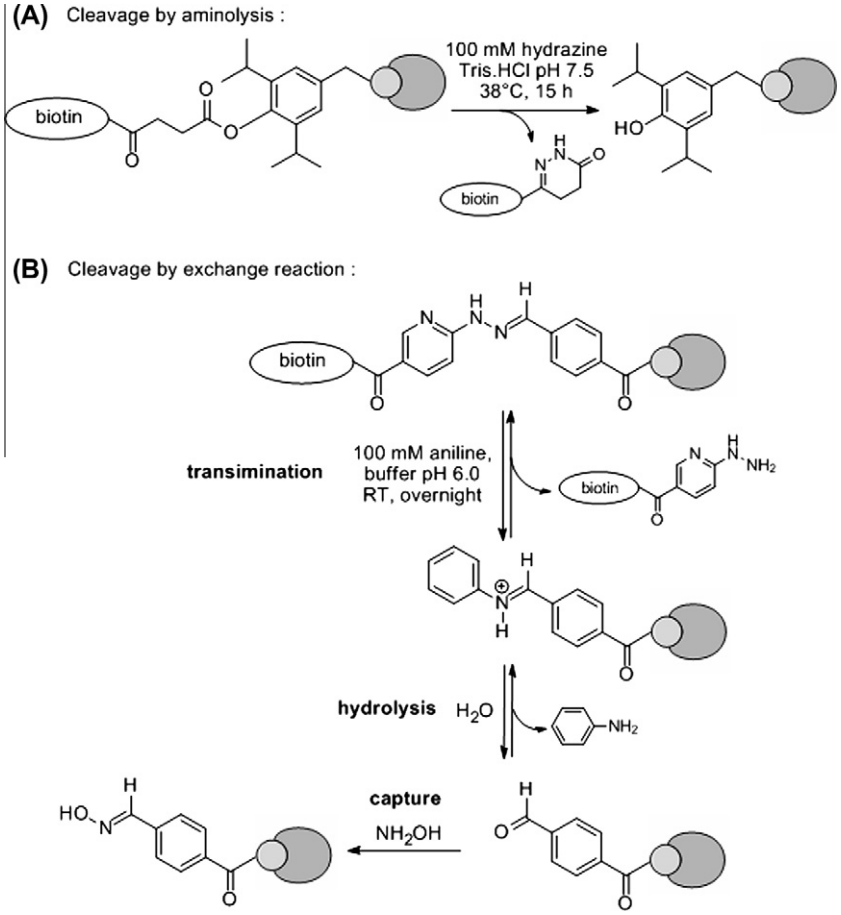
Reduction sensitive linkages have been used in chemical biology for a long time and it is a commonly used class of cleavable linker. However, there are only two types of cleavable linkers sensitive to reductive conditions: disulfide bridges and azo compounds.

4.1. Disulfide bridges

In naturally occurring proteins, disulfide bridges generally play a role in maintaining the protein structure. They are known to be efficiently and rapidly cleaved by mild reducing agents like dithiothreitol (DTT), β-mercaptoethanol or tris(2-carboxyethyl)phosphine (TCEP). In chemical biology, disulfide bridges have been used in a wide range of applications including functional and structural proteomics,[49–54] drug delivery,[55–57] tumor imaging,[59,60] DNA and protein–DNA complex purifications.[61,62] The disulfide-based cleavable linker is commonly used due to its straightforward synthesis and rapid cleavage. However, these linkers have several disadvantages including their instability towards both electrophilic and nucleophilic polar reagents. This subjects disulfide linkers to thiol exchanges and can lead to non-specific cleavage in intracellular conditions.

Pullela et al. designed a fluorescent probe to detect thiols in vitro and in vivo.[63] Fluorescein and rhodamine dyes were introduced on either side of a phenyl disulfide linker, and the fluorescence was turned-on by endogenous thiols cleaving the linker in zebrafish embryos.

Based on extracellular disulfide exchange principle, Zhang et al. developed a prodrug system.[56] The anticancer drug Camptothecin (CPT) is a potent topoisomerase I inhibitor but it is poorly soluble and is instable under physiological conditions. The prodrug strategy adopted targeted CPT to the tumors by linking the drug to neuropeptide substance P (SP) via a disulfide bond. SP can specifically bind to the neurokinin-1 receptor, which is overexpressed in many



Scheme 4. Two amine sensitive linkers: (A) Aminolysis cleavage of levulinoyl ester by hydrazine; (B) Cleavage of bisaryl hydrazone by transamination reaction and capture of the formed aldehyde by hydroxylamine.

different types of cancer, and it is rapidly internalized. Following the internalization of the prodrug, the high glutathione concentration within the cells reduced the linker, and this released the active drug in the tumor cells. This prodrug increased the selectivity of treatment compared to unconjugated drug alone.

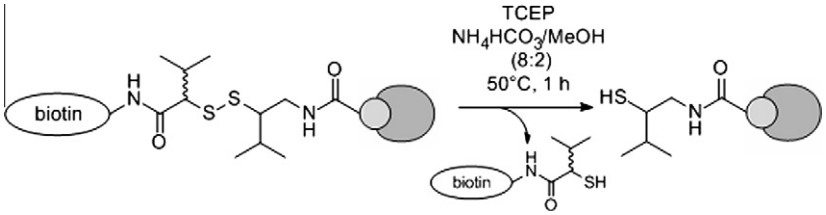
Gartner et al. proposed a hindered disulfide linker for quantitative proteomic applications to overcome base-lability toward thiols (Scheme 5).[64] A catch and release probe was designed containing a biotin group for affinity purification, a cystine-reactive isotope label either side of a disulfide bridge. The cleavable linker was sterically hindered by a carbon framework rendering it inert to thiol exchange or reduction by DTT under alkylating conditions (10 mM DTT/6 M urea/buffer at room temperature for 60 °C). After proteins were immobilized on a solid support, they were eluted in denaturing conditions using 10 mM of TCEP in a mixture of ammonium bicarbonate/methanol (8:2) for 1 h at 50 °C. The authors underlined the critical importance of using the cosolvent to cleave the probe.

4.2. Azo compounds

Azo linkers are very appealing to chemical biologists since they are able to undergo cleavage following treatment with sodium dithionite, a mild and potentially bio-orthogonal reducing agent. The azo compound is reduced into two aniline moieties via an electrochemical reduction mechanism[157] and this allows the use of reducing agents that are commonly used in many biological protocols, such as TCEP, DTT. In chemical biology, azo compounds have been used to cross-link proteins for over a decade and more recently for protein affinity purification.[65–67] Verhelst et al. used this cleavable function to design a protein enrichment probe, which was based on a 4-(2′-hydroxy-phenylazo)-benzoic acid scaffold, with a biotin on one side of the azo and an activity-based probe for cathepsin on the other.[68,69] After capture, the cathepsin proteases were released from streptavidin beads in three dithionite elutions (3 × 25 mM dithionite at pH 7.4 for 15 min) and the cleavage efficiency was greater than 90%. The functional groups surrounding the cleavable bond were modified and have been used in different proteomic studies.[70–73] Landi et al. developed their own linker that could be cleaved with 300 mM dithionite solution at pH 6.5 for 10 min.[74] Hang and co-workers subsequently developed a second generation of azo linkers which elute proteins in a single step using 25 mM of dithionite for 30 min.[75]

To enhance the reactivity of azo compounds towards dithionite at physiological pH and to decrease dithionite concentration, we decided to carry out an extensive structure/reactivity study to fine-tune the azo scaffold.[76] A 2-(2′-alkoxy-4′-hydroxyphenylazo)benzoic acid (HAZA) was identified as an optimal structure for cleavage (Scheme 6). This compound was functionalized with a biotin on one side and an affinity probe targeting Gyrase-B, a subunit of DNA gyrase on the other. After capture and on-bead purification, proteins were rapidly eluted in 5 mM dithionite at pH 7.4 for 5 min.[77] Activity tests demonstrated that these extra-mild conditions permitted the recovery DNA gyrase A2B2 complex in its active form and demonstrated that the protein purification conditions were non-denaturing.

The use of azo reduction linkers is not restricted to functional proteomics, it can also be used to modify proteins on the interior



Scheme 5. Cleavage of hindered DTT resistant disulfide bond by TCEP.

surface of bacteriophages,[78] or to image hypoxia.[79] Kiyose et al. developed a smart FRET-based probe with a QCy5 fluorescent dye and BHQ3 azo based-quencher. The reducing conditions induced by hypoxia cleaved the azo bond in the quencher and turned on fluorescence. The probe was used for fluorescence imaging of hypoxic cells and real-time monitoring of ischemia in the liver and kidney of living mice. Based on this work, azo compounds might become a new platform for tumor drug delivery exploiting the hypoxic conditions of tumor cells.

5. Photocleavable linkers

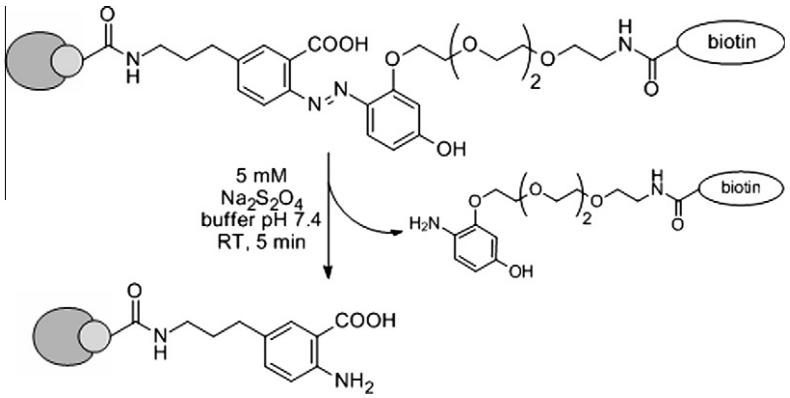
Cleavable reactions triggered by light are highly selective. Moreover, photosensitive groups are stable in different chemical conditions (acidic, basic, etc.). For all these reasons, photocleavable molecules were widely used, as protecting groups in synthetic organic synthesis[158] and as cleavable linkers in SPS.[1] In the literature, we found many different examples for their use in the chemical biology; however, we will present just an overview. For a complete overview of "caged" compounds, we refer to these general reviews.[159–161]

5.1. The *ortho*-nitrobenzyl derivatives

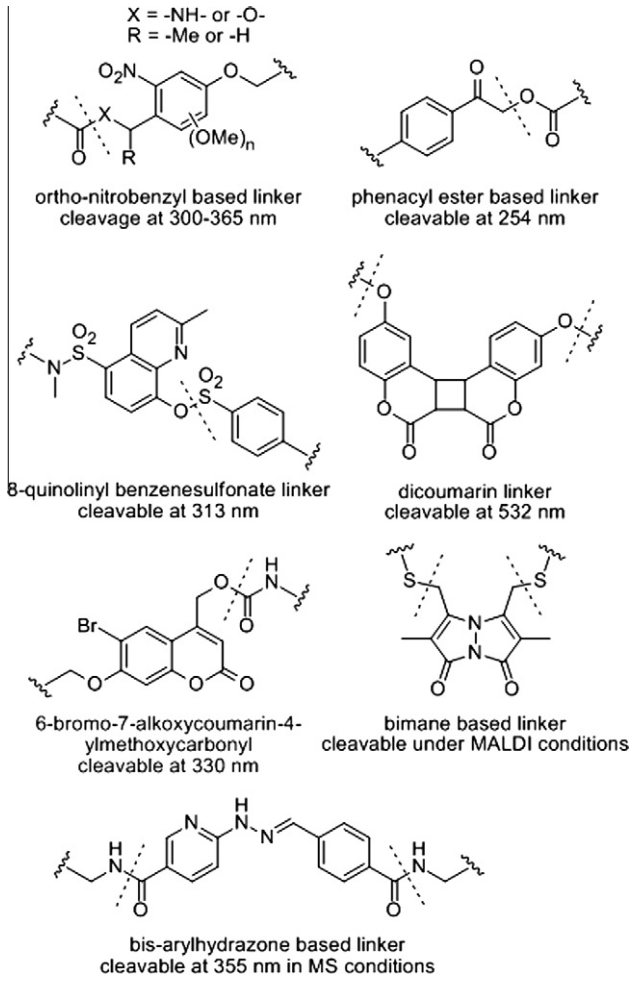
To the best of our knowledge, the most popular type of photolabile linker is the *ortho*-nitrobenzyl alcohol group and its derivatives. Typically, wavelength absorptions are located in the near-UV (300–365 nm) range of the spectrum and they can be fine-tuned by the addition of methoxy groups on the aromatic ring (Scheme 7).[162] Photolysis rates can be increased by the presence of a methyl group on the benzylic carbon,[163] and the reaction generally occurs via a Norrish-type II mechanism. Nitro group excitation leads to intramolecular hydrogen abstraction in γ-position, followed by the formation of azinic acid, which is highly reactive and rearranges into a nitroso compound. One of the biggest drawbacks of *ortho*-nitrobenzyl derivatives is that the release of the nitroso reactive compound can be harmful in biological medium and can induce unwanted side reactions.[160] In spite of this, many groups use this scaffold as a light-sensitive linker to study single nucleotide polymorphism,[80] record enzyme activity by fluorescence,[81] quantitatively analyze proteomes,[82] label proteins with fluorescent tags,[83] study protein–protein or peptide–DNA interactions by mass spectrometry,[84,85] analyze hepatitis C virus RNA polymerase protein,[86] deliver drugs from silica nanoparticules,[87] and determine protein structures.[88–90]

5.2. Phenacyl ester derivatives

Phenacyl ester derivatives can be cleaved by photosolvolysis through a radical scission of a carbon-oxygen bond. It is an alternative cleavable linker to *ortho*-nitrobenzyl derivatives and has higher yields following photolysis, and no reactive products are formed during the reaction (Scheme 7). However, phenacyl esters are sensitive to nucleophiles, unlike *ortho*-nitrobenzyl derivatives.[164] Recently, Orth et al. designed a photolabile probe to capture metabolites on a solid support.[91] This trifunctionnal phenacyl ester linker bears an azide to link a metabolic catcher, a fluorescent dye for HPLC visualization of the trapped metabolites and a biotin group for enrichment. After capture and immobilization on solid support, metabolites were eluted by UV-induced cleavage at 254 nm for 7 min in 69% yield. Unfortunately, this linker cannot be used with all biomolecules, since wavelengths below 300 nm are absorbed by proteins and can cause their degradation. Okamoto et al. used a 4-hydroxy-phenacyl ester derivative to develop a molecule-releasing system based on the molecular-beacon strategy.[92] An oligodeoxynucelotide (ODN) was functionalized at the 5′ end by a triplet quencher and 3′ end with a phenacyl ester and a biotin as the leaving functional molecule. In the initial state, the triplet quencher is in close proximity to the ester bond and inhibits cleavage. In presence of complementary target DNA, hybridization opens up the ODN hairpin-loop structure, which allows photolysis at 312 nm in biocompatible conditions. This study suggested that biotin could be replaced with a bioreactive agent and this molecule could be used in drug release.



Scheme 6. Cleavage of azo compound (HAZA scaffold) by sodium dithionite.



Scheme 7. Examples of photocleavable linkers; (dashed line = sites of cleavage).

5.3. Others photocleavable linkers

Other photolabile linkages were previously used to purify ligand–receptor complexes,[93] to generate photocleavable peptides/proteins[94] or for drug delivery (Scheme 7).[95] We will limit this section to the application of photo-sensitive linkages for MS analysis.

During MS analysis, the cleavage of photo-sensitive cross-linking agents needs to incorporate a label into the cross-linked peptides, which enables the identification of cross-linked peptides from other peptides. Gardner et al. presented two generations of cross-linking agents. The first is based on a triester phosphate group which is cleaved upon IR irradiation by a CO_2 laser.[96] The second is based on a bis-aryl hydrazone linker and is cleaved under UV irradiation at 355 nm.[97] In 2009, Petrotchenko et al. designed a new cross-linking agent: a bimane bisthiopropionic acid *N*-succinimidyl ester (BiPS), which is photocleavable, fluorescent, homobifunctional, amine reactive and isotopically coded.[98] This molecule enables the identification of cross-linked proteins by fluorescence and MS. In this application, cleavage is laser photo-induced and occurs directly in MALDI (Matrix-Assisted Laser Desorption/Ionization) conditions.

6. Electrophile/acid sensitive linkers

Two different modes of electrophilic cleavage are used in chemical biology: acidic sensitive linkers that are sensitive to proton sources, and alkyl 2-(diphenylphosphino)benzoate derivatives sensitive to azide compounds.

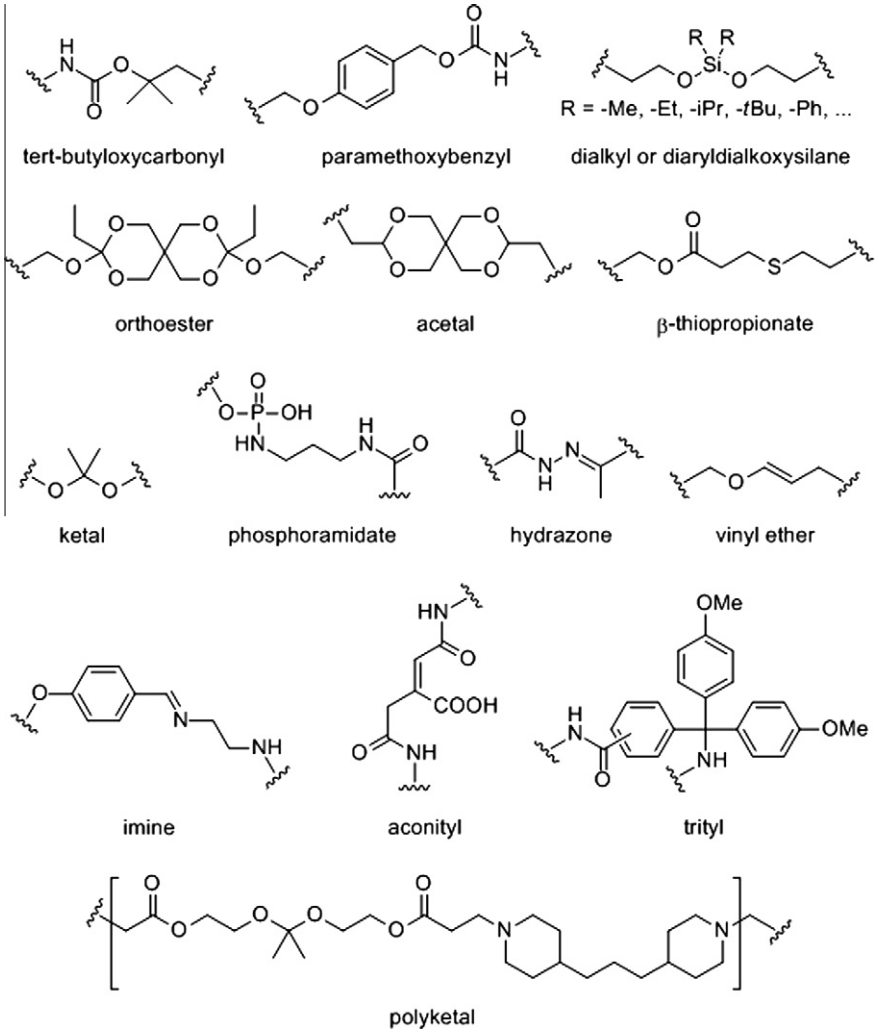
6.1. Acid cleavable linker

Proton sensitive bonds are among the most frequently used cleavable functions in organic chemistry; illustrated by the development of the BOC group which protects amines or the Merrifield resin used in solid phase synthesis. In organic chemistry, the cleavage conditions that can be tolerated are very flexible regarding the acids' reagents, solvents, temperatures and pH. In contrast, biocompatible acid cleavable linkers must be responsive to minor changes in pH. Strong acidic conditions can lead to the denaturation of proteins and DNA. Biocompatible acid cleavable linkers are chosen for their instability near physiological pH and are often different from the classical protecting groups, which are cleaved with strong acids. However, parathoxybenzyl[99,100] or tert-butylcarbamate[101] scaffolds, which are both cleaved by trifluoroacetic acid, were previously used as probes for proteomic expression profiling analysis (Scheme 8). However, their use has been somewhat limited since trifluoroacetic acid denatures proteins and DNA and induce non-specific cleavage.

Recently Szychowski et al. designed an enrichment probe with a diphenyldialkoxysilane cleavable bond, a biotin as a purification tag and an azide as the chemical reactive group.[102] Following the labelling of GFP, 95% of proteins were eluted from the solid support with 5% formic acid (at pH ≈2.5, incubated at room temperature, for 30 min). These elution conditions are milder than those previously described, but they are still denaturing. To the best of our knowledge, no probe has been described to be sensitive to mild acids for protein pull-downs. Only a few papers characterize reversible protein cross-linking probes sensitive to acidic conditions.[103,106] This is due to the difficulties in designing linkers that are stable during protein capture and purification, but are cleaved under mild biocompatible conditions.

Acidic pH-gradients are widely used to trigger cleavage for drug delivery systems or responsive polymeric biomaterials. It is well known that the local acidic conditions are correlated with various diseased states, such as tumors, ischemia and inflammation.[165,166] To ensure that drugs accumulate in these specific areas and not normal tissues, different rates of hydrolysis must be induced by even smaller differences in pH. Selectivity and efficiency of cleavage is based on its specific reaction kinetics. In contrast to classical linkers, this is not a simple turn on/off system stimulated by a chemical agent. In the literature, we found many different examples of acid-sensitive linkers for drug delivery and has been previously reviewed.[167] For example, an acid-sensitive linker was used as a part of a nanoparticle drug delivery system, which was based on the natural polysaccharide pullulan, and the anticancer drug, doxorubicin (Dox).[108] Dox is joined to the pullulan backbone by a pH-sensitive hydrazone bond, and these conjugates self-organize into nanoparticles due to the hydrophibic nature of the drug in water. The drug is released in acidic conditions of pH 5, which surrounds hypoxic tumor cells, but the conjugate is stable at pH 7. In comparison to the unmodified Dox, the pullulan–Dox conjugate has an improved biocirculation, biosafety and cardiotoxicity profile, while the anti-tumor effects remain comparable.

In the field of drug delivery, the most common cleavable structures are: dialkyl and diaryldialkoxysilane,[55] orthoester,[104] acetal,[105] aconityl,[107] hydrazone,[109,110] β-thiopropionate,[111–113] phosphoramidate,[114] imine,[115,116] trityl,[117] vinyl ether,[118] and polyketal (Scheme 8).[119,120] Although a broad spectrum of groups have been used in physiological conditions, it is difficult to choose the best linker for cleavage at a specific pH, because a systematic study



Scheme 8. Examples of acidic sensitive linkers.

comparing the cleavable structure and their pH sensitivities has not yet been published.

6.2. Azide cleavable linker

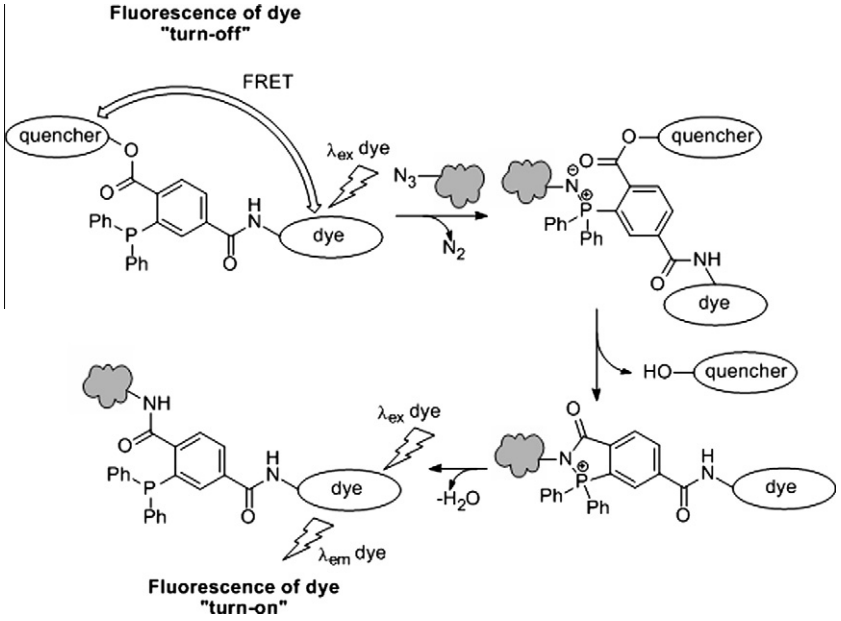
Chemical reactions that can break or form bonds in water can be used as the basis of a cleavable linker, for example the Staudinger ligation.[168,169] This reaction is proceeded by the nucleophilic attack of an alkyl 2-(diphenylphosphino)benzoate derivative on an azide, to form an aza-ylide intermediate (Scheme 9). Then the ester traps the aza-ylide, which leads to the formation of an amide. In this process, the ester acts as a cleavable linker, and the azide as a bioorthogonal chemical agent, which guarantees a chemoselective and bioorthogonal cleavage.

To image azides in living cells, Hangauer et al. designed a FRET-based turn-on fluorogenic phosphine probe.[121] Phosphine was derivatized with a fluorescent dye and the alkoxy residue was functionalized with a quencher, to form a turn-on probe by intramolecular FRET quenching. After labelling cellular glycans with an azide, the derivatized phosphine was incubated with the living cells for 8 h at 37 °C. The subsequent Staudinger ligation released the quencher and turned on the fluorescence. However, this system is hampered by its slow kinetics and the oxidation-sensitive linker.

7. Metal-assisted cleavage

Organometallic compounds are used to catalyze the modification of proteins containing non-natural amino-acids,[170] but their use as cleavage reagent in chemical biology has only been reported a few times. The allyl function is a commonly used protecting group for alcohols in organic synthesis and it is also used as a cleavable linker in DNA sequencing by synthesis.[122,123] Nucleotides are capped on their 3′-OH by a fluorescent dye via an allyl carbamate linkage; this nucleotide can reversibly terminate transcription. Each nucleotide is labelled with a different fluorescent dye. The nucleotide can be incorporated into the growing DNA chain by the polymerase and the capped 3′-OH stops the polymerase reaction. The fluorescence is measured, and the allyl carbamate linkage is cleaved in a deallylation mixture (Thermopol reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$) at 70 °C for 30 s. These harsh cleavage conditions limit the use of this linker to synthesis reactions.

Metal cleavable linkers were also used in the design of peptide nucleic acids (PNAs), which were developed for enzyme-independent DNA/RNA hybridization methods. Two PNAs were synthesized, each containing one half of the complementary sequence to the DNA template; and either contained an ester linker or a chelating pyridylpyrazolyl ligand. In the presence of the DNA



Scheme 9. FRET-based cleavable probe triggered by a Staudinger ligation between a phosphine derivative and an azide.

template, the two PNAs are brought into close proximity and the Cu(II) complexation on the ester linkage leads to 73% cleavage in 30 min.[124] This technique has the disadvantage that a single mismatch within quinoline-derivated PNA/DNA duplex reduced the rate of cleavage fourfold. This problem was overcome by Boll et al. who used a picolinate-ester instead of a 8-hydroxyquinoline ester, and their reaction is slightly more selective than the natural T4 DNA ligase enzyme.[125]

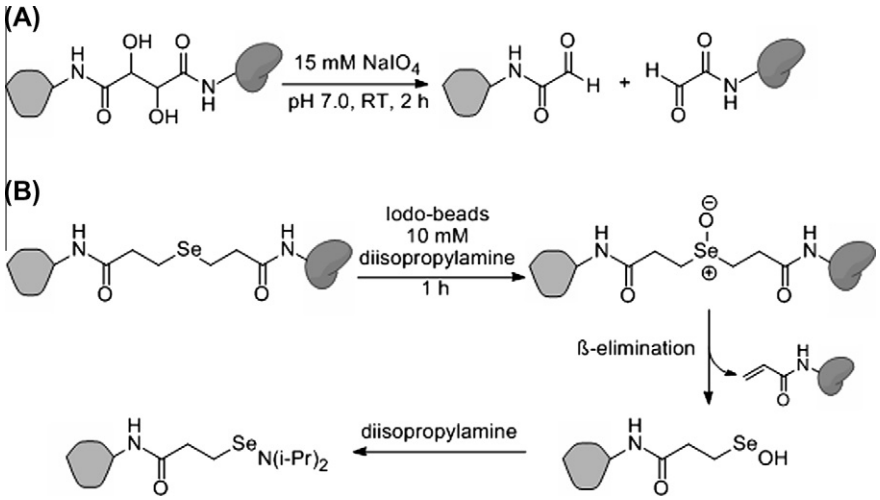
8. Oxidation sensitive linkers

Sodium periodate is undoubtedly the most frequently used biocompatible oxidizing agent due to its ability to cleave vicinal diols to form two aldehydes compounds (Scheme 10). One example of this type of cleavable linker consists of a vicinal diol with a tartaric acid spacer and two functional groups at both ends. Using this structure as a base, Smith et al. designed two disuccimidyl tartarate (DST) reagents to examine the ubiquinone cytochrome *c* reductase protein complex.[126] After cross-linking, both reagents were cleaved with 15 mM sodium periodate at pH 7.0 for 2 h. Since this study, water-soluble DST and DST-modified reagents have been made commercially available and have been used in many different protein cross-linking studies.[127–133] Diepoxybutane is also a linker used in structural biology and forms, after cross-linking, the vicinal diols structure and can be cleaved by sodium periodate.[134–136]

Selenium based linkers contain cleavable bonds sensitive to oxidizing agents, such as sodium periodate or *N*-chlorobenzenesulfonamide immobilized on polystyrene beads (iodo-beads).[137,138] The trigger agent oxidizes the labile bond to selenium oxide, which is then cleaved directly via intramolecular β-elimination or rearrangement. Advantageously, sodium periodate does not react with DNA,[171] or destroy disulfide linkages; consequently protein structure and complexes are frequently maintained (Scheme 10), in contrast to reductive cleavable agents. However, sodium periodate can damage RNA and some proteins; glycoproteins are particularly sensitive.

9. Summary and conclusions

This review outlines seven different classes of cleavable linker used in chemical biology. Although there is no systematic study that comprehensively compares the advantages and disadvantages of the various cleavage strategies, some general observations can be drawn from the literature. The use of enzyme-sensitive linkages provides chemo-selectivity and biocompatibility. However, the specific peptide sequence required by the enzyme will impose certain biophysical restrictions that may limit their use and can be laborious to synthesize. Nucleophile sensitive linkers are mainly used in proteomic studies and can efficiently release proteins from a solid support. Significantly this cleavage strategy presents the opportunity to introduce an additional functionality to the probe via an exchange reaction. However, it appears that nucleophile sensitive linkers require a medium with a basic pH to enhance the nucleophilic character of the reagent. Currently, reducing agent sensitive linkers appear to be the most prominent class of linkers in chemical biology. Disulfide bridges were historically one of the first cleavable linkers, although their sensitivity to nucleophilic compounds and especially thiols results in unwanted cleavages. In the proteomics field, the cleavage of disulfide linkers might also trigger the reduction of proteins' disulfide bridges, changing the protein's structure. Azo compounds, cleaved by sodium dithionite, are an alternative for protein pull-down assays since they are also chemically stable in physiological conditions. Optimized azo-based cleavable linkers were recently described to isolate endogenous protein complexes in non-denaturing conditions. However, the compatibility of sodium dithionite with disulfide bridges has not yet been demonstrated, also azo linkers have not been used to study DNA. Photocleavable linkers are widely used in chemical biology and many different photo labile structures permit efficient cleavage. However, near-UV light is toxic for cells below 400 nm, and wavelengths above 300 nm must be used to prevent protein degradation. Photocleavable groups must be carefully selected if they are to be used in conjunction with photo-reactive groups (eg. diazirine, phenylazide and benzophenone) which are activated in near-UV light. Acid sensitive linkages are very versatile especially since they can be cleaved in mildly acidic medium. A wide panel of acid labile functions was developed to cover the complete range of pH sensitivity from 0 to 6. Using pH to influence the rate of hydrolysis has been used in drug delivery and imaging molecules. For protein purification, acid sensitive linkers usually require a high pH to be cleaved, which likely to lead to non specific cleavage and protein denaturation. Linkers sensitive to oxidative conditions



Scheme 10. (A) Cleavage of a tartaric acid derivative by sodium periodate; (B) cleavage of selenium linker by iodo-beads via a β-elimination and capture of the formed selenic acid by an amine.

can be cleaved by sodium periodate and have many applications in structural biology because it can preserve the protein's structure, including its disulfide bridges.

In conclusion, interest in cleavable linkers is growing due to the rapid development and expansion of chemical biology. Indeed, chemical constraints imposed by the biological conditions cause significant challenges for organic chemists. Reagents, reactions and functional groups have to comply with high selectivity, efficacy and mildness issues linked to proteomics, imaging, and drug delivery applications. This is an interesting time for chemists and biologists to work together to meet and surpass these challenges.

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