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Tag and release: strategies for the intracellular cleavage of protein conjugates

Alice Leonie Baumann[1,2] and
Christian Peter Richard Hackenberger[1,2]

Attaching a functional moiety to a protein is required for a wealth of applications, comprising targeted delivery, controlling of enzyme activity, and prodrug-based therapy. Targeting intracellular processes requires the cellular delivery of the protein. While at first, a stable connection between the protein and the modification is desired, once inside the cell, the conjugate might be cleaved again to restore or activate the function of the individual parts. This can be achieved by employing cleavable linkages in conjugates, which are responsive to chemical or enzymatic stimuli inside cells. In this overview we describe strategies, how such entities can be introduced into proteins and how selective intracellular cleavage can be accomplished.

Addresses
[1] Leibniz-Forschungsinstitut für Molekulare Pharmakologie im Forschungsverbund Berlin e.V. (FMP), Campus Berlin-Buch, Robert-Roessle-Str. 10, D-13125 Berlin, Germany
[2] Humboldt-Universität zu Berlin, Institut für Chemie, Brook-Taylor-Str. 2, D-12489 Berlin, Germany

Corresponding author:
Hackenberger, Christian Peter Richard (hackenbe@fmp-berlin.de)

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Introduction

A protein can gain additional function by conjugating a tag to it, for example, a fluorescent marker for microscopy, a drug in antibody-drug conjugates (ADCs) for targeted therapy [1] or a cell penetrating peptide (CPP) to enhance the intracellular delivery of the protein [2]. The protein conjugate has to be stable until it has reached the desired location. Nevertheless, for several applications it may be desired that the conjugates are cleaved later on, for example, when the ADC has reached the target, and the drug has to be liberated in order to deploy its full activity. To achieve such release in a controlled fashion, selectively cleavable entities are incorporated. In this overview, various designs for such cleavable entities are discussed, with the focus on systems that get cleaved intracellularly (Figure 1). Important aspects in the design of intracellular cleavable entities include high extracellular stability to prevent loss of cargo, for example, during circulation, thereby causing side effects or background signal. Moreover, the linker unit should not impair the properties of the protein conjugate for the given application, while granting for efficient and specific release at a given intracellular destination. Last but not least the conjugate has to be incorporable into the protein via a practical route.

In this review we show how intracellularly cleavable entities can be introduced into proteins by means of bioconjugation and how they can selectively be cleaved afterwards. Cleavable entities have also been exploited for protein caging strategies to control protein function (as for example by the groups of Chen and Deiters [3,4]); however, these examples exceed the scope of this review. Notably, other excellent recent reviews exist on the subject of controlled intracellular cleavage and release [5–7]. Consequently, we focus on applications with protein conjugates.

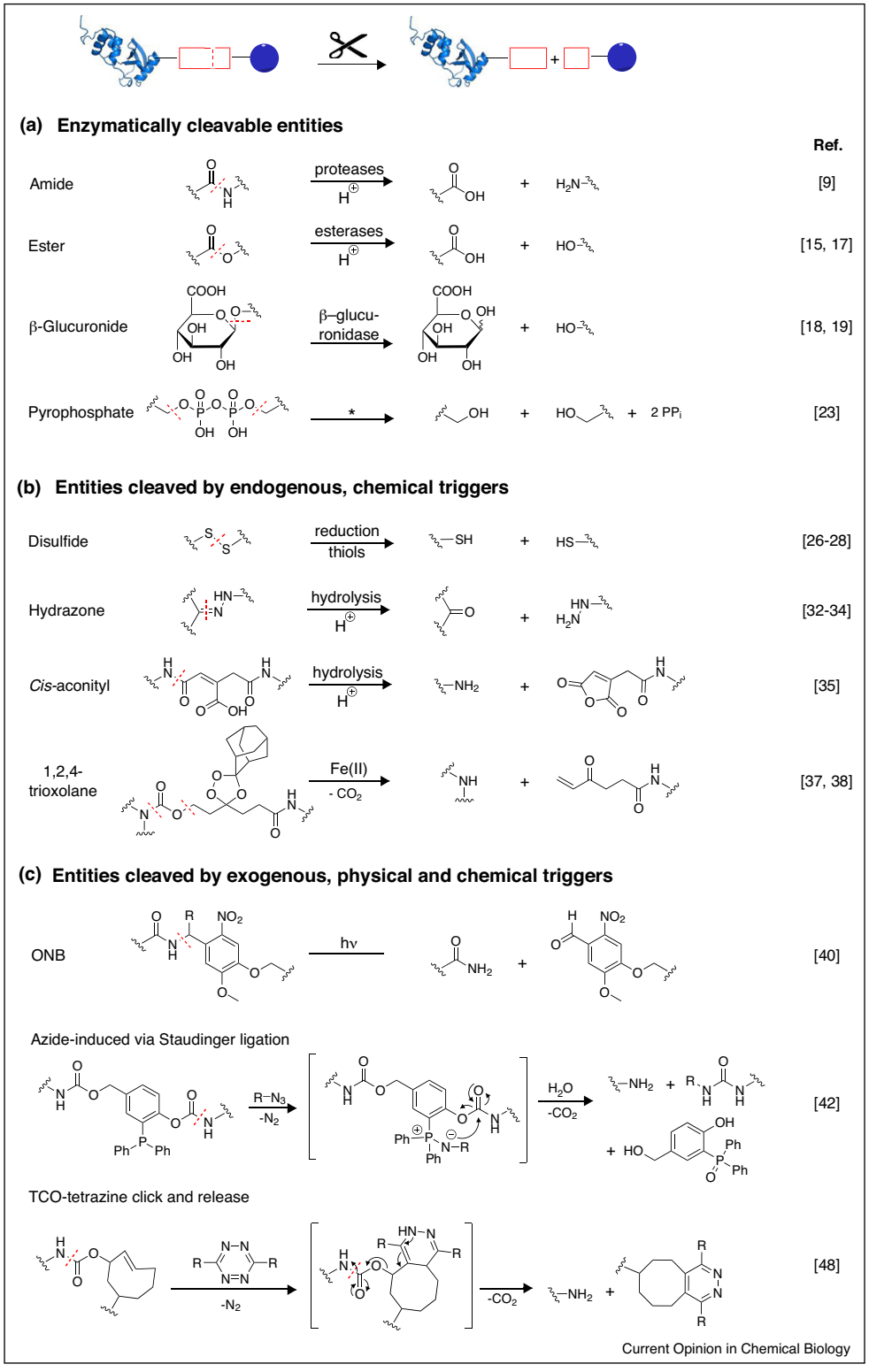
Enzymatically cleavable entities

Enzymatically cleavable linkers are particularly popular since the high intracellular abundance of enzymes ensure specific release (Figure 1a). Also, some enzymes are overexpressed in diseased cells, which can be exploited for drug conjugates.

Peptide linkers containing short recognition sequences get hydrolyzed in endosomes and lysosomes by proteases. They can conveniently be accessed by solid phase peptide synthesis (SPPS) and functionalization is usually made easy via coupling to a carboxylic acid or amine functionality.

A highly popular enzymatic cleavage sequence is the dipeptide valine-citrulline (Val-Cit) [8], which gets cleaved by the cysteine-protease cathepsin B. Cathepsin B is mainly located intracellularly in lysosomes [9] and overexpressed in many aggressive forms of cancer [10], making drug-cathepsin substrate conjugates good prodrug candidates. While cathepsin B has a relatively broad substrate scope, the Val-Cit linker is preferred in

Figure 1



Overview of intracellularly cleavable entities. *The cleavage mechanism for pyrophosphates has not been elucidated yet. ONB, *ortho*-nitrobenzyl, TCO, *trans*-cyclooctene.

conjugates because it displays a favorable balance between intracellular protease cleavage and plasma stability [11]. This system has been widely used for the construction of ADCs, also in combination with a self-immolative 1,6-linker as for instance in the generation of the conjugate brentuximab-vedotin (Adcetris®) [12]. While the Val-Cit linker shows good stability in human plasma, it is less stable in mouse plasma due to the presence of the extracellular carboxylesterase 1c. Anami *et al.* have recently reported on a Glu-Val-Cit sequence, which shows enhanced stability in mouse plasma [13]. Other cathepsin B recognition sequences have been exploited in a number of prodrug strategies, for example, the tetrapeptide Gly-Phe-Leu-Gly. While other proteases with intracellular activity have been exploited, still most prodrug approaches focus on cathepsin B due to its prevalent expression in intracellular lysosomes [14].

Carboxylic ester linkers are widely employed for different release applications. Ester bonds are susceptible to hydrolysis under acidic conditions in endosomes and lysosomes and by hydrolytic proteins including esterases [15] and cytochrome P450 [16]. Given this broad spectrum of release mechanisms, ester-based linker designs are problematic for targeted release strategies, since they are more prone to hydrolysis in serum than peptide-based linkers. Nevertheless, ester-based linkages have still proven useful in a number of applications, when their extracellular stability is carefully controlled. The Raines group for example used a trimethyl lock variant of an ester-cleavable system to release GFP from the cell surface-targeting ligand upon delivery into cells [17].

β-glucuronide-containing linkers are cleaved by β-glucuronidase, an enzyme present in lysosomes [18] and overexpressed in some tumors [19]. The first proposal of using beta-glucuronidase-responsive linkers in a prodrug strategy came from Tietze *et al.* in 1988 [20]. In recent years, numerous prodrugs were investigated based on this approach, often in combination with a self-immolative linker between the drug and the carbohydrate [21]. Burke and Lyon have recently shown that glucuronide-based linkers are suitable for reducing the hydrophobicity of highly loaded ADCs when combined with PEG units [22]. The construction of a β-glucuronide-containing linker requires several synthetic steps, rendering this method not as straightforward as others. An attractive feature of this release strategy is that the enzyme concentration outside cells is low, thereby ensuring high specificity.

A **pyrophosphate linker** has recently been developed by Kern *et al.* [23•]. Upon internalization, the pyrophosphate diester gets cleaved rapidly through the endosomal–lysosomal pathway. The enzymes involved in this process have not yet been identified. In their study they showcase that this linker can be used to construct ADCs; it showed excellent stability in human plasma, greater aqueous solubility than traditional linkers and fast release of payload in lysosomes. A limitation of this strategy is the rather sophisticated synthesis of the linker by phosphorimidazolidate coupling.

Chemically cleavable entities based on endogenous triggers

As opposed to enzymatically cleavable entities, release can also be triggered by chemical stimuli inside cells, for example, by exploiting differences in pH or in the redox environment between the outside and the inside of the cell. The main strategies described below rely on reductive cleavage in the cytosol and on acid-mediated cleavage in endosomes and lysosomes (see Figure 1b).

Reductively cleavable entities

The concentration of thiols is much higher inside than outside cells due to the presence of glutathione, cysteine or other thiol-containing biomolecules. Moreover, certain redox enzymes are present in intracellular compartments and have the ability to reduce disulfide bonds [24]. Consequently, **disulfide linkers** are efficiently cleaved in the reducing environment inside cells, while usually showing good stability outside.

Disulfide linkers can be readily synthesized from a wide range of thiol-containing starting materials using activated mixed disulfides or by direct oxidation [25]. Disulfide linkers have been exploited frequently for the intracellular release of a variety of cargos. Our group has recently demonstrated that full-length proteins, including fluorescent properties and nanobodies, can be delivered into cells when connected to cell-penetrating peptides [26•,27]. In both examples, a disulfide linker was used to connect the proteins with the CPP, which eventually resulted in traceless release and targeted intracellular localization of the proteins upon thiol exchange.

In an elegant recent work by Pillow *et al.*, it was demonstrated that small thiol-containing molecules can be directly attached to a cysteine residue of engineered antibodies via a disulfide bridge. They identified attachment sites, which resulted in antibody conjugates that were highly stable during circulation but showed rapid release once entered the cell [28]. In a study by Kellogg *et al.*, the relationship between the lability of the disulfide linker and the *in vivo* potency of an antibody-drug conjugate was investigated [29]. They looked at varying levels of steric hindrance around the disulfide bond when introducing adjacent methyl groups and observed that an ADC with a partially hindered disulfide linker (and hence increased disulfide bond stability) displayed the best efficacy. This result suggests that the release rate of a drug influences the cell killing activity and can be tuned by the respective linker design. Along these lines, Zhang *et al.* investigated the influence of linker immolation on

the cell killing potency of a variety of substituted disulfide linkers and found that efficient release was observed for methyl-substituted and cyclobutyl-substituted disulfide linkers [30].

Acid-cleavable entities

While the extracellular pH, for example in the blood stream, typically shows values between 7.2 and 7.4, the pH in intracellular compartments such as endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0) is considerably lower [31]. This variation can be exploited for the design of acid-cleavable linker systems that allow specific release of cargo at these locations. Given that many ligands are taken up by receptor-mediated endocytosis and therefore end up in endosomes, acid-sensitive linker strategies became popular for a wide range of applications.

Widely used acid-sensitive moieties for targeted release are **hydrazones**. Hydrazones are cleaved preferentially at pH < 5 but are fairly stable at physiological pH [32]. They are formed between an aldehyde or ketone and a hydrazine. Hydrazone-linkers have been applied for numerous anticancer therapeutic molecules [33], including ADCs. The first clinically approved ADC (anti-CD33 calicheamicin conjugate Mylotarg®) contained a hydrazone linker [34]. This ADC had been temporarily withdrawn from the market (and reintroduced again in 2018), as it caused too many side effects, likely due to instability of the hydrazone linker during circulation. Nevertheless, the stability of hydrazone linkers can be finely tuned by chemical substitutions, similarly as for the disulfide linkers described above.

Another acid-sensitive moiety is ***cis*-aconityl**, which was identified to have potential for targeted drug release in 1981 [35]. While most amides are highly stable toward hydrolysis, *cis*-aconityl is responsive to low pH environments. It has been used to connect the drug doxorubicin with a polymer-RGD-peptide conjugate [36]. However, this linker strategy has not been widely employed, probably due to synthesis considerations (incorporation into peptides by solid-support synthesis is made difficult due to acidic cleavage conditions) or the non-traceless nature of its release components.

Metal-cleavable entities

Recently, Spangler *et al.* have introduced a **ferrous iron-cleavable linker** based on a 1,2,4-trioxolane scaffold [37••] and showed in a proof-of principle study its potential as a linker for ADCs [38]. The idea is based on the observation that altered iron metabolism and elevated iron levels are associated with cancer [39]. The linker in its reported form suffers from instability when attached to an antibody (however not in cell culture experiments in the absence of an antibody). The reason for this remains unclear at this point and requires further investigation. Nevertheless, the concept of using a relatively unexploited endogenous trigger such as iron is an interesting idea and has the potential to open up a new dimension for linker design.

Cleavage induced by exogenous triggers

As opposed to endogenous triggers, *exogenous* triggers allow for designing bioorthogonally cleavable systems. By using complementary unnatural moieties, a higher degree of selectivity and temporal control of cleavage can be achieved (see Figure 1c). Particularly for prodrug approaches, where premature drug loss can lead to side-effects, highly specific release systems are of great importance. Common exogenous triggers comprise light and small molecules.

Light-triggered cleavage is attractive because it allows additionally for spatial control of cleavage by shining light locally at the site of the target. Photo-cleavable linkers that are amenable to release applications are based on light-responsive aromatic rings comprising *o*-nitrobenzyl (ONB), coumarin and quinolone [5]. In 2005 the Muir group developed a light-cleavable protein conjugate by means of expressed protein ligation [40]. For this, ONB-cysteine derivatives of a fluorophore or a lipid were used, which enabled the ligation of these functionalities to the protein of interest. Upon delivery of the resulting protein conjugates into cells, UV irradiation was applied to release the proteins again. Chen *et al.* have recently presented an alternative conjugate, which is cleavable by near-infrared (NIR) light instead (808 nm) [41•]. They used a carbon nanotube, which facilitates both the intracellular delivery of an attached protein and its subsequent release, which is mediated by heat generated from the irradiation of the nanotube with NIR light.

Also **small organic molecules** can trigger specific release, which is particularly powerful when initiated via a bioorthogonal reaction as for instance the Staudinger ligation [42]. However, the required phosphine reagents are prone to oxidation and for most *in vivo* applications the efficiency of these reactions is too low.

In recent years, the field of bioorthogonal decaging using small molecule triggers has seen great advances, foremostly fueled by the finding that the rapid inverse electron demand Diels-Alder (IED-DA) reaction can be used for a bioorthogonal triggered release. Researchers around Robillard adapted the well-known reaction between *trans*-cylcooctene (TCO) and tetrazine [43,44] to develop a click and release reaction [45]. Upon reaction with tetrazine, the TCO group undergoes a rearrangement, which results in the removal of the TCO group from the amine moiety along with the liberation of CO_2. The Chen group observed that differently substituted tetrazine derivates resulted in varying yields of eliminated products. Tetrazines bearing an electron-withdrawing (EWG) and a small, non-EWG substituent give the best combination between rapid cycloaddition and efficient

release [46]. Recently, Weissleder *et al.* further looked into the release mechanism of the tetrazine-TCO click product. They found that this step is pH-sensitive and showed that a carboxylic acid tetrazine derivative greatly enhanced the release efficiency [47••]. Furthermore, methylation of the carbamate nitrogen prevents the formation of an undesired 'dead-end' isomer of the click product. Using such *N*-methyl derivatives, complete release was obtained.

In the seminal click and release report by Rossin *et al.*, the drug doxorubicin-TCO conjugate was given to cultured cells [45]. Upon addition of tetrazines they observed increased cytotoxicity, demonstrating thereby successful click and release *in cellulo*, notably in a non-targeted way.

To take the click and release approach further, Rossin *et al.* have demonstrated targeted drug release from an ADC targeting cancer cells in mice [48]. However, technically this is not an example for an intracellular cleavage as the ADC gets cleaved extracellular but they observe the uptake of the drug into the tumor cells after cleavage. A problem with this approach is the systemic distribution of the trigger component (tetrazine), which can cause off-target effects. This is a difference to the previously discussed endogenous triggers, which are present in defined compartments. However, such off-target effects can be prevented by the use of a clearing agent, which reacts and clears residual ADC in the blood before the activator is administered. Two other groups have recently reported a conceptually different approach to achieve selective, local activation. They showed that local activation of systemically administered small-molecule TCO-prodrugs is possible *in vivo* when using tetrazine-modified supramolecular assemblies, such as hydrogels or peptide-based assemblies. The latter concentrate and activate the prodrug at the target site, for example, at a bacterial infection or tumor site [49,50].

While both strategies are powerful, the need for a clearing agent or the implantation of a polymer to achieve local activation still adds a lot of complexity to the overall protocol. The group of Robillard further optimized the click and release system in 2018 [51]. They devised a new TCO functionalized ADC consisting of a diabody without the Fc region. Because of its decreased size this ADC shows enhanced penetration into tumor tissue. In addition, a PEG linker between the diabody and the toxin accelerates clearance of the ADC from the blood and thereby circumvents the need of a clearing reagent.

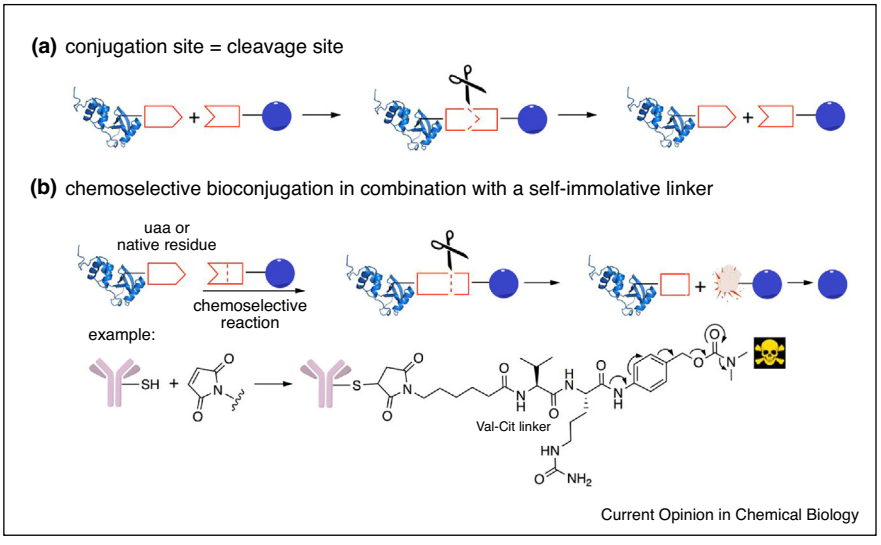
Still, it remains to be demonstrated that the click and release strategy is also suitable to achieve selective intracellular cleavage for delivered protein conjugates.

Nevertheless, this new form of cleavable ADC linker is potentially more specific than current technologies since it does not depend on differences between healthy and diseased cells. Also, this chemistry allows expanding the scope of cleavable ADCs to non-internalizing targets, a challenging task when relying on endogenous triggers.

Incorporation of cleavable entities into proteins

The incorporation of cleavable entities into a protein can be achieved by various bioconjugation strategies. Usually, the conjugation site is different from the cleavage site. An exception to this are disulfide protein conjugates and

Figure 2



Cleavable entities can be introduced via a number of different bioconjugation strategies into proteins; in some cases by direct attachment **(a)** but often in combination with a linker **(b)**.

ester conjugates, in which the latter is generated between a diazo compound and a carboxylic acid [52] (Figure 2a). Well-established protocols for disulfide synthesis (see chapter 3) allow for obtaining tracelessly cleavable conjugates. However, this requires the presence of accessible thiol moieties on both parts and may be problematic when several thiols are present. For non-thiol containing moieties, the attachment to a protein requires mutations or other conjugation chemistry.

Outside this scenario conjugation methods can be combined with self-immolative linkers, thus enabling the traceless release of the attached moiety. For example, upon cleavage of the Val-Cit linker, 1,6-elimination of the spacer liberates the free cytotoxic drug in the ADC Adcetris® (see Figure 2b). For conjugation strategies, one possibility is to use a handle that reacts chemoselectively with certain native amino acid residues. In order to render the conjugation site-specific, one can introduce an unnatural functionality into a protein, for example via amber suppression or chemoenzymatic methods and use bioorthogonal chemistry in a subsequent step to modify it. For both strategies, a plethora of methods exist that have been extensively described in other excellent reviews [e.g. Ref. 53].

Conclusions

Efficient and specific intracellular cleavage of protein conjugates is greatly dependent on the cleavable entity. From the systems discussed herein, disulphide-based and peptide-based linkers as well as the recently engineered TCO-tetrazine system have received the most attention. These entities exhibit high extracellular stability and enable rapid and selective intracellular cleavage. Disulfide linkers are attractive for applications where cytosolic release of cargo is desired and they are easy to install. In contrast, peptide linkers have proven particularly useful for endosomal and lysosomal release of cargo. As for enzymatic approaches, we believe that there is still a great potential to discover new, also compartment-specific cleaving enzymes, which would fuel new linker designs and pave the way for specific subcellular drug release and turn-on probes for imaging applications. Particularly interesting will be the investigation of diseased cells, where changes in enzyme activities can be used as triggers for prodrug strategies. It will be challenging however to come up with new concepts relying on endogenous, chemical triggers, as the number of distinct differences in the chemical environment between inside and outside the cell that allow for selective cleavage is limited. Much more innovation is expected in the direction of using exogenous, bioorthogonal triggers such as tetrazine. With them, temporal control of cleavage can be achieved, along with outstanding cleavage selectivity, a crucial consideration in drug delivery. A main challenge with such exogenous triggers remains in achieving local and in particular selective intracellular cleavage. To realize this, one has to make sure that the protein conjugate is only present at the desired cleavage site (e.g. at diseased cells) before administering the cleavage trigger. Promising developments toward this end have been made recently by the Robillard group as discussed above. As for selective intracellular cleavage, both components would additionally have to be delivered inside the cell. Furthermore, for cases where only specific cell types or compartments want to be addressed (e.g. in drug release applications), the protein conjugate has to be delivered in a targeted way.

Overall, the development of intracellularly cleavable entities has paved the way for controlled release strategies and protein activity manipulations, foremost important to targeted therapy but also in basic research. We believe that the improvement of the current systems, and likely the development of new ones, will further advance the field of chemical biology and continue to provide important tools for therapy.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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