MINIREVIEW: ADDRESSING THE RETRO-MICHAEL INSTABILITY OF MALEIMIDE BIOCONJUGATES

This minireview explores published strategies to counteract the retro-Michael instability of the thiosuccinimide linkage, a limiting factor of the efficacy and safety of antibody-drug conjugates.

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Declaration

We, the authors, confirm here that all work reported herein is our own work, or the source of the work has been indicated.

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Table of Contents

Abstract

Bioconjugation, the modification of biological macromolecules such as proteins, is an up and coming area in the field of chemical biology. Antibody-drug conjugates (ADCs), combining the antigen-selectivity of natural antibodies with the cytotoxic potency of small molecule drugs, are a powerful therapeutic technology. Four such constructs are currently on the market for cancer therapy. However, the conjugation methodology employed in these therapeutics is far from ideal. Herein we provide an overview on methods that attempt to increase the safety and efficacy of ADCs via “self-hydrolysing maleimides” or by improving the stability of maleimide-conjugates by other means. We find that some very promising reagents have been reported, however the mechanism by which these reagents act is not clear, thus limiting rational design.

Keywords: Bioconjugation, Organic Chemistry, Chemical Biology, Protein modification

1.1 Introduction to Bioconjugation

Protein bioconjugation has emerged in recent years as an important area of research within the field of chemical biology. Exploiting the high fidelity of protein-protein interactions, these constructs can have a variety of medicinal and biotechnological applications, including protein half-life extension [1], emulation of post-translational modification [2] and exploration of new treatment options for cancer [3].

In the area of cancer research, an emergent class of new therapeutics are antibody-drug conjugates (ADCs). These constructs combine the exquisite antigen-selectivity of antibodies with the potent cytotoxic properties of small-molecule drugs. The method provides an edge in efficacy over unmodified antibody therapy alone, whilst reducing off-site toxicity (and thus side-effects) compared to conventional small molecule chemotherapy enabling the use of more powerful drugs [4–6].

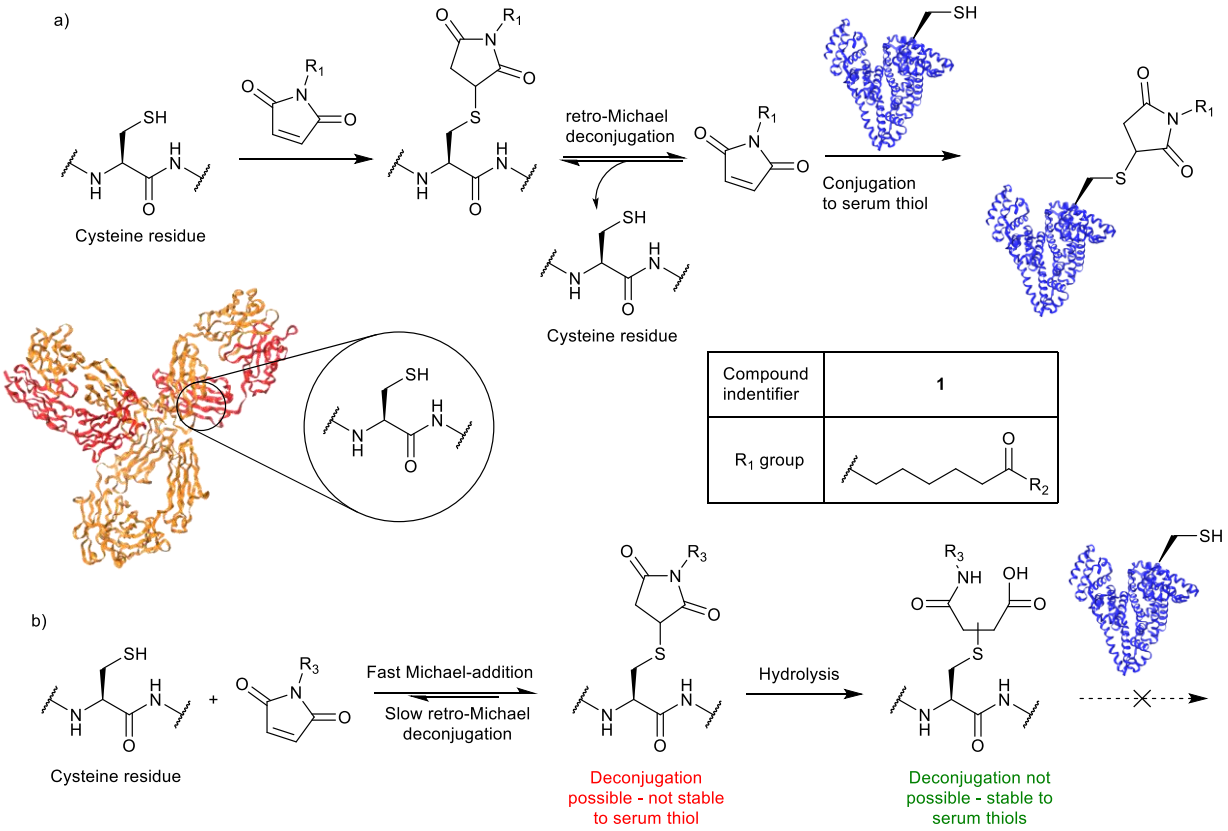
There are currently four ADCs approved for use by the food and drug administration (FDA), Brentuximab vedotin (Adcetris®), Trastuzumab emtansine (Kadcyla®), Inotuzumab Ozogamicin (Besponsa®), and Gemtuzumab Ozogamicin (Mylotarg®) with more than 60 currently under clinical evaluation [7]. The lessons learned from the development of these ADCs, and many failed ADCs, highlight the importance of optimising the attachment method of drug to protein. Kadcyla®, Besponsa® and Mylotarg® are constructed *via* modifying the antibody's surface accessible lysine residues. However, as lysine residues are abundant in proteins (*ca.* 3.6% in terrestrial organisms) [8], this method leads to highly heterogeneous conjugates when targeting typical drug to antibody ratios (DARs) of 2-4 [9–11]. Cysteine modification, as seen with Adcetris®, has gained popularity in recent years due to the high nucleophilicity, selectivity towards soft electrophiles, and low natural abundance of the sulfhydryl group-bearing amino acid residue in naturally occurring proteins (*ca.* 0.15% in terrestrial organisms) [8,12]. In Adcetris®, the 4 accessible interchain disulfide bonds are partially reduced to liberate cysteines that can be site-selectively modified. Whilst this still results in heterogeneous conjugates when targeting typical DARs (partial reduction is not selective for the hinge or Fab interchain disulfides), it is far less heterogeneous than modifying lysine residues.

Typically, maleimides have been used extensively for cysteine modification as they react rapidly and selectively with thiols. Functionalising maleimides by adding substituents on the *N*-atom is also facile. In the instance of Adcetris®, and many other ADCs under evaluation, a maleimide linkage is used to modify the solvent accessible cysteines liberated from partial reduction of disulfide linkages on an IgG1 antibody. However, it has recently come to light that the thioether linkage undergoes deconjugation through a retro-Michael pathway, leading to loss of cargo and reduction in efficacy. The maleimide-cargo conjugate can then be bound to other plasma thiols (*e.g.* human serum albumin) leading to off-site toxicity and reduced efficacy (Scheme 1a) [13–15].

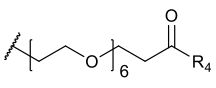
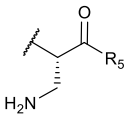
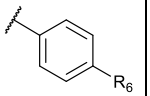
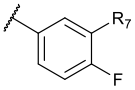
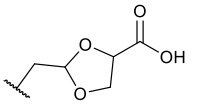
A slew of methods has been developed to circumvent this problem of instability inherent in maleimide-cysteine conjugation. Of great interest in the field is the concept of "self-hydrolysing maleimide" reagents. These compounds undergo rapid hydrolysis to the corresponding succinamic acid post-conjugation, thus eliminating the retro-Michael deconjugation pathway and resulting in more effective/stable bioconjugates [16–23]. Alternatively, researchers have employed distinct thiol reactive groups, [24–27] or tweaked the traditional maleimide scaffold to obviate the issue, *e.g.* adding leaving groups to the double bond [28] or using exocyclic olefinic maleimides [29]. Herein a review of the area will be given.

1.2 Introduction to the concept of “Self-hydrolysing maleimides”

Although it has been known for many years that on hydrolysis, maleimide reagents lose their reactivity towards thiol-bearing functionalities, [30,31] it was only recently that Shen and co-workers proposed that post-conjugation hydrolysis increases the stability of thiol-maleimide conjugates [13]. Using antibodies with cysteines engineered in various unnatural positions, they showed that local protein micro-environment has a powerful effect on the stability of the bioconjugate. They proposed that local positively charged residues promote rapid thiosuccinimide ring hydrolysis, and thus limit thiol-exchange mediated loss of cargo (Scheme 1b). In a preliminary safety study, they also show that more stable conjugates lead to less liver toxicity in rats.



Compound indentifier	1
R_1 group	

Compound indentifier	2	3	4	5	8
R_3 group					

Scheme 1

Hydrolysis of maleimide linkers confers stability to retro-Michael deconjugation. Crystal structures of antibody [37] and human serum albumin [38] adapted from the RSC PDB using Maestro.

In the years following the Shen *et al.* paper [13] in 2012, the development of methods to create maleimide reagents that hydrolyse rapidly after conjugation, without the requirement for a specific local microenvironment, were explored.

2. Methods to increase the rate of thiosuccinimide hydrolysis

In 2014, Tumey and co-workers observed that an ADC prepared *via* maleimide-thiol conjugation using a maleimide reagent bearing six polyethylene glycol (PEG) units (Scheme 1, maleimide **2**) hydrolysed whilst in a neutral storage buffer. In contrast, an analogous ADC constructed using a maleimidocaproyl linker (Scheme 1, maleimide **1**) showed no hydrolysis. They proposed that co-ordination of water to the proximal PEG-oxygen of the linker was responsible for the increased rate of hydrolysis. The rate of hydrolysis of the succinimides in the ADC created with PEG maleimide **2** was evaluated at 37 °C and shown to progress to complete hydrolyse by mass spectrometry over 14 h in borate buffered saline (BBS) at pH 9.2. However, in PBS at pH 7.4, only 30% hydrolysis was observed over 16 h. Moreover, and perhaps as expected, on comparing the unhydrolysed maleimidocaproyl-bearing ADCs to the hydrolysed maleimide-PEG derived ADCs, the latter showed improved plasma half-lives and efficacies in mice [16].

Also in 2014, Lyon *et al.* [17] showed that a maleimide reagent incorporating an amino group at a specific position (Scheme 1, maleimide **3**) underwent rapid hydrolysis when unconjugated. They proposed that the reported half-life of around 25 mins under mild conditions (pH 7.4 and 22 °C) was facilitated by intramolecular base catalysis. The stability of ADCs prepared with "self-hydrolysing" maleimide **3** and maleimide **1** was appraised. The hydrolysis half-life of the bioconjugate bearing compound **3** was estimated to be 2.6 h and 2.0 h for the light and heavy chain, respectively. In contrast, no hydrolysis was observed at 24 h for the ADC bearing the maleimidocaproyl linker **1**. Over two weeks in an *N*-acetyl cysteine containing buffer at pH 8 and 37 °C their "self-hydrolysing" ADC showed no measurable drug loss, while the control ADC lost *ca.* half of its drug component. The comparison showed similar results in rat plasma and in rats *in vivo*. The group also showed that the more stable ADC led to lower off-site bone marrow toxicity in rats as well as substantially increased anti-tumour activity.

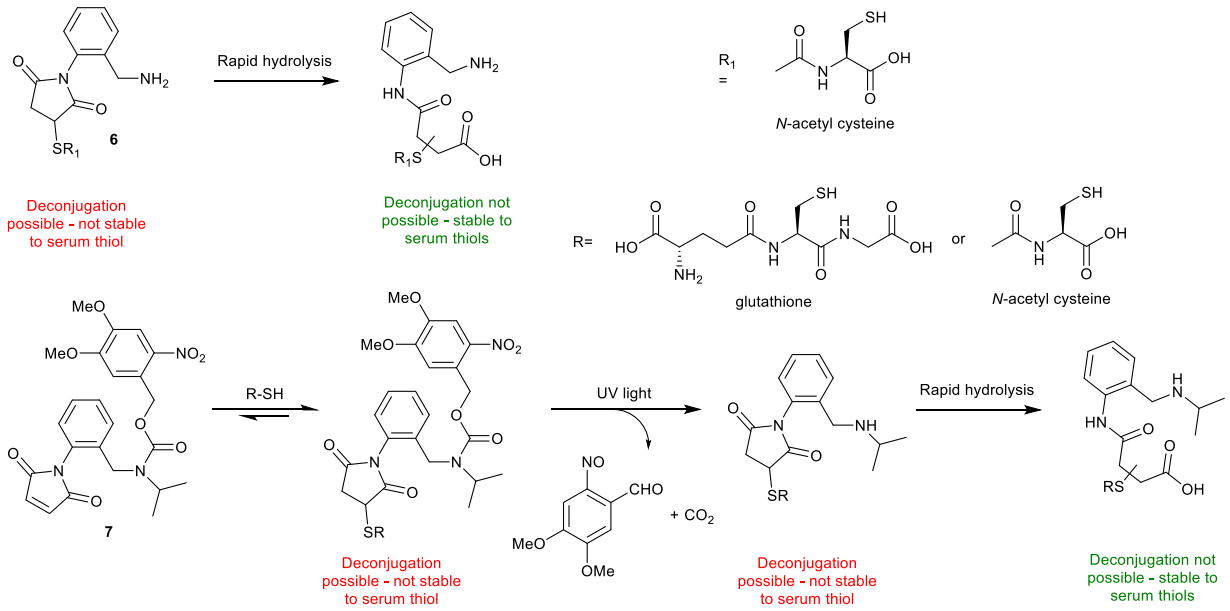
A study of thiosuccinimide hydrolysis by Fontaine *et al.* offered a different explanation of the kinetics of the aforementioned self-hydrolysing reactions [18]. They compared the rates of hydrolysis and thiol-exchange for around twenty maleimide based conjugates containing varyingly powerful electron withdrawing groups (EWGs) and observed a linear correlation between Taft σ* polar substituent constants and hydrolysis rates. They proposed that the effect observed by Lyon *et al.* was due to the electron withdrawing effect of the protonated amino group and not due to base catalysis. They showed that 4° amines, which cannot act as base catalysts but have large positive σ*s, also increase the rate of hydrolysis at a comparable rate. They proposed that the beneficial effect of PEG spacers found by Tumey *et al.* also arose from an electron withdrawing effect, rather than from co-ordination. They also found that EDGs (such as alkyl and carboxylate) slowed hydrolysis, further substantiating their claims.

Furthermore, they calculated the sensitivities to electron withdrawal of both the hydrolysis reaction and the thiol-exchange reaction by invoking the Taft polar sensitivity factor ρ*. The ρ* for the former reaction was found to be 1.0 while the ρ* for the second process was calculated as 0.48, indicating that succinimide hydrolysis has a higher sensitivity to electron withdrawing groups.

In line with these findings Christie *et al.* [19] reported that unconjugated *N*-phenyl maleimides (Scheme 1 maleimide **4**) hydrolyse ~5.5 and react with thiols ~2.5 times faster than *N*-alkyl maleimides at physiological pH, with the half-life of the former process being *ca.* 55 mins. Adding an electron withdrawing fluorine atom to the phenyl ring decreases the $t_{1/2}$ to 28 mins (Scheme 1, maleimide **5**). Slower hydrolysis rates were observed post-conjugation; *N*-alkyl thiosuccinimides having a 27 h half-life compared to 1.5 h for the *N*-aryl variant and 0.7 h for the *N*-fluorophenyl conjugate at pH 7.4, 37 °C.

On incubation in PBS containing β-mercaptoethanol or in mouse serum, the stability of ADCs prepared with *N*-aryl maleimides was shown to be substantially better than those prepared with *N*-alkyl maleimides. The former retained 90–100% conjugation, in both cases over a 200 h period, with the latter suffering from 60–70% deconjugation. Perhaps as expected, increased efficacy was observed for the more stable conjugate in a cell killing assay [19].

Following these findings, a method combining the electron withdrawing ability of a phenyl group and the activity (acid catalysis as claimed by the publishing group) of an amino group was reported [20]. The *N*-acetyl cysteine adduct of *o*-aminoethyl- phenylmaleimide (Scheme 2, maleimide **6**) was shown to completely hydrolyse over a 2 h period at room temperature and pH 7 post-conjugation to *N*-acetyl cysteine. To eliminate intramolecular macrocyclisation between the amino group and the carboxylate group of the thiosuccinimide ring, an *N*-isopropyl variant of the linker was prepared. The $t_{1/2}$ of the *N*-acetyl cysteine conjugate of this linker was found to be 20 mins at room temperature at pH 7, while under the same conditions the corresponding conjugate of *N*-aminoethyl maleimide was observed to have a half-life of 3.6 h. After demonstrating the cysteine selectivity and rapid hydrolysis of their scaffold on proteins, a method to increase the shelf-life of the linker molecule was approached. A photocleavable 6-nitroveraltryl-oxycarbonyl (NVOC) group was added to the amino group of the reagent (Scheme 2, maleimide **7**), thus masking its catalytic activity. A major improvement in shelf-life was observed. To test the scaffold, a bioconjugation reaction with cysteine-containing tripeptide glutathione and *N*-acetyl cysteine was carried out on ice, to generate the bioconjugate in 10 mins. The constructs were then irradiated with UV light for 50 mins, and complete conversion to the ring-opened bioconjugate was observed by HPLC analysis.



Scheme 2

Kalia et al. method for UV light triggered maleimide self-hydrolysis.

A popular method for coupling thiols to amines is the succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) heterobifunctional reagent, however, the thiosuccinimide linkage of this has been shown to be prone to thiol-exchange [21]. A study undertaken to improve SMCC solubility found that by replacing the cyclohexyl group with a 1,3-dioxane ring, the rate of thiosuccinimide hydrolysis increased. The dioxane-bearing probe hydrolysed completely over 29 h, while only trace amounts of the hydrolysed cyclohexyl probe were observed via a fluorescence resonance energy transfer (FRET) assay [22].

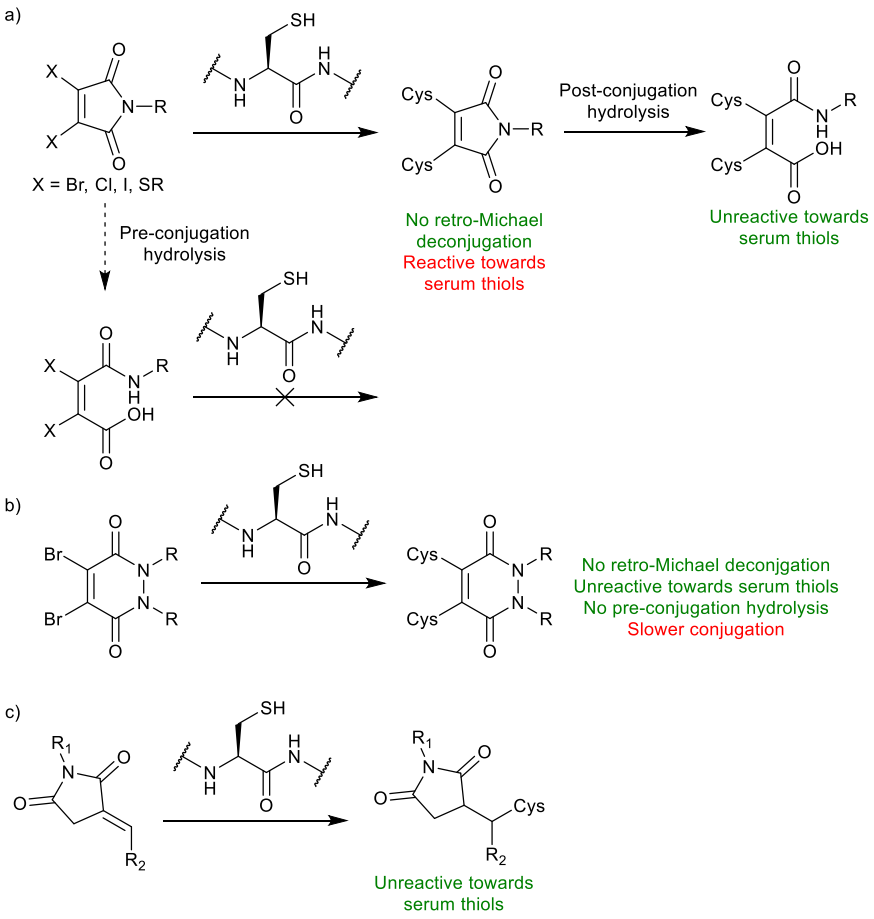
Following on from these findings, Tobaldi and co-workers investigated the use of a reagent with a dioxolane ring attached to a maleimide ring *via* a methylene bridge (Scheme 1, compound **8**) for use in bioconjugation [23]. In PBS (10% DMSO), at 37 °C and pH 7.4, the dye-conjugate of this molecule showed complete ring hydrolysis in less than 20 h. Surprisingly, in human plasma, at the same temperature, the hydrolysis was considerably slower, showing close to 65% hydrolysis over 30 h. Next, thiol-exchange rates were measured, and the construct was found to undergo no thiol-exchange with albumin when hydrolysed prior to incubation, and only modest thiol-exchange when not pre-hydrolysed, plateauing at <30% after 7 days. The authors offer no mechanistic explanation for the increased rate of hydrolysis for the dioxo linkers, but both the water-to-oxygen co-ordination theory of Tumey *et al.* [16] and the electron withdrawing effect based explanation of Fontaine *et al.* [18] could be invoked.

3. Alternative solutions to retro-Michael deconjugation

While the aforementioned self-hydrolysing maleimides are a promising route towards preventing retro-Michael deconjugation, it should be noted that other strategies exist to prevent this altogther. A simple and inexpensive solution is to incorporate a leaving group (*i.e.* a bromine atom) on the vinylic bond of the maleimide scaffold, forming an unsaturated thio-succinimide linkage post conjugation that is mechanistically stable to the retro-Michael process [28]. However, hydrolysis is still considered essential for this strategy as the unsaturated thio-succinimide moiety is susceptible to additional thiol exchange, resulting in suboptimal *in vivo* stability without hydrolysis.

Most recently, reported by Forte *et al.* [32], substituted maleimide conjugation can be considered an intricate balance between rate of cysteine conjugation and pre-conjugation hydrolysis (ideally quick and slow respectively). Forte *et al.* also provided evidence to suggest varying the leaving group on the maleimide affects both the conjugation and hydrolysis rates with diiodomaleimides presenting the most ideal profile of rapid conjugation and slow pre-conjugation hydrolysis [32]. It is also noted from research presented from Morais *et al.* [33] that varying the dibromo maleimide *N*-directed handle had a direct influence on maleimide hydrolysis. This work highlighted functional groups (*e.g.* aromatics) that exhibit a pre-conjugation hydrolysis rate similar to the typical cysteine conjugation rate, resulting in low conjugation yields (Scheme 3a). A similar class of molecules called bromopyridazinediones exploit the same principles as substituted maleimides, but pose a hydrolytically stable alternative for cysteine functionalisation [34]. Furthermore, the conjugated pyridazinedione scaffold is stable *in vivo* (blood) with no further hydrolysis required (Scheme 3b) [35,36]. It is noted however, that this moiety is less reactive towards cysteine than the maleimide scaffold, and so quantitative formation of the bioconjugate can take longer (up to 16 h).

Additionally, a strategy based on exocyclic olefinic maleimides has been reported, [29] where having the double bond exocyclic to the maleimide ring offers a substantial improvement in the stability of the conjugate towards thiol-exchange (Scheme 3c). The authors of the study attribute this increased stability to reduced mesomeric stabilisation of their scaffold compared to traditional endocyclic maleimides. The synthesis of a wide range of such compounds has been reported through a Wittig-like reaction offering good yields of exocyclic olefinic maleimides.



Scheme 3

Non-classical maleimide-based solution to the retro-Michael deconjugation mediated instability of the thiosuccinimide linkage. a) "Next generation" maleimides. b) Bromopyridazinediones. c) Exocyclic olefinic maleimides.

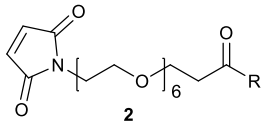
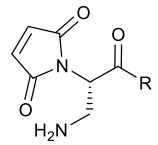
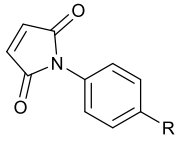
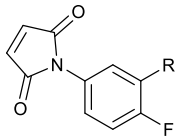
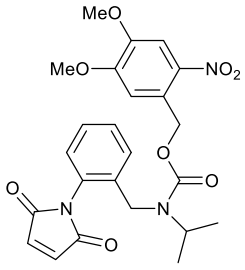
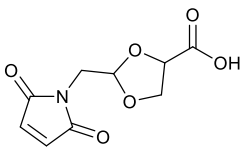
4. Conclusion

To conclude, several promising solutions to the retro-Michael instability of the thiosuccinimide linkage have been proposed. Unfortunately, as no study comparing these different methods exists to date, it is difficult to make a substantive/conclusive statement about the relative merit of these approaches. It would be interesting and beneficial to the scientific community for such a study to be carried out, potentially incorporating an even wider array of bioconjugation techniques. This would allow identification of the most promising candidates, so the scientific community may focus its efforts on these. As of now, based on available data, no clear "best approach" can be identified. All methods mentioned above have their advantages and drawbacks, and the "best fitting" approach for now at least must be identified on a case-by-case basis.

Nonetheless, whilst multiple approaches to self-hydrolysis have been reported (Table 1), it can be argued that the photocaged maleimide linker reported by Kalia et al. (maleimide 6) can be considered as an evolution of the concept. It combines multiple positive effects on hydrolysis rate as reported above (i.e. phenyl ring and amino group) whilst also obviating the issue that reagents that hydrolyse fast post-conjugation can also be expected to hydrolyse fast pre-conjugation, which is detrimental to efficiency. This reagent has an extended shelf-life and retains the potential for a rapid rate of hydrolysis, the exact timing of which can be controlled by UV irradiation [20]. In terms of mechanistic explanations for the effect of self-hydrolysis, no consensus has been reached, with Lyon and co-workers suggesting a base-catalysed effect of the proximal amino group, [17] while Fontaine

et al. convincingly argued that the inductive electron withdrawing effect of the protonated amino group is responsible for the increased rate of hydrolysis [18]. Christie and co-workers have argued that the mesomeric electron withdrawing effect of the phenyl group is at play in their method, [19] and Kalia *et al.* proposed that their aminoethyl phenyl maleimide reagent combines both the effects of mesomeric electron withdrawal and acid catalysis. To rationally design even more efficient reagents, a firm mechanistic understanding of the phenomenon would be required. Thus, experiments designed to increase our understanding of the mechanism of thiosuccinimide hydrolysis would be beneficial. However, even in their current state, these reagents undoubtedly provide an interesting solution to the thiosuccinimide instability problem, and the recent "triggered" hydrolysis method could pave the way for next generation thiol-maleimide conjugates.

Table 1
Summary of self-hydrolysing maleimide methods reported to-date.

Maleimide structure	Hydrolysis rate of bioconjugate	Additional notes
 **2**	30% hydrolysis over 16 h in PBS pH 7.4	
 **3**	2 – 2.6 h half-life at pH 7.4	
 **4**	1.5 h half-life at pH 7.4, 37 ˚C	
 **5**	0.7 h half-life at pH 7.4, 37 ˚C	Was found to not hydrolyse by Kalia *et al.* [20]
 **7**	Complete hydrolysis after 50 min of UV irradiation at 0 ˚C	No hydrolysis occurs on storage
 **8**	Complete hydrolysis after 20 h in PBS pH 7.4, 37 ˚C	

Acknowledgements / Declaration of Contributions

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