Thiol-based Michael-type addition. A systematic evaluation of its controlling factors

Arianna Gennari, Jennifer Wedgwood, Enrique Lallana, Nora Francini, Nicola Tirelli

PII: S0040-4020(20)30844-9

DOI: https://doi.org/10.1016/j.tet.2020.131637

Reference: TET 131637

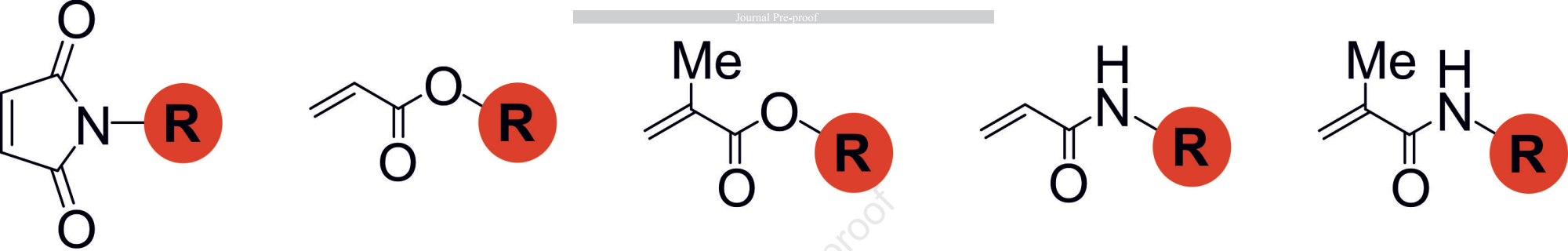
To appear in: *Tetrahedron*

Received Date: 1 May 2020

Revised Date: 31 August 2020

Accepted Date: 22 September 2020

Please cite this article as: Gennari A, Wedgwood J, Lallana E, Francini N, Tirelli N, Thiol-based Michael-type addition. A systematic evaluation of its controlling factors, *Tetrahedron*, https://doi.org/10.1016/j.tet.2020.131637.



REACTIVITY WITH THIOLS

STABILITY OF THE ADDUCTS

Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Thiol-based Michael-type addition. A systematic evaluation of its controlling factors

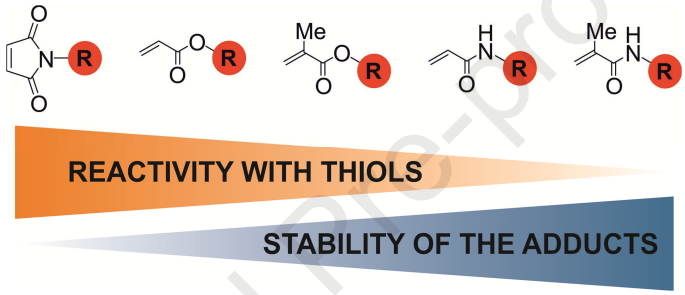
Leave this area blank for abstract info.

Arianna Gennari[a] , Jennifer Wedgwood[b, §] , Enrique Lallana[b] and Nicola Tirelli[a, b] *

[a] *Laboratory of Polymers and Biomaterials, Fondazione Istituto Italiano di Tecnologia, via Morego 30, 16163 Genova, Italy*

[b] *Division of Pharmacy & Optometry, School of Health Science, The University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom*

[§] *Current address: Servier Laboratories Ltd, Sefton House, Sefton Park, Bells Hill, Stoke Poges, Buckinghamshire SL2 4JS*



Tetrahedron
journal homepage: www.elsevier.com

Thiol-based Michael-type addition. A systematic evaluation of its controlling factors

Arianna Gennari[a] , Jennifer Wedgwood[b, §] , Enrique Lallana[b] , Nora Francini[a] and Nicola Tirelli[a, b] *

[a] *Laboratory of Polymers and Biomaterials, Fondazione Istituto Italiano di Tecnologia, via Morego 30, 16163 Genova, Italy*
[b] *Division of Pharmacy & Optometry, School of Health Science, The University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom*
§ Current address: Servier Laboratories Ltd, Sefton House, Sefton Park, Bells Hill, Stoke Poges, Buckinghamshire SL2 4JS

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Bioconjugation; Hammett equation; Retro Michael; Acrylates; Methacrylates; Acrylamides; Methacrylamides; Maleimides; Cysteine

ABSTRACT

This paper is about the factors controlling kinetics and product stability of this popular bioconjugation reaction. We demonstrate that a) thiol pKa, i.e. the amount of thiolates, is the only determinant of the reaction kinetics for the nucleophile; b) product degradation occurs primarily via hydrolysis (not thiol exchange), and is more prominent for the most rapidly reacting electrophiles. In terms of molecular design, acrylamides and low pKa thiols appear as the reaction partners that provide the best compromise for stability and reaction rate.

* Corresponding author. e-mail: nicola.tirelli@iit.it

1.

Bioconjugation is a major research theme since the 70s, best known for linking antibodies to drugs[1] or prodrugs, or therapeutic proteins to poly(ethylene glycol) (PEG)[2] or other polymers.[3]

Michael-type addition is a most popular bioconjugation reaction,[4] most commonly with the use of thiols as nucleophiles and electron-poor double bonds as electrophiles. For clarity, Michael-type addition differs from Michael addition, where the nucleophile is a (stabilized) carbanion, and from thiol-ene reactions, where thiols add through a free radical mechanism onto non-electron- poor olefins. Thiol-based Michael-type addition's popularity for bioconjugation and beyond (e.g. in thiol recognition,[5] surface functionalization,[6] synthesis of polymers[4] or biomaterials[7] etc.) is due to a) the mild reaction conditions, b) the absence of byproducts and c) its bio-orthogonal character, i.e. the reaction has hardly any competition by other biologically occurring nucleophiles. This selectivity has a kinetic origin: thiols have an appreciable acidity, hence anionic and thus strongly nucleophilic thiolates are present already at neutral pH; the more acid the thiol, the more rapid the reaction,[8] a feature also shared with disulfide formation.[9] There are, however, still areas of poor mechanistic understanding for this reaction, which to date often hinder the accurate prediction of e.g. thiol reactivity[10] or of the stability of the products . For example, we still do not know if thiol pKa is one or the main controlling factor of the reaction kinetics, and how this may depend on the structure of the acceptor. Another point to clarify is which one of the two main degradative paths and to which extent can undermine the stability of the conjugation (Figure 1); it is known that sulfur in γ position,[11] even more when oxidized as sulfoxide or sulfone, [12,13] accelerates ester hydrolysis, but there appears to be no quantitative relation nor extension to other hydrolysable groups; it is also known that retro-Michael-type addition can occur, allowing for an exchange with more reactive/more concentrated thiols, but this has been shown only on maleimides.[14,15]

Here we carried out a comprehensive investigation on the effects of the Michael-type donors' and acceptors' structures, as well as the reaction environment on the rate constant and the stability of the final Michael-type adducts.

2. Results and discussion

We have employed a small library of α,β-unsaturated acceptors (Figure 2), varying strength of the electron-withdrawing group (ester, amide, maleimide), hindrance on the double bond (CH_3 vs. H) and polarity of the side chain potentially linking a payload (alcohol vs. amide). Since amino- or NHS-ester-terminated heterobifunctional linkers are routinely used in conjugation reactions which both result in amide bond formation, five of the seven Michael-type acceptors (AcAEA, AcAEMA, AcAEAm, AcAEMAm and AcAEMi) featured a terminal N-acetyl group, thereby better mimicking the structures (and thus, the kinetic and hydrolysis properties) of these Michael-type acceptors. The other two commercially available compounds (HEA and HEMA) simply contained a terminal hydroxyl group to serve as controls. Using these compounds, we have determined A) the rate constant for the addition of two model thiols, i.e. 3-mercaptopropionic acid (**3-MPA**) and *N*-acetylcysteine (**NAC**), separately analysing thiol and thiolate reactivity; B) the stability of their products towards hydrolysis and exchange with the most common thiol in biological fluids, i.e. glutathione (**G-SH**). It is noteworthy that the three thiols used in this study differ in size, polarity and above all acidity, pka values being >11 (**3-MPA**), 10-11 (**NAC**) and 8-9 (**G-SH**) (for their determination through the thiolate UV absorption, see Supporting Information, sections S1.3.2. and have taken proper care of the reduction in thiol concentration due to disulfide formation (see Supporting Information, section S2.2 and Table S2).

By measuring the thiol concentration as a function of time (Figure 3A, left), it is possible to calculate the effective rate constant k_{eff} for the various Michael-type addition reactions (slopes of the graphs in Figure 3A, right); as it is apparent in the 3-MPA / HEA example, the reaction is more rapid at high pH (higher concentration of thiolates) and at high acceptor concentration. k_{eff} can then be used to calculate a kinetic constant independent of the initial concentrations of the reactants, k_{obs} (Figure 3B); this highlights that at any given pH, as it should be expected, the reaction is faster for acceptors bearing esters,

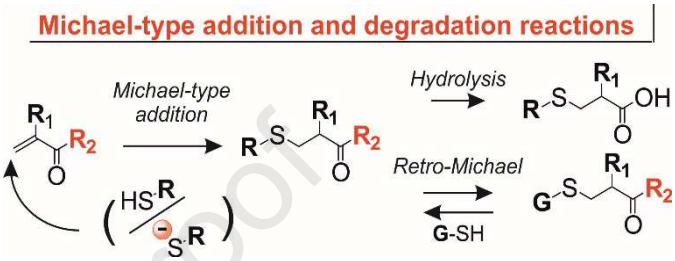


Figure 1. Thiol-based Michael-type addition (left) and the two degradation reactions that its products may undergo (right).

slower with amides or methacrylates, and slowest for methacrylamides, which combine steric hindrance with the poor electronegativity of amides (see also Supporting Information, Section S2.3 and Table S3). Please note that the maleimide-based AcAEMi was excluded from this analysis, because of its almost instantaneous reactivity with both thiols, and also because of its interference with the Ellman's reagent (see Supporting Information, section S2.5). For similar reasons, we have not considered vinyl sulfones: if on one hand they react with thiols so rapidly to be kinetically selective over acrylates, on the other hand this reactivity is marred by the parasite addition of OH^- at even mildly basic pH.

NAC is a stronger acid than 3-MPA; this means that, at a given pH, NAC has a higher proportion of thiolates and therefore

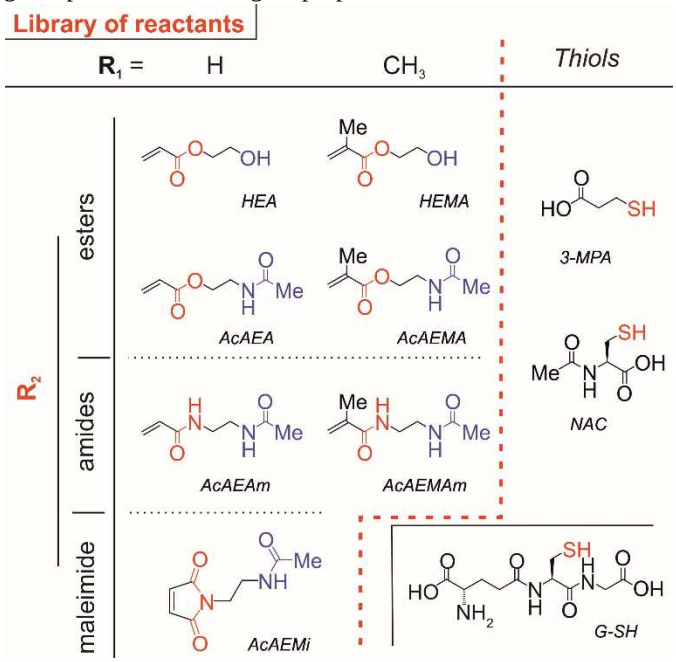


Figure 2. Summary of the reactants used in this study: two nucleophiles (NAC and 3-MPA) and a small library of acceptors. A third thiol (G-SH) was used in retro-Michael studies.

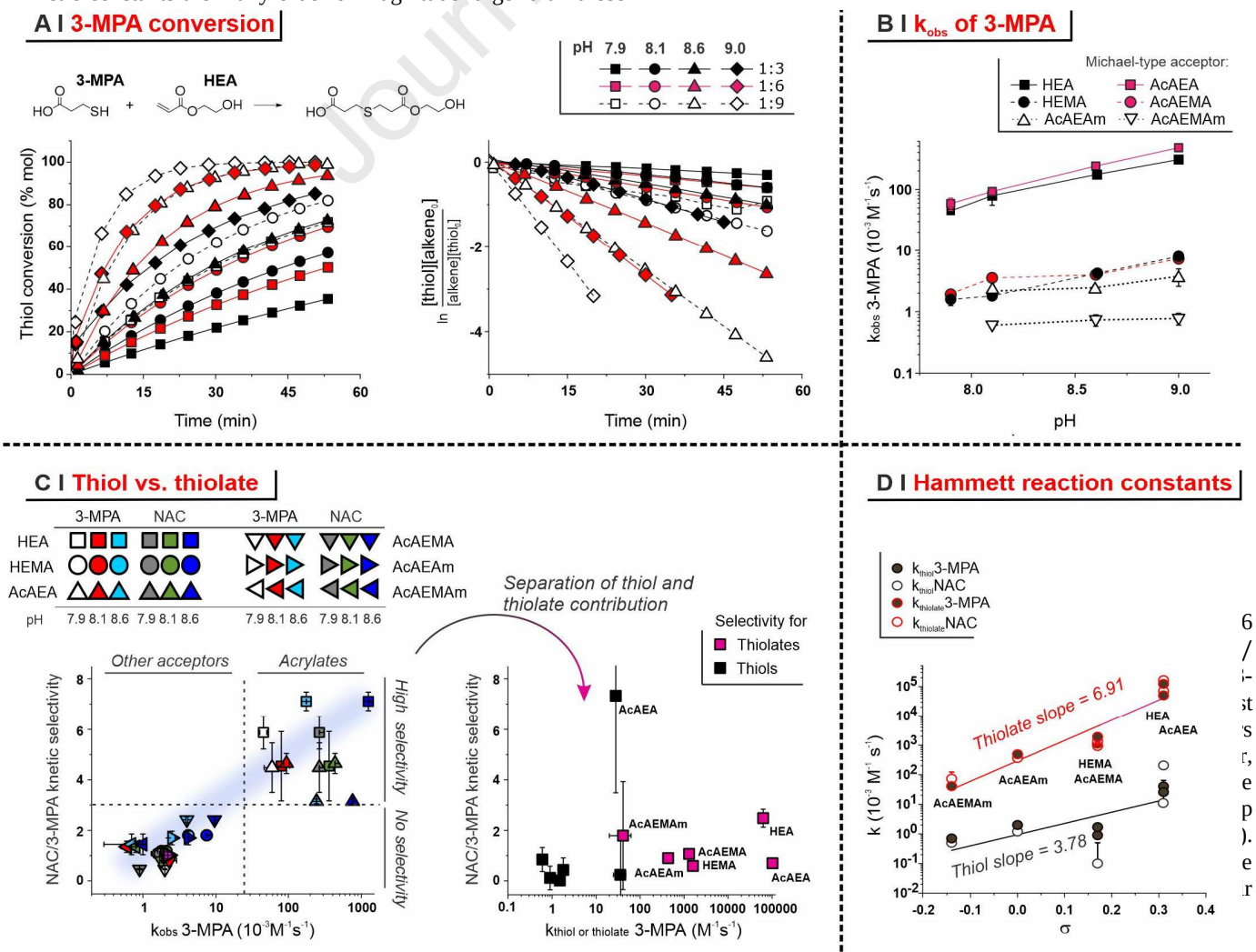
very apparent with acrylates, but marginal or simply not observed for all other acceptors (Figure 3C, left); this may raise questions about the general applicability of selectivity and may even seem somehow counter-intuitive: a tenet of organic chemistry is that selectivity is expected in slower reactions. This effect, however, is a simple result of the effect of the thiol/thiolate ratio on the reaction kinetics. Thiols are present in two nucleophilic forms, i.e. protonated thiols and deprotonated thiolates, which are in a pH-controlled equilibrium and are differently charged and reactive. For any given thiol, it is therefore possible to discriminate the largely pH-independent contributions of the two forms to the overall reaction kinetics ($k_{thiolate}$ and k_{thiol}, respectively), with coefficients that reflect the ionization degree α of the group. It then becomes apparent that a) in a thiolate form, 3-MPA and NAC react with the same speed with all acceptors (no 3-MPA / NAC selectivity; see purple squares in Figure 3C, right), and b) they do so also in their thiol forms (black squares), albeit with a slower kinetics and with the presence of one outlier (AcAEA, with very large error bars). Therefore, the observed selectivity is merely a reflection of the different amounts of thiolates (more reactive nucleophiles; compare red and black squares) produced by thiols with a different pKa.

The situation can also be described through a group contribution approach, similar to what already used for other Michael-type additions:[17,18] if we plot both thiolate and thiol kinetics constants vs. Hammett constant σ (one of the two parameters of the Hammett equation) calculated considering the substituent on the α,β-unsaturated Michael-type acceptors, the experimental points for the 3-MPA and NAC in a thiol form are aligned and distinct from those (equally aligned) of them as thiolates. This means that the reaction constant ρ (the slope) depends on whether the thiol group is protonated or not, but not on identity, size or polarity of the nucleophile. It is also noteworthy that, although thiolate kinetic constants are many order of magnitude larger than those

pH << pKa, e.g. in a slightly acidic environment.

Another point worth of clarification is the stability of the products of Michael-type addition, and whether hydrolysis and retro-Michael-type/thiol exchange or both (Figure 4A) can differently affect products with different structures. We have synthesized adducts of all acceptors with NAC (for synthetic details, see Supporting Information, section S1.4.1-6). NAC was chosen because it can mimic the structure and hence the chemical behaviour of cysteine-containing bioconjugates (proteins, peptides). We monitored the disappearance of these adducts at pH 7.4 and 7.9 (Figure 4B, top) in the presence of a competitive thiol (G-SH), which is about one order of magnitude more acid than NAC (see Supporting Information, Table S1) and was used in a 10 molar excess. All compounds showed a pseudo-first order behaviour, whose degradation rate constant (k_{degrad}) can be obtained as the slope of a plot of $ln([adduct]/[adduct]_0)$ vs. time (see Supporting Information, Table S4). As already known from literature, maleimide adducts were the most unstable, which is due to the occurrence of retro-Michael followed by exchange with G-SH and/or hydrolytic ring-opening (see Supporting Information, section S2.4).[14,15] It Is worth noting that after ring-opening, the resulting succinimide thioethers reportedly undergo no further thiol exchange, suggesting the rapid retro-Michael to be a peculiarity of maleimide derivatives.[14]

For the products of all other acceptors, their kinetic stability was in the order acrylates < methacrylates < acrylamides ≈ methacrylamides (Fig 3B, bottom), i.e. less sterically hindered esters react rapidly, amides are the most stable. This order would suggest a hydrolytic degradation mechanism, and indeed HPLC-MS identified hydrolysis products (Figure 4C, see also Supporting Information, section S2.6) as the only additional compounds present in the reaction environment even after 2 weeks; no G-SH-containing product was detected for any of the adducts. This points to hydrolysis as the main degradative path



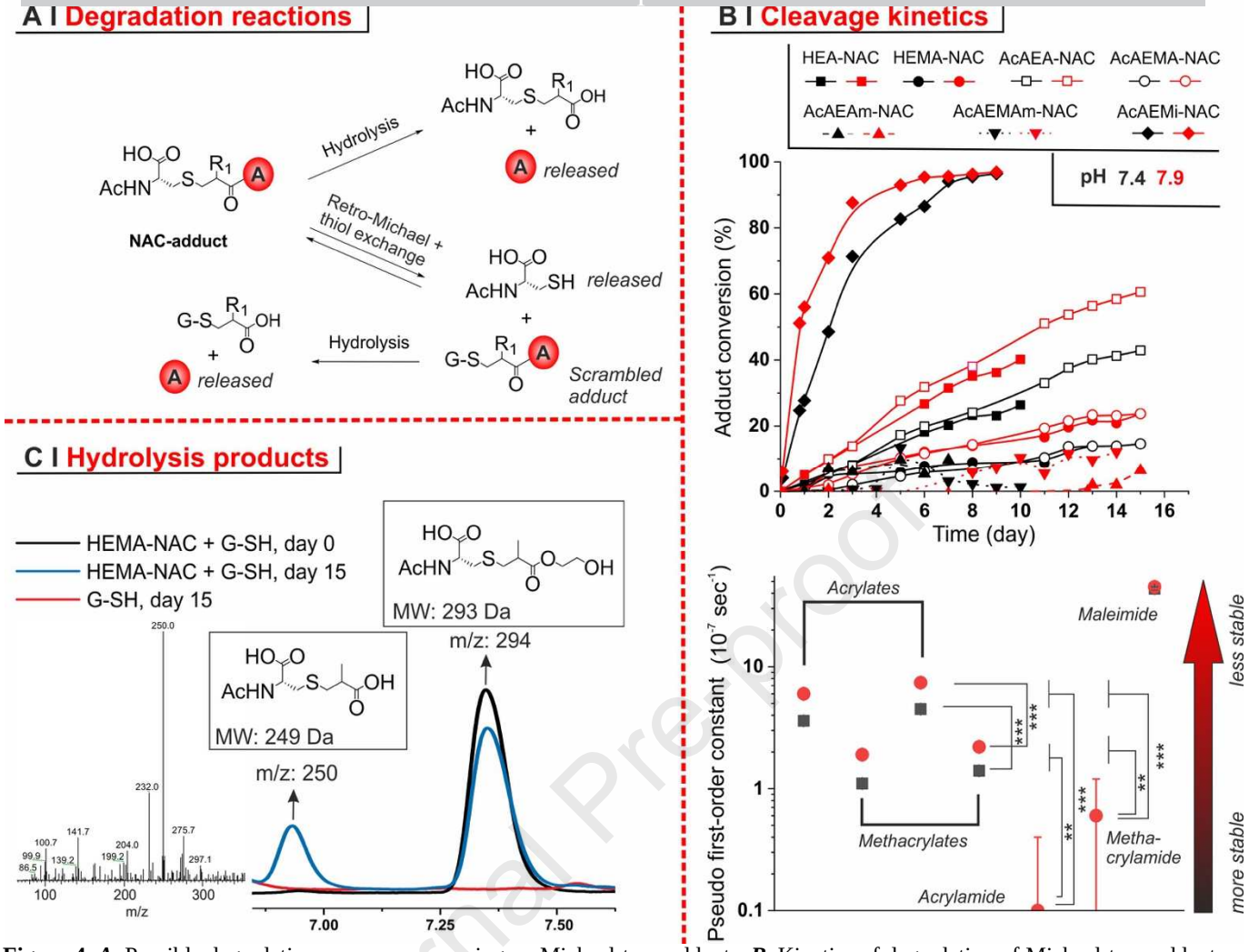


Figure 4. ***A***. Possible degradation processes occurring on Michael-type adducts. ***B***. Kinetics of degradation of Michael-type adducts (*top*; please note that lines are just guides for eyes) with 10 eq. of G-SH at pH 7.4 and 7.9 and 37 °C, and corresponding pseudo-first-order constants (*bottom*). ***C***. RP-HPLC/ESI analysis of HEMA-NAC, showing the appearance of a hydrolyzed product (see Supporting Information Figure S5-6 for the mass spectra of the hydrolyzed products of HEA-NAC, HEMA-NAC, AcAEA-NAC and AcAEMA-NAC). Statistical analysis through a 2-way ANOVA; ** - p<0.01,*** - p<0.001

for non-maleimide Michael-type acceptor, and to the less reactive but more stable (meth)acrylamide derivatives as a rational choice of acceptor.

3. Conclusions

In short, this study has provided two important design criteria: 1) whatever the chemical structure of the thiol, its acidity is the primary controller of the reaction kinetics, 2) unsaturated amides endow their constructs with the highest kinetic stability against hydrolysis, although suffering of a somehow sluggish reaction kinetics (tens of hours to completion). If this is an issue, it can be overcome by lowering the thiol pKa through appropriate engineering of its structure (e.g. adding vicinal cationic sites)[9,8] ; the *caveat* of this approach is that more acidic thiols are also more prone to disulfide formation, so additional attention has to paid to avoid their oxidation.

4. Experimental section

Determination of rate constants:

All Michael-type addition reactions of **3-MPA** or **NAC** onto various acceptors were carried out in a 96-well plate at 30 °C, at various pH (7.9, 8.1, 8.6, 9.0), using a 80:20 % v/v solvent mixture of 100 mM Tris buffer/EtOH and three different thiol/Michael-type acceptor molar ratios (1:3, 1:6, 1:9 for the more reactive **HEA** and **AcAEA**, and 1:30, 1:60, 1:90 for the less reactive **HEMA**, **AcAEMA**, **AcAEAm** and **AcAEMAm**). Please note that the presence of EtOH is necessary to ensure that all reaction partners (in particular the rather hydrophobic 3-MPA and HEMA) are fully soluble in the medium. After the reactions were quenched with diluted HCl at different time points, the Ellman's test (see Supplementary Information section S1.3.1) was employed to measure the unreacted thiol concentration in each well. The unreacted thiol concentration $[thiol]_t$ was corrected by the amount of disulfides produced at any time point (see Supplementary Information section S1.3.2) and then used to calculate the unreacted double bond concentration $[alkene]_t = [alkene]_0 - ([thiol]_0 - [thiol]_t)$, under the assumption that the alkenes undergo solely Michael-type addition. The two residual concentrations can be combined with the respective initial concentrations $[thiol]_0$ and $[alkene]_0$ in a general equation for the time-dependent reagent consumption, as expressed through an effective kinetic constant k_{eff}; please note that the latter can also be (and is in our cases) a negative number.

$$ln\left(\frac{[thiol][alkene_0]}{[thiol_0][alkene]}\right) \quad (1)$$

Since the standard definition for an observed rate constant (k_{obs}) is as provided in Equation 2,

$$k_{obs} = (k_{thiolate} - k_{thiol}) \cdot \alpha \quad (2)$$

$$= \frac{1}{([thiol_0] - [alkene_0])} ln\left(\frac{[thiol][alkene_0]}{[thiol_0][alkene]}\right)$$

Then it is possible to use the determined values of k_{eff} to calculate those of k_{obs}, which are independent of the initial concentrations of the reagents. Please note that, in order to do so, the initial concentrations of the two reagents cannot be identical.

$$k_{eff} = k_{obs}([thiol]_o \quad (3)$$

In addition, the reactions involving **AcAEMi** were evaluated via RP-HPLC (see section 1.2), since previously obtained results from Ellman's tests with **AcAEMi** demonstrated that the maleimide-containing compound showed reactivity towards thiol-based nucleophilic reaction products (TNB^{2-}), thereby invalidating this test for the determination of the reactants' concentrations (see Supplementary Information section S1.6.1.2 and section S2.4 and Figure S4).

We here describe the reaction between **HEA** and **3-MPA** at pH 7.9 as an example. A thiol stock solution (2 mM) in water and three HEA solutions at different concentration (6, 12 and 18 mM) in a 60:40 % v/v solvent mixture of Tris buffer (100 mM, pH 7.9) and EtOH were prepared (note: ethanol ensures complete solubilisation of the Michael-type acceptors). 100 µL aliquots of each acrylate solution and 300 µL aliquots of the thiol solution were then pipetted into individual wells of a 96-well plate, and left to incubate at 30 □C for 30 min in the microplate reader. The Michael-type addition reactions were initiated by adding 100 µL of the thiol solution to the first three wells containing 100 µL of 6, 12 and 18 mM acrylate solutions (resulting in a final thiol:acrylate molar ratio of 1:3, 1:6 and 1:9, respectively). The reactants were mixed thoroughly by pipetting and the reaction was then continuously incubated at 30 □C. These steps were repeated for the other aliquots of acrylate solutions every 5 min for 45 min. Thereafter, the reactions were quenched by the addition of 100 µL of a 250 mM HCl (aq) solution, after which the unreacted thiol concentration in each well was immediately evaluated via Ellman's test.

The numerical value for the substituent constant σ relative to each Michael acceptors was approximated as the sum of the σ_{para} values relative to the two substituents (various carboxylic derivatives, methyl or H) on the double bond, which were obtained from Carey F.A., Sundberg R.J. (1990) Study and Description of Organic Reaction Mechanisms. In: Advanced Organic Chemistry. Springer, Boston, MA. Please note that for what pertains to carboxylic groups, we considered their contribution similar to that of an acetoxy residue for **HEA**, **HEMA**, **AcAEA** and **AcAEMA,** and to that of an acetamide for **AcAEAm** and **AcAEMAm**, both in para position of an aromatic system (respectively σ=0.31 and 0.0); the values of methyl and proton were respectively -0.14 and 0.0.

Discrimination of thiol/thiolate rate constants:

Knowing the pKa (Supporting Information sections S1.3.2) the rate constants for deprotonated and protonated thiols ($k_{thiolate}$ and k_{thiol}, in $M^{-1} \cdot s^{-1}$) can be obtained through Equation 4:

(4)

where α is the molar fraction of deprotonated thiol,

$$\alpha = \frac{[thiolate]}{[thiol]+[thiolate]} = \frac{K_a}{[H^+]+K_a}.$$

$$ln([adduct]) = -k_{deg} \cdot t + ln([adduct]_0)$$

Knowing the pKa values (Supporting Information Table S1) of 3-MPA and NAC in the reaction environment, it is therefore possible to separately obtain the rate constants from a plot of k_{obs} vs. α, k_{thiol} being the intercept and thus allowing the calculation of $k_{thiolate}$ from the slope of the graph.

Stability of the Michael-type adducts:

Analysis via RP-HPLC: Michael-type adducts were dissolved at a concentration of 1 mM in phosphate buffer (50 mM, pH 7.4 or 7.9) containing 10 mM G-SH, and all samples incubated at 37 °C. At regular intervals, 400 µL aliquots of the samples were collected and added to 100 µL of a 1.0 M HCl solution to reduce the pH and quench the Retro-Michael-type addition reactions. Quenched samples were stored at -20 °C until analyzed using RP-HPLC to calculate the concentrations of intact adducts. The rate constants and half-life for the degradation kinetics of the Michael-type adducts in the reducing environments were then determined using the pseudo-first order rate law of Equation 5 and 6, respectively.

(5)

$$t_{1/2} = \frac{\ln(2)}{k_{deg}} \quad (6)$$

where *[adduct]* and $[adduct]_0$ are the concentrations of the Michael-type adducts at time *t* and at time *0*, respectively; k_{deg} is the degradation rate constant for the adducts; $t_{1/2}$ is the half-life of the degradation process.

Acknowledgments

Dr. Wedgwood is indebted to the Engineering and Physical Sciences Research Council (EPSRC) for a Ph.D. studentship within the North-West Nanoscience (NoWNano) Doctoral Training Centre (EPSRC Grant No. EP/G03737X/1). Dr. Lallana and Dr. Gennari received funding through the EU FP7 ReLiver (Grant Agreement No. 304961), and Univax (Grant Agreement No. 601738), respectively.

The authors wish to thatnk Mrs Anna Daghetti (Università degli Studi di Milano, Milan, Italy) for HPLC-MS analysis and Dr Richard d'Arcy (Vanderbilt University: Nashville, TN, US) for the helpful discussion.

Supplementary Material

Supplementary material that can be found online.

References

[1] Verma, S.; Miles, D.; Gianni, L.; Krop, I. E.; Welslau, M.; Baselga, J.; Pegram, M.; Oh, D. Y.; Dieras, V.; Guardino, E.; Fang, L.; Lu, M. W.; Olsen, S.; Blackwell, K.; Grp, E. S. *N. Engl. J. Med.* **2012,** *367*, 1783-1791.

[2] 1451-1458.
[3] Vicent, M. J.; Duncan, R. *Trends Biotechnol.* **2006,** *24*, 39-47.
[4] Mather, B. D.; Viswanathan, K.; Miller, K. M.; Long, T. E. *Prog. Polym. Sci.* **2006,** *31*, 487-531.
[5] Yin, C. X.; Huo, F. J.; Zhang, J. J.; Martinez-Manez, R.; Yang, Y. T.; Lv, H. G.; Li, S. D. *Chem. Soc. Rev.* **2013,** *42*, 6032-6059.
[6] Heggli, M.; Tirelli, N.; Zisch, A.; Hubbell, J. A. *Bioconjugate Chem.* **2003,** *14*, 967-973.
[7] Lutolf, M. P.; Hubbell, J. A. *Biomacromolecules* **2003,** *4*, 713-722.
[8] Lutolf, M. P.; Tirelli, N.; Cerritelli, S.; Cavalli, L.; Hubbell, J. A. *Bioconjugate Chem.* **2001,** *12*, 1051-1056.
[9] Geven, M.; Luo, H. Y.; Koo, D.; Panambur, G.; Donno, R.; Gennari, A.; Marotta, R.; Grimaldi, B.; Tirelli, N. *ACS Appl. Mater. Interfaces* **2019,** *11*, 26607-26618.
[10] Folikumah, M. Y.; Neffe, A. T.; Behl, M.; Lendlein, A. *MRS Adv.* **2019,** *4*, 2515-2525.
[11] Redondo, J. A.; Navarro, R.; Martinez-Campos, E.; Perez-Perrino, M.; Paris, R.; Lopez-Lacomba, J. L.; Elvira, C.; Reinecke, H.; Gallardo, A. *J. Polym. Sci., Part A: Polym. Chem.* **2014,** *52*, 2297-2305.
[12] Crielaard, B. J.; Rijcken, C. J. F.; Quan, L. D.; van der Wal, S.; Altintas, I.; van der Pot, M.; Kruijtzer, J. A. W.; Liskamp, R. M. J.; Schiffelers, R. M.; van Nostrum, C. F.; Hennink, W. E.; Wang, D.; Lammers, T.; Storm, G. *Angew. Chem., Int. Ed.* **2012,** *51*, 7254-7258.
[13] Boyatzis, A. E.; Bringans, S. D.; Piggott, M. J.; Duong, M. N.; Lipscombe, R. J.; Arthur, P. G. *J. Proteome Res.* **2017,** *16*, 2004-2015.
[14] Baldwin, A. D.; Kiick, K. L. *Bioconjugate Chem.* **2011,** *22*, 1946-1953.
[15] Baldwin, A. D.; Kiick, K. L. *Polym. Chem.* **2013,** *4*, 133-143.
[16] Chatani, S.; Nair, D. P.; Bowman, C. N. *Polym. Chem.* **2013,** *4*, 1048-1055.
[17] Um, I. H.; Lee, E. J.; Seok, J. A.; Kim, K. H. *J. Org. Chem.* **2005,** *70*, 7530-7536.
[18] Vlasov, V. M. *Monatsh. Chem.* **2016,** *147*, 319-328.

Highlights for

Thiol-based Michael-type addition. A systematic evaluation of its controlling factors

Arianna Gennari[a], Jennifer Wedgwood[b, §], Enrique Lallana[b], Nora Francini[a] and Nicola Tirelli[a,b,*]

[a] *Laboratory of Polymers and Biomaterials, Fondazione Istituto Italiano di Tecnologia, via Morego 30, 16163 Genova, Italy*
[b] *Division of Pharmacy & Optometry, School of Health Science, The University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom*

- Thiolate concentration is the determinant of thiol Michael-type reactivity.
- Retro-Michael (= thiol exchange) appears to be relevant only for maleimides.
- Hydrolysis is more likely for acceptors that accelerate the reaction (e.g. esters).
- Acrylamides + acidic thiols offer the best combination of stability + reactivity.

* Corresponding author. e-mail: nicola.tirelli@iit.it

§ Current address: Servier Laboratories Ltd, Sefton House, Sefton Park, Bells Hill, Stoke Poges, Buckinghamshire SL2 4JS

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: