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Introduction

Maleimides represent one of the most widely used functional groups for conjugation chemistry.^{1–4} This is due to the extremely favourable kinetics of their reaction with thiols, which ensures high yields of the desired conjugates are generated rapidly whilst minimising competing side-reactions. This has led to their use in fields ranging from therapeutic bioconjugates,^{5–7} to multifunctional polymers and a diverse array of materials.^{8,9} Despite this widespread use, maleimides suffer several limitations which have been widely noted. Most significantly, the reaction generates succinimides **1** that are unstable over prolonged time *in vivo*, undergoing retro-conjugate additions and subsequent trapping with endogenous thiols such as glutathione.^{10–13} To overcome this, the succinimides must be hydrolysed post-conjugation, which can lead to a reduction in the yield of the conjugates,^{14,15} prolonged reaction times and undesirable exposure to high pH conditions. Furthermore, the hydrolysis generates two regioisomeric maleamic acids **2a** and **2b**, which when combined with the formation of a stereocenter in the initial conjugate addition, results in four isomeric products (Fig. 1A).

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One-pot thiol–amine bioconjugation to maleimides: simultaneous stabilisation and dual functionalisation†

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Maleimide chemistry is widely used in the site-selective modification of proteins. However, hydrolysis of the resultant thiosuccinimides is required to provide robust stability to the bioconjugates. Herein, we present an alternative approach that affords simultaneous stabilisation and dual functionalisation in a one pot fashion. By consecutive conjugation of a thiol and an amine to dibromomaleimides, we show that aminothiomaleimides can be generated extremely efficiently. Furthermore, the amine serves to deactivate the electrophilicity of the maleimide, precluding further reactivity and hence generating stable conjugates. We have applied this conjugation strategy to peptides and proteins to generate stabilised trifunctional conjugates. We propose that this stabilisation-dual modification strategy could have widespread use in the generation of diverse conjugates.

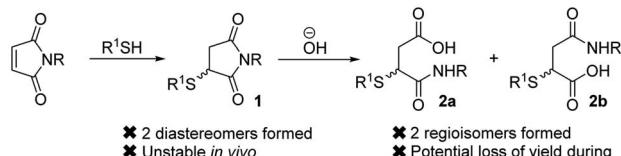
To overcome these limitations we, and others, have reported extensively on the use of dibromomaleimides (DBMs),^{16–23} and related substituted maleimides known collectively as next generation maleimides (NGMs).^{20,24} These reagents retain the favourable kinetics of maleimides^{25,26} whilst avoiding the formation of stereoisomers, and through the addition of two thiols allow the construction of dual conjugates (Fig. 1B). Whilst this bis-thiol conjugation has found widespread use in the bridging (also known as stapling) of disulfide bonds it has found less application to intermolecular conjugates, as the dithiomaleimide products **3** are still thiol reactive and thus the control of stoichiometry is challenging to prevent scrambling of the thioethers. Furthermore, hydrolysis post-conjugation is required to confer *in vivo* stability, which still generates a mixture of two regioisomeric maleamic acids.^{10,11}

We hypothesized that a completely different mechanism for maleimide conjugate stabilization might prove viable, overcoming the limitations of imide hydrolysis whilst offering new opportunities for the construction of multifunctional conjugates. The addition of an amine to a dibromomaleimide-thiol conjugation reaction would afford an aminothiomaleimide **4** (Fig. 1C), in which the electron donating amino group would deactivate the maleimide to further nucleophilic attack. The product would thus be stable, precluding the requirement for post-conjugation hydrolysis and generating a single isomer (Fig. 1C). Furthermore, the amine would represent an additional site for functional attachment, thus representing an ideal scaffold for the construction of triconjugates.

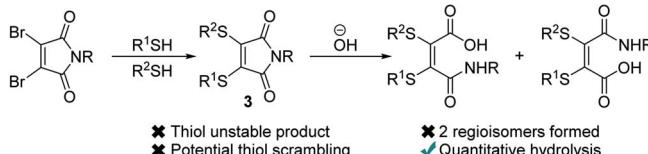
It has previously been shown that dibromomaleimides (DBMs) react selectively with thiols over amines, hence the



A) Maleimide thiol bioconjugation



B) Dibromomaleimide thiol-thiol bioconjugation



C) This work: thiol-amine bioconjugation



Fig. 1 (A) Thiol conjugation with classical maleimides. (B) Dual thiol conjugation using Next Generation Maleimides (NGMs). (C) Dual thiol-amine conjugation with simultaneous stabilisation described herein.

initial addition of thiol was expected to occur rapidly and selectively.^{16,21} Amine addition to dihalomaleimides has been reported to generate aminohalomaleimides, suggesting that whilst the reaction is slower than for thiols it is still viable.²⁷ Interestingly, thiol addition to aminobromomaleimides has been found to occur only under high temperatures (80 °C),²⁸ indicating that the amino group does indeed dramatically reduce the maleimide's electrophilicity. Whilst promising indicators for the desired reaction sequence, far milder reaction conditions would be required for this reaction to be broadly applicable as a bioconjugation strategy. For protein conjugates the reactions would have to take place at, or near, room temperature, at high dilution and be effective in aqueous buffered conditions in which most amines would be substantially protonated.

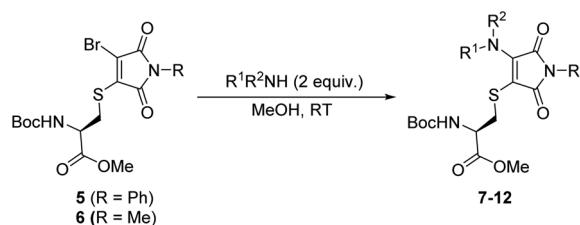
Results and discussion

Bromothiomaleimides **5** and **6** were synthesized as test substrates for initial tests of amine additions. Enticingly all amines trialled conjugated efficiently in methanol to generate the desired aminothiomaleimides **7–12** in excellent yields (Fig. 2), with both *N*-aryl and *N*-alkyl substituents. As expected, the more nucleophilic secondary alkyl amines, such as piperidine and pyrrolidine, reacted more efficiently than aryl amines (*ca.* 10 min *vs.* 24 h, see ESI Table S1† for more details).

Whilst this study shows the promising potential of this dual conjugation sequence in organic solvent, we moved on to test aqueous buffered conditions. Analysis of the relative rates of amine addition under aqueous conditions (pH 7.8, 4.7 mM, with 2 equiv. of amine) was carried out using NMR, *via* the integration of the starting material and product *N*-methyl peaks (Fig. 3). We observed that aniline ($pK_{aH} = 4.87$) offered

a substantial rate enhancement over pyrrolidine ($pK_{aH} = 11.31$), confirming that protonation was substantially attenuating reactivity of the secondary amine. The pyrrolidine reaction was also observed to generate undefined side products by crude NMR analysis, and was therefore ruled out as a viable reagent. In contrast, incorporating a *para*-methoxy group onto the aniline, which subtly raises the pK_{aH} (*p*-anisidine, $pK_{aH} = 5.36$), afforded the most reactive amine under these conditions. *p*-Anisidine represented the best balance between nucleophilicity and pK_a for undertaking these reactions at near neutral pH,^{29,30} yielding a single product and reaching completion in 185 min. It is notable that the aminothiomaleimide products were observed to be fluorescent in THF, which has been reported for related aminothiomaleimides by O'Reilly and coworkers and in which they also note that this fluorescence is not observed in protic solvents.²⁸ The aminothiomaleimide **11** was then assessed for stability by prolonged incubation in: pH 7.4 buffer, pH 5 buffer and also 7 mM thiol solution (2-mercaptoethanol). The resulting NMR data confirmed the product as completely stable in all conditions (monitored for 6–8 days, see ESI Fig. S23–S26†). The *p*-anisidine conjugation to bromothiomaleimides was thus shown to be an extremely promising new bioconjugation reaction, occurring under mild aqueous conditions and generating stable products.

To trial this strategy in protein bioconjugations, wild-type human serum albumin (HSA) was chosen as an initial target; it contains a single cysteine (Cys-34) and HSA-conjugates are of clinical interest as a leading platform for half-life extension.^{31–34} A useful feature of this chemistry is the ability to monitor the



R	R ¹ R ² -(Amine)	Structure	Yield	Product no.
Ph	Piperidine		98%	7
Ph	Pyrrolidine		96%	8
Me	Pyrrolidine		92%	9
Me	Aniline		82%	10
Me	<i>p</i> -anisidine		90%	11
Me	<i>N</i> -Me- <i>p</i> -anisidine		88%	12

Fig. 2 Reactions of bromothiomaleimides **5** and **6** with amines in methanol.



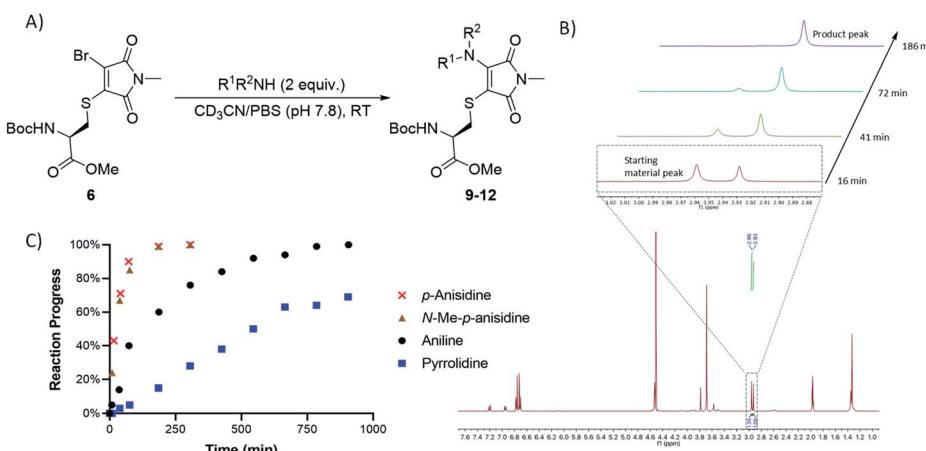


Fig. 3 (A) General reaction scheme for NMR time course reactions. (B) Methyl peak development observed in time course ¹H NMR study for reaction between *p*-anisidine and bromothiomaleimide 6. (C) Graph showing the progress of 4 key amine reactions under buffered conditions (see ESI Table S3†).

progress of both thiol and amine additions by UV-vis spectroscopy, as distinct λ_{max} values are associated with the bromothiomaleimide (ε_{375} 2910 M⁻¹) and the red-shifted aminothiomaleimide products (ε_{415} 4250 M⁻¹). The conjugation of DBM 13 to the protein was complete after just 20 min, confirmed by UV (Fig. 4), and subsequently LCMS analysis (ESI Fig. S7†). *p*-Anisidine (50 mM) was then added to the reaction mixture and the absorbance was monitored at 415 nm, taking 180 min to plateau. LCMS analysis confirmed that the amine addition had taken place (Fig. 4). It was unsurprising to observe relatively long reaction times on HSA, with high concentrations of amine required to ensure the reaction went to completion; this is likely due to the buried nature of the Cys-34 cleft, and the fact that a proximal nucleophilic Tyr-84 has been proposed to intercept dihalomaleimide–HSA conjugates, as both halide atoms are lost from the backbone of the DBM during conjugation.²⁰

To preclude these specific issues associated with wild-type HSA, we shifted our focus onto an HSA mutant (mHSA) with an alternative cysteine location (C34A + K93C), kindly provided by Albumedix.³⁵ It was pleasing to observe in this case that the bromothiomaleimide was present in the LCMS analysis of the intermediate prior to amine addition, indicating that there were no reactions with other nucleophilic residues near the conjugation site (ESI Fig. S8†). Interestingly, *p*-anisidine addition was rapid (~20-fold faster compared to WT HSA, see Fig. 4) for mHSA intermediates. A pseudo-first order analysis of the reaction with UV-vis spectroscopy was carried out and enabled calculation of the second order rate constant k_2 for the *p*-anisidine addition to conjugate 15, with $k_2 = 0.5226 \pm 0.0247 \text{ M}^{-1} \text{ s}^{-1}$ (ESI Fig. S36†). Interestingly, this was ~16-fold faster than the $k_2 = 0.0367 \pm 0.0059 \text{ M}^{-1} \text{ s}^{-1}$ calculated for the reaction of *p*-anisidine to single cysteine conjugate 6 using NMR analysis (ESI Fig. S33†). The reason for this rate acceleration on the protein is unclear, but could be postulated to be influenced by reduced organic solvent concentration in the protein reaction (7% vs. 42%), or an effect of the local amino-acid environment.

Crucially, the amine addition possesses kinetics “on the protein” towards the faster end of the strain-promoted azide–alkyne cycloaddition (SPAAC) scale and more than two orders of magnitude faster than the Staudinger ligation.^{36–42}

The high reactivity of the mHSA intermediate suggested that the amine could be reacted at much lower concentrations. Furthermore, we postulated that the rate difference between thiol and amine additions would enable an *in situ* reaction, in which DBM 13 (3 equiv.) was added to a mixture of the protein and the *p*-anisidine (5 equiv.). This proved effective after being left overnight at RT (ESI Fig. S13†), demonstrating this thiol–amine dual conjugation to be a rare example of an efficient multi-component reaction on a protein. Finally, conjugate 17 was assessed for thiol stability (glutathione, GSH). Treatment with 10 μ M GSH (representative of extracellular concentrations),^{43–46} pH 7.4, 37 °C for 7 days, showed stability by LCMS analysis (ESI Fig. S29†). A further experiment was carried out to approximate the harsh intracellular GSH concentrations (4 mM GSH, pH 6.8, 37 °C)^{43–46} and showed little change after 24 h (ESI Fig. S31†). In summary, *p*-anisidine addition was shown to offer an alternative approach to hydrolysis for maleimide bioconjugate stabilisation. It offers the further advantages of taking place under mild conditions, can be accelerated simply by increasing the amine concentration and can be carried out step-wise or *in situ*.

A further enticing opportunity offered by this conjugation methodology was the possibility of generating triconjugates, if a heterobifunctional amine based on the *p*-anisidine core was used. To this end, two variants of *p*-anisidine, alkyne 18 and azide 22 were synthesised that would enable construction of clickable triconjugates. These scaffolds would then facilitate further modification as desired through the plethora of well-defined click chemistry currently available, depending on the nuances of both protein and probe in use. Importantly, functional analogues 18 and 22 displayed very similar reaction rates with the bromothiomaleimide 11 as *p*-anisidine (ESI Fig. S2†).

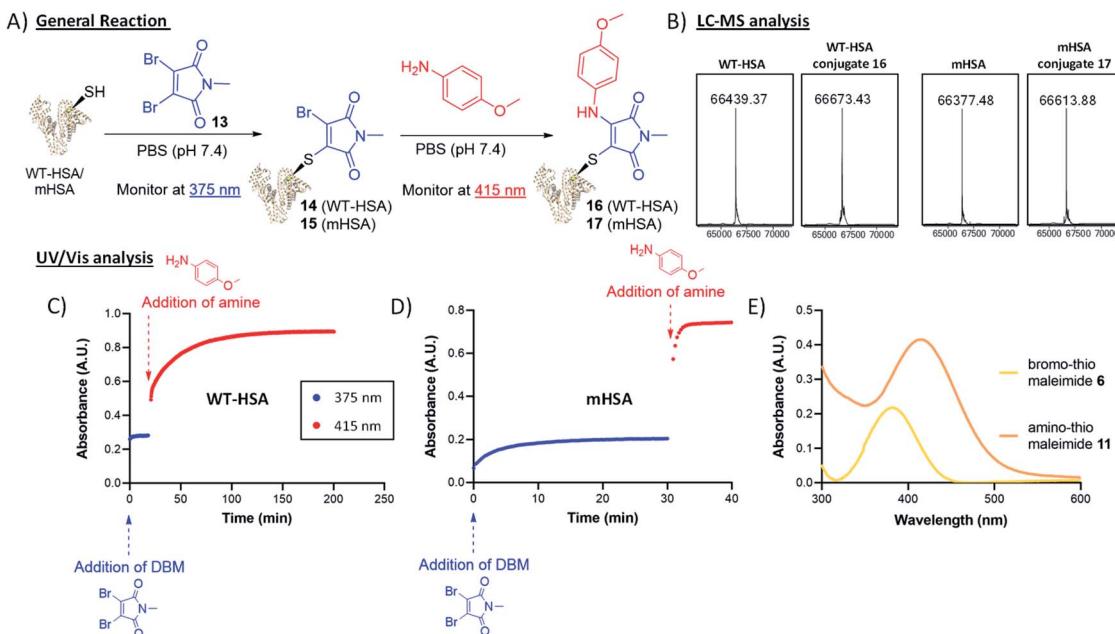


Fig. 4 Thiol–amine dual bioconjugation on wild type HSA (WT-HSA) and mutant HSA (mHSA); (A) general reaction scheme, (B) LCMS analysis of native WT-HSA, mHSA and final conjugates; expected mass for conjugate 16: 66 671 Da, observed mass: 66 673 Da; expected mass for conjugate 17: 66 613 Da, observed mass: 66 614 Da, (C) and (D) UV/Vis analysis of thiol–amine bioconjugation on WT-HSA and mHSA respectively; the absorbance of the resultant bromothiomaleimide was monitored at 375 nm and upon reaction completion, *p*-anisidine was added in the conjugation, where the progress was monitored at 415 nm, (E) UV/Vis analysis of bromothiomaleimide 6 and amino-thiomaleimide 11.

Our first test of this dual conjugation strategy was trialled on a peptide. Cell Penetrating Peptides (CPPs) have garnered interest as a means to achieve payload internalisation in a cell.^{47–49} P218R is an optimised cell-penetrating peptide designed by Dixon *et al.* and represents an extremely challenging modification target due to the abundance of lysine and arginine residues present (9 and 11 residues respectively) in a peptide of 3.8 kDa.⁵⁰ Interestingly, the rapid kinetics of the thiol conjugation enabled efficient and rapid dual modification of the CPP *via* an *in situ* protocol (40 equiv. amine–alkyne **18**, 1.2 equiv. DBM **19**, 30 min at 37 °C, see Fig. 5), indicated by crude LCMS data (ESI Fig. S20†) and immediate colour change upon addition of DBM **19**. Subsequent HPLC purification afforded conjugate **20** (Fig. 5). Work with modified analogues of **20** is underway for use as imaging agents.

The creation of clickable triconjugates was also carried out on two different proteins. DBM **21**, functionalised with biotin as a model handle (used widely in affinity purification and biological assays) was conjugated to mHSA, before amine–azide **22** (20 equiv.) was added and left at room temperature. The dual-functionalization occurred in 2 h at room temperature, with a small amount of unmodified and doubly modified HSA present as the only other detectable species (Fig. 6). Green Fluorescent Protein (GFP) was chosen as a second single cysteine containing protein to further appraise this dual conjugation strategy. Gratifyingly, conjugation of DBM **21** to the thiol was complete in just 5 minutes and amine–azide **22** in a further 95 minutes, with LCMS analysis showing a high degree of conversion (Fig. 6). To avoid the use of toxic copper in Cu¹–

catalysed click reactions,³⁶ modification of conjugates **23** and **25** was achieved with dibenzocyclooctyne–tetramethylrhodamine (DBCO–TAMRA) *via* SPAAC. This successfully yielded the desired fluorescent-biotinylated dual conjugates **24** and **26** (ESI Fig. S15 and S18†). These procedures serve as examples to show the simplicity of forming useful triconjugates with thiol–amine dual conjugation. Similar bifunctional biotin–TAMRA conjugates have recently been used to observe protein modification in human cells.^{51,52}

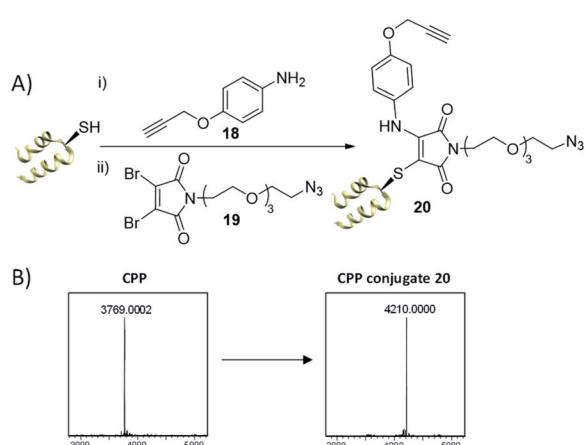


Fig. 5 (A) Dual thiol–amine conjugation on CPP. CPP (0.9 mM) in PB (10 mM, pH 6.5), amine–alkyne **18** (40 equiv.), DBM **19** (1.2 equiv.) left at 37 °C for 30 min, (B) LCMS data; expected mass for conjugate **20**: 4208 Da, observed mass: 4210 Da. See ESI Fig. S19–S22† for more detail.

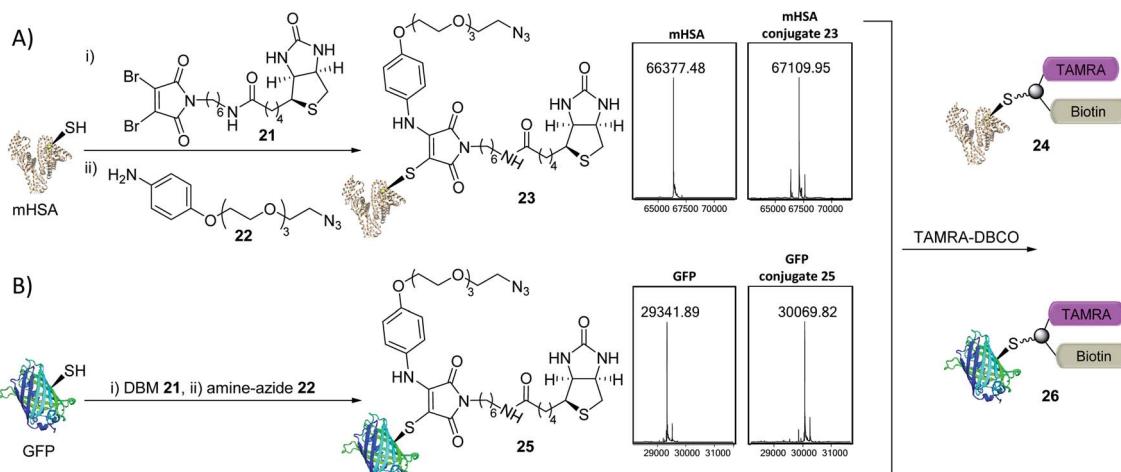


Fig. 6 Trifunctional conjugate formation with mHSA and GFP (LCMS analysis shown). (A) (i) DBM 21 (1.5 equiv.), mHSA (100 μ M) in PBS pH 7.4 at RT for 45 min; (ii) amine-azide 22 (20 equiv.), RT for 2 h; (iii) TAMRA-DBCO (10 equiv.), RT for 16 h. (B) (i) DBM 21 (2.0 equiv.), GFP (40 μ M) in PBS pH 7.4 at 25 $^{\circ}$ C for 5 min; (ii) amine-azide 22 (30 equiv.), 25 $^{\circ}$ C for 95 min; (iii) TAMRA-DBCO (24 equiv.), RT for 5 h. See ESI Fig. S14–S18† for more detail.

Conclusion

We have demonstrated a dual thiol–amine bioconjugation strategy that provides distinct advantages over the hydrolysis of maleimide conjugates. This method simultaneously confers thiol-stability and enables addition of an extra functional handle. Unlike maleimide conjugation and hydrolysis, this method does not form regio- or stereoisomeric products. The dual conjugation reactions may be followed in real time using UV-vis spectroscopy owing to the formation of the amino-thiomaleimide scaffold (ϵ_{415} 4250 M^{-1}). More specifically, we have demonstrated the use of *p*-anisidine-based functional amines that react with the bromothiomaleimide backbone and create trifunctional conjugates on both proteins and peptides. We have observed that the environment local to the cysteine of interest can affect the rate of amine addition and have shown that post-conjugation functionalisation of the amino-thiomaleimide through click chemistry provides an efficient route to such triconjugates. This thiol–amine coupling is also demonstrated to be a rare example of an efficient multi-component bioconjugation reaction on a protein.

Conflicts of interest

K. Nicholls is an employee of Albumedix Ltd. V. Chudasama and J. R. Baker are Directors of the spinoff ThioLogics.

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References

- 1 J. M. J. M. Ravasco, H. Faustino, A. Trindade and P. M. P. Gois, *Chem.-Eur. J.*, 2019, **25**, 43–59.
- 2 K. Renault, J. W. Fredy, P. Y. Renard and C. Sabot, *Bioconjugate Chem.*, 2018, **29**, 2497–2513.
- 3 P. Adumeau, S. K. Sharma, C. Brent and B. M. Zeglis, *Mol. Imaging Biol.*, 2016, **18**, 1–17.
- 4 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, London, 3rd edn, 2013.
- 5 F. Li and R. I. Mahato, *Mol. Pharm.*, 2017, **14**, 1321–1324.
- 6 A. Beck, L. Goetsch, C. Dumontet and N. Corvaia, *Nat. Rev. Drug Discovery*, 2017, **16**, 315–337.
- 7 J. R. Junutula, H. Raab, S. Clark, S. Bhakta, D. D. Leipold, S. Weir, Y. Chen, M. Simpson, S. P. Tsai, M. S. Dennis, Y. Lu, Y. G. Meng, C. Ng, J. Yang, C. C. Lee, E. Duenas, J. Gorrell, V. Katta, A. Kim, K. McDorman, K. Flagella, R. Venook, S. Ross, S. D. Spencer, W. Lee Wong, H. B. Lowman, R. Vandlen, M. X. Sliwkowski, R. H. Scheller, P. Polakis and W. Mallet, *Nat. Biotechnol.*, 2008, **26**, 925–932.
- 8 E. Dolci, V. Froidevaux, C. Joly-duhamel, R. Auvergne, B. Boutevin and S. Caillol, *Polym. Rev.*, 2016, **56**, 512–556.
- 9 Z. Yang, Y. Guo, S. L. Ai, S. X. Wang, J. Z. Zhang, Y. X. Zhang, Q. C. Zou and H. X. Wang, *Mater. Chem. Front.*, 2019, **3**, 571–578.
- 10 B.-Q. Shen, K. Xu, L. Liu, H. Raab, S. Bhakta, M. Kenrick, K. L. Parsons-Reponte, J. Tien, S.-F. Yu, E. Mai, D. Li, J. Tibbitts, J. Baudys, O. M. Saad, S. J. Scales, P. J. McDonald, P. E. Hass, C. Eigenbrot, T. Nguyen, W. A. Solis, R. N. Fuji, K. M. Flagella, D. Patel, S. D. Spencer, L. A. Khawli, A. Ebens, W. L. Wong, R. Vandlen, S. Kaur, M. X. Sliwkowski, R. H. Scheller,



P. Polakis and J. R. Junutula, *Nat. Biotechnol.*, 2012, **30**, 184–189.

11 A. D. Baldwin and K. L. Kiick, *Bioconjugate Chem.*, 2011, **22**, 1946–1953.

12 P. A. Szijj, C. Bahou and V. Chudasama, *Drug Discovery Today: Technol.*, 2018, **30**, 27–34.

13 S. D. Fontaine, R. Reid, L. Robinson, G. W. Ashley and D. V. Santi, *Bioconjugate Chem.*, 2015, **26**, 145–152.

14 L. N. Tumey, M. Charati, T. He, E. Sousa, D. Ma, X. Han, T. Clark, J. Casavant, F. Loganzo, F. Barletta, J. Lucas and E. I. Graziani, *Bioconjugate Chem.*, 2014, **25**, 1871–1880.

15 M. E. B. Smith, M. B. Caspersen, E. Robinson, M. Morais, A. Maruani, J. P. M. Nunes, K. Nicholls, M. J. Saxton, S. Caddick, J. R. Baker and V. Chudasama, *Org. Biomol. Chem.*, 2015, **13**, 7946–7949.

16 M. Morais, J. P. M. Nunes, K. Karu, N. Forte, I. Benni, M. E. B. Smith, S. Caddick, V. Chudasama and J. R. Baker, *Org. Biomol. Chem.*, 2017, **15**, 2947–2952.

17 M. W. Jones, R. A. Strickland, F. F. Schumacher, S. Caddick, J. R. Baker, M. I. Gibson and D. M. Haddleton, *J. Am. Chem. Soc.*, 2012, **134**, 1847–1852.

18 Y. Cui, Y. Yan, Y. Chen and Z. Wang, *Macromol. Chem. Phys.*, 2013, **214**, 470–477.

19 J. K. Y. Tan, J. L. Choi, H. Wei, J. G. Schellinger and S. H. Pun, *Biomater. Sci.*, 2015, **3**, 112–120.

20 N. Forte, M. Livanos, E. Miranda, M. Morais, X. Yang, V. S. Rajkumar, K. A. Chester, V. Chudasama and J. R. Baker, *Bioconjugate Chem.*, 2018, **29**, 486–492.

21 M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick and J. R. Baker, *J. Am. Chem. Soc.*, 2010, **132**, 1960–1965.

22 J. T. Husband, A. C. Hill and R. K. O'Reilly, *Polym. Int.*, 2019, **68**, 1247–1254.

23 S. Ramesh, P. Cherkupally, T. Govender, H. G. Kruger, F. Albericio and B. G. De La Torre, *Chem. Commun.*, 2016, **52**, 2334–2337.

24 A. Wall, K. Nicholls, M. B. Caspersen, S. Skrivergaard, K. A. Howard, K. Karu, V. Chudasama and J. R. Baker, *Org. Biomol. Chem.*, 2019, **17**, 7870–7873.

25 Z. Chen, S. D. Boyd, J. S. Calvo, K. W. Murray, G. L. Mejia, C. E. Benjamin, R. P. Welch, D. D. Winkler, G. Meloni, S. D'Arcy and J. J. Gassensmith, *Bioconjugate Chem.*, 2017, **28**, 2277–2283.

26 F. F. Schumacher, M. Nobles, C. P. Ryan, M. E. B. Smith, A. Tinker, S. Caddick and J. R. Baker, *Bioconjugate Chem.*, 2011, **22**, 132–136.

27 A. B. Mabire, M. P. Robin, W. Quan, H. Willcock, V. G. Stavros and R. K. O'Reilly, *Chem. Commun.*, 2015, **51**, 9733–9736.

28 Y. Xie, J. T. Husband, M. Torrent-Sucarrat, H. Yang, W. Liu and R. K. O'Reilly, *Chem. Commun.*, 2018, **54**, 3339–3342.

29 F. Brotzel, C. C. Ying and H. Mayr, *J. Org. Chem.*, 2007, **72**, 3679–3688.

30 A. Dirksen, T. M. Hackeng and P. E. Dawson, *Angew. Chem., Int. Ed.*, 2006, **45**, 7581–7584.

31 B. Elsadek and F. Kratz, *J. Controlled Release*, 2012, **157**, 4–28.

32 R. E. Kontermann, *Expert Opin. Biol. Ther.*, 2016, **16**, 903–915.

33 D. Sleep, J. Cameron and L. R. Evans, *Biochim. Biophys. Acta, Gen. Subj.*, 2013, **1830**, 5526–5534.

34 M. T. Larsen, M. Kuhlmann, M. L. Hvam and K. A. Howard, *Mol. Cell. Ther.*, 2016, **4**, 1–12.

35 K. K. Schelde, K. Nicholls, F. Dagnæs-Hansen, K. Bunting, H. Rawsthorne, B. Andersen, C. J. A. Finniss, M. Williamson, J. Cameron and K. A. Howard, *J. Biol. Chem.*, 2019, **294**, 3735–3743.

36 N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046–15047.

37 J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793–16797.

38 X. Ning, J. Guo, M. A. Wolfert and G. J. Boons, *Angew. Chem., Int. Ed.*, 2008, **47**, 2253–2255.

39 M. F. Debets, J. S. Prins, D. Merkx, S. S. Van Berkel, F. L. Van Delft, J. C. M. Van Hest and F. P. J. T. Rutjes, *Org. Biomol. Chem.*, 2014, **12**, 5031–5037.

40 J. Dommerholt, F. P. J. T. Rutjes and F. L. van Delft, *Top. Curr. Chem.*, 2016, **374**, 1–20.

41 M. B. Soellner, B. L. Nilsson and R. T. Raines, *J. Am. Chem. Soc.*, 2006, **128**, 8820–8828.

42 A. S. Cohen, E. A. Dubikovskaya, J. S. Rush and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 8563–8565.

43 M. Fernández, A. Shamsabadi and V. Chudasama, *Chem. Commun.*, 2020, **56**, 1125–1128.

44 S. Wang, H. Ma, J. Li, X. Chen, Z. Bao and S. Sun, *Talanta*, 2006, **70**, 518–521.

45 L. Ducry and B. Stump, *Bioconjugate Chem.*, 2010, **21**, 5–13.

46 S. Santra, C. Kaittanis, O. J. Santiesteban and J. M. Perez, *J. Am. Chem. Soc.*, 2011, **133**, 16680–16688.

47 K. Kurrikoff, M. Gestin and Ü. Langel, *Expert Opin. Drug Delivery*, 2016, **13**, 373–387.

48 F. Wang, Y. Wang, X. Zhang, W. Zhang, S. Guo and F. Jin, *J. Controlled Release*, 2014, **174**, 126–136.

49 C. Bechara and S. Sagan, *FEBS Lett.*, 2013, **587**, 1693–1702.

50 J. E. Dixon, G. Osman, G. E. Morris, H. Markides, M. Rotherham, Z. Bayoussef, A. J. El Haj, C. Denning and K. M. Shakesheff, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, E291–E299.

51 M. Broncel, R. A. Serwa, P. Cieplak, E. Krause, M. J. Dallman, A. I. Magee and E. W. Tate, *Angew. Chem., Int. Ed.*, 2015, **54**, 5948–5951.

52 E. M. Storck, J. Morales-Sanfrutos, R. A. Serwa, N. Panyain, T. Lanyon-Hogg, T. Tolmachova, L. N. Ventimiglia, J. Martin-Serrano, M. C. Seabra, B. Wojciak-Stothard and E. W. Tate, *Nat. Chem.*, 2019, **11**, 552–561.