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Programmable Chemical Switch based on Triggerable Michael Acceptors

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Developing engineerable chemical reaction that is triggerable for simultaneous chemical bond formation and cleavage by external cues offers tunability and orthogonality that is highly desired in many biological and material applications. Here, we present a chemical switch that concurrently captures these features in response to chemically and biologically abundant and important cues, viz., thiol and amine. This thiol/amine-triggerable chemical switch is based on a Triggerable Michael Acceptor (TMAc) which bears good leaving groups at its α-position. The acceptor undergoes a "trigger-to-release" process where thiol/amine-addition triggers cascaded release of leaving group and generates a less activated acceptor. The newly generated TMAc can be further reversed to liberate original thiol/amine by a second nucleophile trigger through "trigger-to-reverse" process. Within the small molecular volume of the switch, we have shown five locations can be engineered to achieve tunable "trigger-to-release" kinetics and tailored reversibility. The potential of engineerable bonding/debonding capability of the chemical switch is demonstrated by applications in cysteine-selective and reversible protein modification, universal self-immolative linker, and orthogonally addressable hydrogel.

Introduction

As two of the most abundant and important nucleophiles that are widely involved in many chemical and biological processes, thiol and amine moieties have drawn tremendous research interest.[1-3] The significance of these reactions has been found in a variety of applications, ranging from drug discovery to protein modification, drug delivery, and materials preparation.[4-12] Highlighted by thiol-X click reaction and activated ester based amidations, considerable efforts have been devoted to developing efficient C-S/C-N bond formation chemistry with these functionalities.[13-23] On the other hand, triggered debonding reactions with high fidelity to either produce thiol/amine or using them as an external cue to liberate other molecular identities under mild condition have been very few, though its importance is well recognized.[24-29] Triggerable debonding chemistry can be broadly classified into two categories: i) trigger-to-reverse and ii) trigger-to-release. The former approach is based on reversible reactions, where a coupling product can be chemically, thermally or mechanically decoupled and reversed to regenerate the original starting materials.[26,30] The reversibility and dynamic nature of the reversible reaction offers unique access to introduce interesting features such as malleability, self-healing, shape-memory adaptability, stress relaxation and responsiveness into new functional materials and even the opportunity to regulate function of biomolecules.[25,31-34] In comparison, trigger-to-release strategy uses an efficient bond formation reaction to trigger the cascaded cleavage of preinstalled functionalities of interest.[35] The ability to incorporate various releasable functionalities with this approach has great potential in the design of cleavable traceless linkers for antibody drug conjugates, neuromodulator delivery strategies, triggered catalysis and bioimaging.[35-40] Thiol/amine associated reactions that concurrently capture the key advantageous features of trigger-to-release and trigger-to-reverse processes are not well-studied. Combining these features offer the opportunity to bring about new function in areas that are independently represented in both these strategies. Moreover, many of the reported reactions in either of the strategies lack the structural handles to instill programmable reaction kinetics and customizable reversibility. For example, water-soluble allylsulfones have been used to execute double Michael addition reactions, where disulfide bonds in proteins can be replaced with a functional handle.[41] This modification capability has been used further to introduce biorthogonal functionalities onto protein surfaces. [42]To fully realize the potential of these types of modifications in a much larger variety of applications, the reactivity of these functional groups with other common bio-nucleophiles and their reversibility need to be fully

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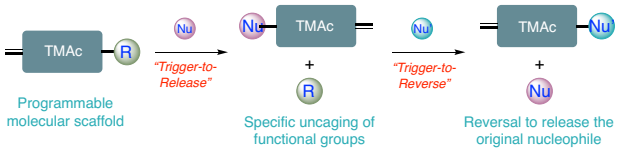

Scheme 1. Schematic representation of a scaffold with programmable features in the trigger-to-release and trigger-to-reverse process

understood. Herein, we present a class of Triggerable Michael Acceptors (TMAc) that not only exhibits the combination of advantages of both trigger-to-reverse and trigger-to-release processes with programmable reactivity and reversibility, but also offers the capability for both chemical bond formation and cleavage as represented in Scheme 1. Considering the indispensable roles played by these nucleophiles, we envision that developing reactions featuring the above capabilities for thiols and amines will allow us to build a molecular portfolio based on a single platform for a variety of chemical, biological and materials applications. In addition to demonstrate the unprecedent structural control over the two processes, we also showcase the utility of the reaction as a bonding and debonding chemical switch in cysteine selective and reversible protein modification, universal self-immolative linker, and preparation of orthogonally addressable hydrogel.

Results and discussion

Design of engineerable chemical switch for bonding and debonding

We have designed a chemical switch that can undergo a trigger-to-release process and a structure-dependent trigger-to-reverse process in the presence of certain nucleophiles. The general structure of the TMAc switch is represented by **A1** in Fig 1A. The molecule is simply an activated Michael acceptor, bearing a good leaving group (X) at the α-position. We envisioned that nucleophilic addition of **N1** to **A1** which is already favored can be significantly enhanced due to the elimination of leaving group; the subsequent generation a less activated TMAc, **A2**, should result in a relatively stable product. We envisaged several advantages to this very simple, but underexplored reaction: (i) the leaving group 'X' can be used to mask and then provide triggered release of a variety of functional groups, with implications in the activated release of a drug molecule or a sensing reporter (trigger-to-release); (ii) the product **A2** can be structurally customized to be either inert or susceptible to a stronger nucleophile **N2** that causes the release of the originally conjugated nucleophile **N1** (trigger-to-reverse); and (iii) within the relatively small molecular volume, there exists five different locations (R1-R4, and X) that impact on the kinetics of these reactions, thus offering a fundamental understanding of the structural factors that underlie these two processes.

For the initial test of reaction hypothesis, molecule **1**, an acrylate with a quaternary ammonium leaving group was synthesized and evaluated for its reaction with two nucleophiles, 2-(2-methoxyethoxy)ethanethiol and benzylamine. ^{1}H NMR showed that the reaction of **1** with the thiol (0.5 equiv.) is completed in 3 minutes (Fig S1) and that the product is indeed due to the Michael-type addition (Fig 1B and Fig S2). Interestingly, reaction of **1** with benzylamine was much slower at pH 7.4, where the reaction took ~50 minutes for completion. This is attributed to the weaker nucleophilicity of amines under these conditions. Also, the product here is the dialkylated product with no discernible monoalkylated product (Fig S3&S4), likely because the secondary amine product of the first step is a much better nucleophile than the primary benzylamine. These differences in the kinetics and the nature of products formed from thiol and amine nucleophiles provided us with an additional handle to understand the structural factors that could influence the behavior of the molecular switch.

Trigger-to-release process with tunable reaction kinetics

To understand the factors that could influence the trigger-to-release kinetics, we systematically varied R^1, R^2, R^3, R^4 and X groups on **A1** (Fig 2A). The stoichiometry between **A1** and the

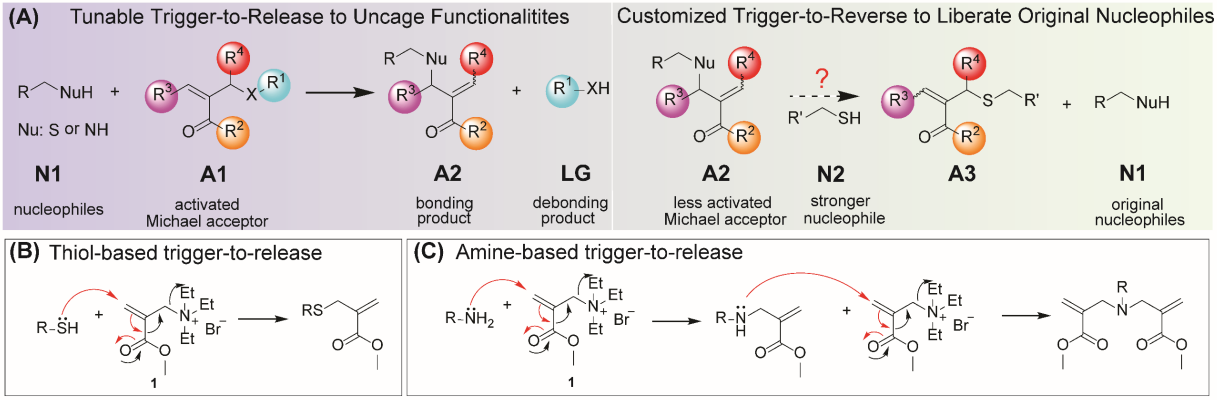

Fig 1. Chemical switch for bonding and debonding using Triggerable Michael Acceptors (TMAc). (A) Schematic illustration of trigger-to-release as a universal strategy for uncaging of functional groups (left) and trigger-to-reverse process, the reversibility of which can be structurally customized (right). (B) Proposed thiol-based trigger-to-release. (C) Proposed amine-based trigger-to-release

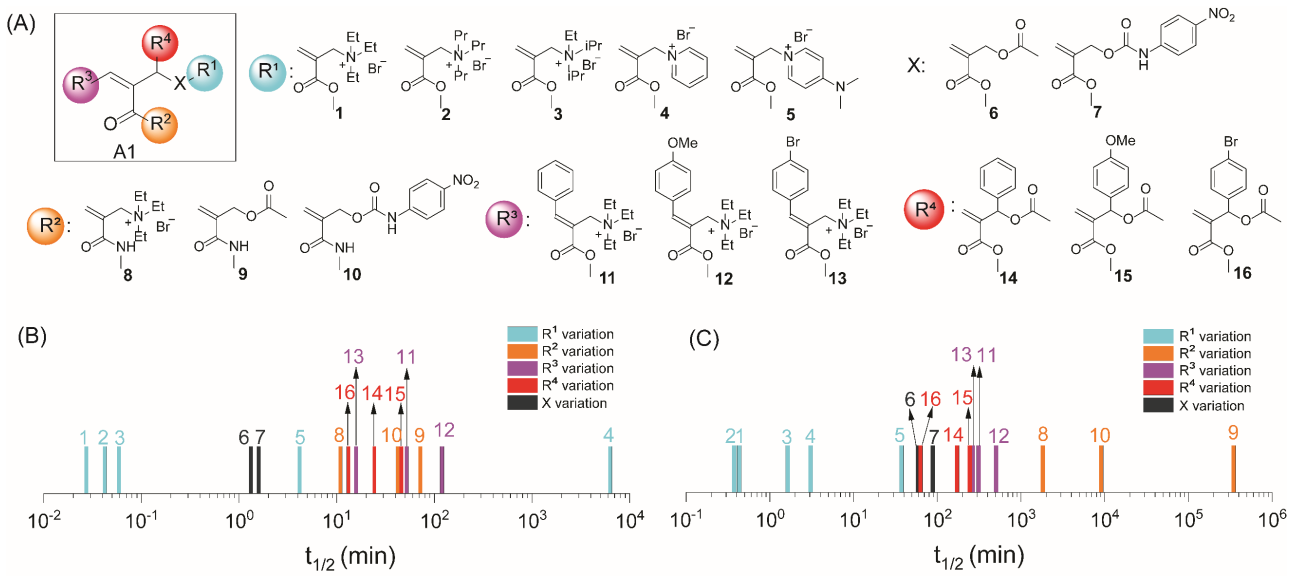

Fig 2. Structure-property relationship investigation on trigger-to-release kinetics. (A) Structure variations of **A1**. (B) Substitution effect on thiol-based trigger-to-release kinetics described by the half-life of thiol. (C) Substitution effect on amine-based trigger-to-release kinetics described by the half-life of amine. Note: The half-life of thiol and amine was extrapolated from the kinetics curves obtained by NMR (Fig S5). The reaction conditions were identical within a comparison group (color-coded) and can be found in Fig S5.

nucleophiles was maintained as 2:1 in all experiments. Similarly, the reaction conditions such as solvent, concentration and pH were identical within a comparison group (color-coded group in Fig 2). However, these conditions might be different between two different comparison groups, either because of solubilization issues associated with a specific set of **A1** derivatives or due to the need to slow down the reaction in order to achieve measurable kinetics. For this reason, whenever needed, we also carried out at least one molecule in two different reaction conditions to bridge reaction rates in two comparison groups. Reaction kinetics was measured by NMR, and then the half-life of thiol or amine was extrapolated from kinetics curve (Fig S5) to compare the influence of structural factors on the **A1** reactivity. First, the thiol-addition reactions for **1**-**5** with aliphatic and aromatic ammonium leaving groups were studied (Fig 2B & Fig S5B, S5H). The reactions were completed within just a few minutes for molecules **1**-**3**, when the leaving group was an aliphatic ammonium moiety. However, the reaction was much slower for **4** and **5**, which contains aromatic ammonium leaving groups. This difference is attributed to the fact that the positive charge in aromatic ammoniums is more stabilized by delocalization. By extension, **5** would be less reactive than **4**, as the electron-donating dimethylamino group can better stabilize the charge. However, we observed that **5** was faster than **4**. The observation is likely due to the basicity of the released DMAP molecule, which likely accelerates the reaction in MeOH-d4 where the pH of reaction mixture could not be maintained. This assertion is supported by the drastic rate enhancement observed in the presence of extrinsic DMAP in **4**/thiol reaction (Fig S6). To further investigate this, we analyzed the reactivity of **4** and **5** towards benzylamine, where pH of solution was maintained in deuterated MeOH/buffer mixed media (Fig 2C & Fig S5E). Indeed, the reactivity of **4** toward amine is higher than **5** here. Influence of the leaving group was observed to be similar in both thiol and amine-conjugation. Additionally, the reaction kinetics of the Michael addition can be significantly influenced by the polarity of solvent because of the stabilization of the developing of transition state. To test this hypothesis, thiol-based trigger-to-release reaction of **1**, **4**, **8** was carried out in both MeOD and buffer solution. As shown in Fig S7, kinetics in buffer solution is much faster than that in MeOD except for compound **1**, in which the reactivity is too high to be distinguished in the two solvents. In all these reactions, we were also concerned about any potential solvent-dependent interferences from the air oxidation of thiols. To understand this, oxidation of thiol in both MeOD and buffer was monitored by NMR. We do not see any discernible changes in the stability of thiols over the period of this study, which indicates that thiol oxidation has negligible influence on the obtained Michael addition kinetics (Fig S105, 106). Overall, this study provided the initial indication that the leaving group can significantly influence the kinetics of the trigger-to-release process.

To further test the diversity of leaving groups, we varied leaving group (X) from quaternary ammonium (**1**) to carboxylate (**6**) to carbamate moieties (**7**), where the released product would be a tertiary amine, carboxylic acid or a primary amine respectively. For direct comparison, reaction of **1** with thiol was also performed in the MeOH-d4/buffer mixed solution where reaction of **6** and **7** were carried out. The reaction for **1** was too fast to distinguish reactivity difference between pure MeOH-d4 and mixed solvent within the detectable time scale (Fig S7). Indeed, both with the thiol and the amine addition reactions, the relative reactivities correlate (**1**>**6**≥**7**) with the leaving ability

of 'X' (Fig 2B&2C, Fig S5C, S5F, S5I). Considering the electronic influence on reactivity, we hypothesized next that the reactivity can be altered by varying the electrophilicity through variations in the R^2 moiety. Accordingly, we synthesized acrylamide molecules **8**, **9**, and **10** as the weak electronic acceptor analogs of **1**, **6**, and **7** respectively. Indeed, the acrylamides were found to be considerably slower than the corresponding acrylates (Fig 2B, 2C & Fig S5C, S5F, S5I).

Next, the influence of R^3 substitution was investigated using **11**-**13**, the reactivities of which were substantially lower compared to the unsubstituted olefin **1** (Fig 2B&2C, Fig S5D& S5G). In addition to the steric barrier for nucleophilic attack, the extended conjugation could also deactivate the double bond. In such a scenario, an electron-donating substituent should make the molecule less reactive. Indeed, we found the reactivity order here to be bromophenyl > phenyl > methoxylphenyl, for both thiol and amine reactions. Interestingly, only mono-addition product was observed for the latter, which also supports the steric-based inhibition of this reaction (Fig S8, S82, S83, S86, S87, S90, & S91). When substituted phenyl groups are incorporated onto the allylic position as the R^4 substitution, electron-withdrawing bromo moiety was found to accelerate the reaction (Fig 2B, 2C & Fig S5D, S5G). This observation was taken to suggest that the reaction goes through an enolate intermediate and the step involving the leaving group has an early transition state structure with a developing negative charge at the benzylic position.

Engineering the capability of trigger-to-reverse process to liberate original nucleophiles

Both reversible and irreversible conjugate reactions have implications and the preference depends on the specific applications.[31,43-45] In this context, we evaluated the structural factors that dictate reversibility of trigger-to-release products. Possible products from thiol and amine-based trigger-to-release reactions with **A1** molecules are shown in Fig 3A. Reversibility, i.e. the release of the originally conjugated nucleophile, was studied in the presence of second nucleophile by following the generation of original thiol and amine by time-dependent NMR measurement.

First, when **17** was treated with eight equivalents of ethanethiol (**T1**), generation of **18** is expected along with the released diethyleneglycolthiol (**T2**) (Fig 3B&3D). Interestingly, concurrent formation of other species **26**-**28** was also observed, which can be understood based on the Michael addition-elimination mechanism (Fig S9&S10). Solely, from the deconjugate perspective, 70% of the originally conjugated **T2** was released in this reaction. Lack of complete release of **T2** is attributed to its reaction with **18** to generate **26**. The fact that ethanethiol (**T1**) also reacts with **18** to form **28**, relative to **26**, suggests that the product distribution is simply determined by the ratio of **T1** and **T2** nucleophiles. This is understandable as the reaction is under equilibrium and this assertion was confirmed by decreasing the amount of **T1**, where an increase in the percentage of **26** was observed (Fig S11).

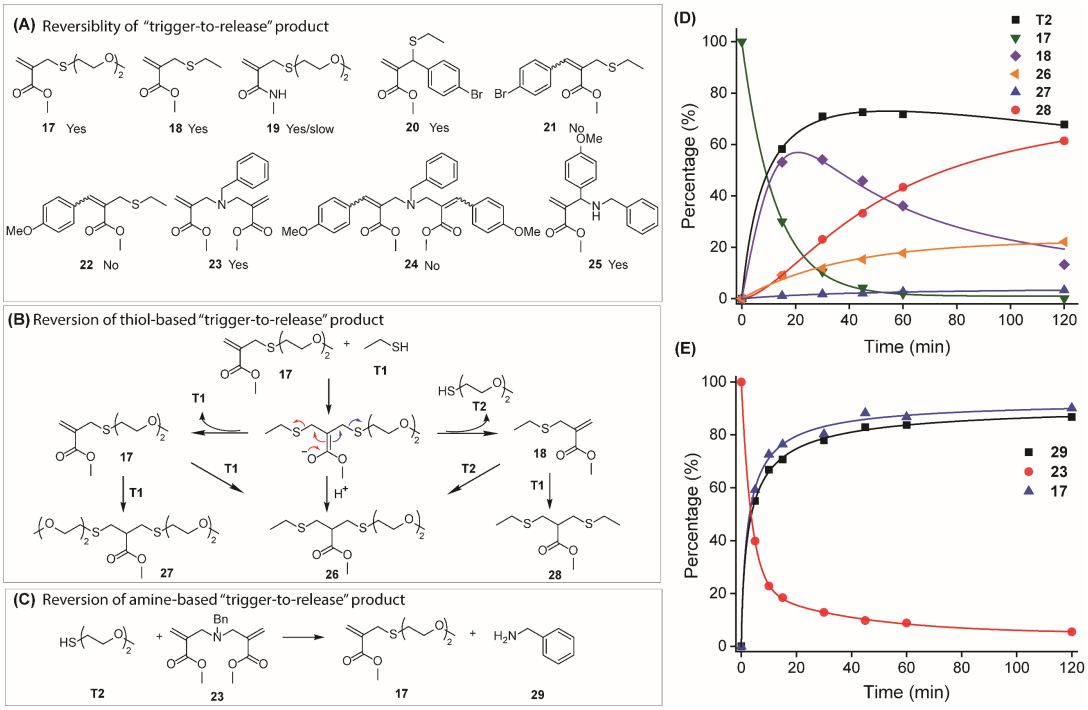

Fig 3. Customizing reversibility of trigger-to-release product. (A) Structures of thiol- and amine-based trigger-to-release product. (Occurrence of triggered reversion is indicated by "Yes"). (B) Triggered reversion of thiol-based trigger-to-release product, **17**. [**T1**] / [**17**] =8. (C) Triggered reversion of amine-based trigger-to-release product, **23**. [**T2**] / [**23**] =2. (D) Percentage of existing species in the course of trigger-to-reverse process of **17**. (E) Percentage of existing species in the course of trigger-to-reverse process of **23**.

Similarly, thiol triggered reversal of the amine-conjugated product, **23** was also investigated (Fig 3C). More than 80% of benzylamine (**29**) was released in the presence of just 2 equivalents of **T2** (Fig 3E & S12). The rather clean conversion, even with low amounts of thiol, is attributed to the lack of nucleophilicity of the protonated benzylamine at pH 7.4. Further studies on thiol-triggered reversal of other compounds show that **18**, **19**, **20**, and **25** can also be reversed to release the original thiol or amine (Fig S13-S16 & S20). Interestingly, though thiol-triggered deconjugate of **19** was observed, the reaction rate was very slow which can be explained by the decreased electrophilicity of the double bond due to the conjugation effect of amides. This inspired us to further take advantage of structural tunability of **A1** to engineer the reversibility of thiol/amine trigger-to-release reaction. We hypothesized that thiol-triggered reversal of the conjugate reaction will be profoundly suppressed by a phenyl-substitution on double bond, because the substitution not only reduces its electrophilicity, but also increases the steric hinderance of nucleophilic addition. To test this rationale, compounds **21**, **22**, and **24** were synthesized by conjugating ethane thiol or benzyl amine to corresponding **A1** molecules and were subjected to thiol-triggered deconjugate reaction. Indeed, **21**, **22**, and **24** were all inert to thiol and generation of original nucleophiles (ethane thiol or benzyl amine) was not observed (Fig S17-S19). Additionally, the trigger-to-reverse process is also promoted by polarity of reaction medium suggested by the observation of accelerated reaction in phosphate buffer than in pure MeOD (Fig S107, 108). Overall, the results above demonstrate that the capability for reversing both thiol and amine conjugate reactions can be conveniently engineered by simply varying the **A1** structure.

Chemoselective and reversible protein modification by trigger-to-release and trigger-to-reverse

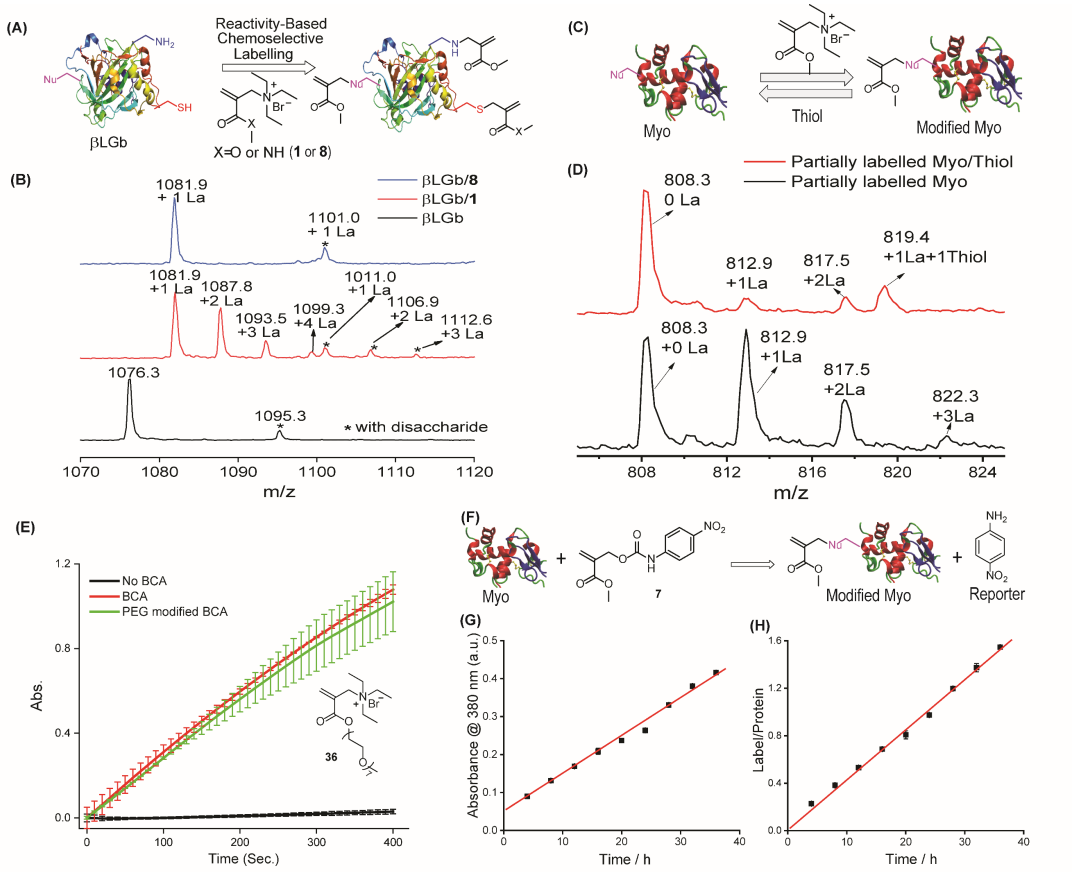

Fig 4. Protein modification with TMAc chemical switch. (A) Schematic illustration of cysteine-selective labeling of βLGb driven by reactivity difference between **1** and **8**. (B) Mass spectra of unlabeled βLGb (bottom) and βLGb labeled with **1**(middle), and **8** (top). Note: The spectra show only one charge state (+17) and the full spectra are shown in Fig S22. 1 La stands for 1 modification. Peak highlighted by * corresponds to βLGb with disaccharide PTM. (C) Schematic illustration of reversible modification of Myoglobin using compound **1**. (D) Mass spectra of myoglobin modified with **1** and further incubated with and without thiol. Note: One charge state is shown and spectra with all charge states shown in Fig S31. 1 La stands for 1 modification. (E) Influence of PEG modification using 36 on BCA activity evaluated by chromogenic assay. (F) Use the conjugate-to-release strategy to spectroscopically monitor modification of myoglobin by following absorbance of reporter, 4-nitroaniline. (G) 4-nitroaniline absorbance evolution during myoglobin modification with compound **7**. (H) The number of modifications on myoglobin corresponding to (G).

As demonstration of versatility, we investigated chemical modification of proteins as it provides a powerful tool for expanding their functions and profiling activity.[46] Here, we envision that structural tunability of TMAc can provide an opportunity to tailor its trigger-to-release reactivity in order to selectively react with thiol for cysteine-selective protein modification. Considering proteins' delicate nature, modification reactions must be fast and free of organic solvents. The ammonium version of **A1** is both reactive and water soluble and therefore was investigated for the modification. Specifically, acrylamide **8** exhibits a clearly distinguishable reactivity between thiols and amines in aqueous medium (Fig S21), and it is far less reactive than ester **1**. Both of molecules were tested for the modification of β-lactoglobulin B (βLGb), which has one cysteine and fifteen lysine residues. As illustrated in Fig 4A, we envisaged that modifications on multiple nucleophilic residues would occur with the highly reactive **1**, while **8** would selectively modify cysteine. βLGb was fully labelled within 5 and 40 minutes, when treated with excess of **1** and **8** respectively, as discerned from the disappearance of the unmodified protein peaks in ESI-MS (Fig 4B & S22). The mass shift in the concurrently appearing new peaks correspond to one to four modifications in βLGb, when treated with **1**. Multiple modifications suggest that **1** is non-selective in labeling the protein. In contrast, only a single new peak corresponding to the protein with one modification was found in βLGb labelled with **8**. To further pinpoint modification sites on βLGb, modified proteins were digested and subjected to MS/MS analysis (Fig S23-S30). Only the free cysteine site was modified with **8**. However, modifications of other nucleophilic residues including lysine, tyrosine and threonine were found with **1**. Interestingly, the primary amine of lysine was not double-labeled, as seen with small molecule nucleophiles. This is likely due to the competition from other nucleophilic residues.

As with the small molecules above, the reversibility of the covalent modification of the proteins was also tested, as this possibility has potential implications as "catch and release" strategy for protein purification or protein function manipulation. To test for this possibility, myoglobin (Myo) was modified with **1**, followed by incubation with 10 equivalents of thiol for 2 hours (Fig 4C). Multiple modifications were observed on myoglobin, when treated with **1** (Fig 4D & Fig S31). Modified proteins were subsequently reversed to unmodified myoglobin after incubating with a thiol, as shown by the intensity reduction of modified protein peaks accompanied by an increase in that of the unmodified protein peak. The extent of recovery is consistent with that observed in the small molecule study above. To assess the influence of the covalent modification on protein structures, Myo with ~3 modifications was investigated for changes CD spectroscopy, melting temperature and absorption spectrum of heme region (Fig S32&S33). The lack of any significant change in Myo's physical properties shows that the modification has negligible effect on protein structures. Similarly, PEG modified bovine carbonic anhydrase (BCA) (with ~3 modifications using **36**) does not exhibit any effect on its enzymatic activity (Fig 4E &S34).

Additionally, note that our method also offers the ability to simultaneously monitor protein labeling within this reaction format itself when the released leaving group is a reporter (Fig 4F). To test this idea, we tested the modification of Myo with **7**, where a nucleophile induced conjugate-to-release reaction would form 4-nitroaniline, the absorbance of which can report on the progress of protein modification. The correlation between the molecular release and the extent of protein modification is indeed clear (Fig 4G, H).

Utilization of TMAc as a universal self-immolative linker to uncage pre-installed functionalities The ability to attach a functional molecule to a scaffold and then readily recover it in the presence of a chemical trigger has implications, including in triggerable catalysis, sensing, cellular manipulation and cleavable traceless linker for prodrug design.[36, 38, 40, 47-51] In order to fully recognize this potential, the scope of the released functionalities from trigger-to-release process must be broad. We show here (Fig 5A & Fig S35-S44), for the first time, that a wide variety of functionalities including carboxylic acid, thiol, sulfonamide and alcohol can be released, in addition to 1°, 2°, 3°, and aromatic amines in response to a thiol trigger. Release of these molecules was quantitative, except with the thiol (~70%) and the alcohol (~20%). Considering the potential application of this strategy in triggerable drug release, we are fully aware that the model molecules investigated here could be too reactive and that the thiol reactive by-product can be an issue after drug release. However, note that the structure property relationship established on trigger-to-release reactivity and trigger-to-reverse capability offers us a useful guideline to design a less reactive linker that can generate an irreversible by-product. To further illustrate the scope of trigger-to-release approach, we incorporated a carboxylic acid-based drug (bexarotene) and a 2°-amine based drug molecule (desmethyltamoxifen) on to a DNA aptamer and a polymer respectively through a much less thiol-reactive acrylamide linker and investigated thiol-triggered release of these drugs (Fig 5B & Fig S49). Relevance of releasing drug molecule in response to a thiol trigger can be understood by the fact that reducing intracellular environment of most cells is enabled by the thiol functionality of the cysteine-bearing tripeptide, glutathione. The concentration of this peptide inside cells is about three orders of magnitude higher than that outside the cells. To check if thiol-triggered uncaging strategy can be translated to these prodrug molecules, both **33** and **34** were subjected to glutathione or diethylene glycol thiol treatment at relevant concentration. Thiol-triggered release of corresponding parent drugs from **33** and **34** was clearly evident (Fig S45-49) by the appearance of their signature peaks from NMR or mass spectrometry.

Next, azide-functionalized prodrug **34** was conjugated to AS1411 aptamer which is modified with a complementary reactive DBCO group through strain-promoted alkyne azide cycloaddition (SPAAC). The conjugation was characterized by mass spectrometry (Fig 5C). Successful conjugation of **34** to aptamer is supported by the observation of molecular weight shift of 729.84 which is identical to the molecular weight of **34**.

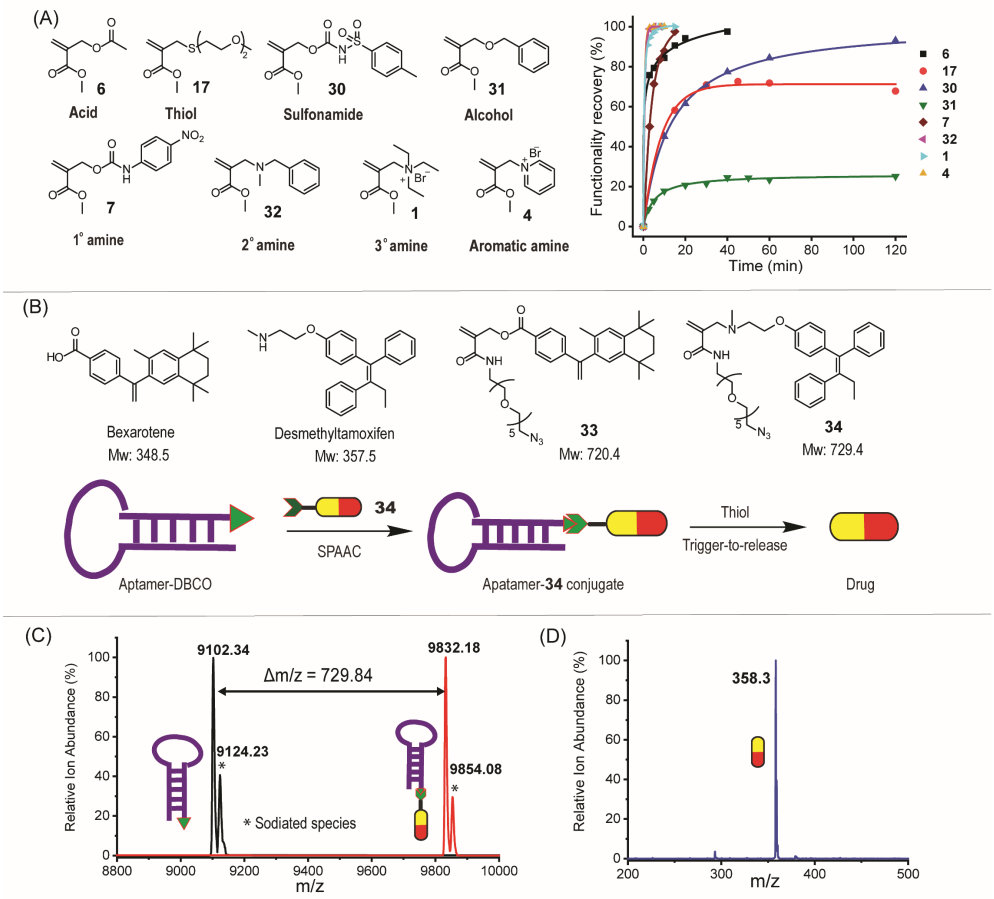

Fig 5. Trigger-to-release as a universal strategy for uncaging functionalities. (A) Functionalities can be masked and uncaged from the chemical switch (left) and efficiency of thiol-triggered recovery of these functionalities. (B) Caging bexarotene and desmethyltamoxifen with amide-based clickable TMAc (up) and preparation of aptamer-**34** conjugation and thiol-triggered release of desmethyltamoxifen from conjugate (bottom). (C) Mass spectrometry analysis of aptamer-DBCO (black) and aptamer-**34** conjugate (red). (D) Mass spectrometry analysis of released small molecule from aptamer-**34** conjugate.

Release of parent drug, desmethyltamoxifen, from aptamer-**34** conjugate in the presence of thiol was followed by LC-MS. Mass analysis (Fig 5D) shows that molecular weight of released small molecules is identical to that of desmethyltamoxifen suggesting the thiol-induced drug release from the conjugate. Using the same strategy, we also conjugated **33** to DBCO modified polyethylene glycol to form a polymer-drug conjugate, where the release of bexarotene molecules from the conjugate was also be triggered by thiol (Fig S49&S50). As a proof of concept study, we show here that the chemical switch can serve as a general platform to uncage drugs with different functionalities without linker chemistry redesign. Note that to make the system suitable for delivery applications, structural engineering on the linker is still required to achieve optimal release kinetics and on-target thiol-triggered release. The primary objective of this work is to simply illustrate the potential utility of the methodology developed here.

Orthogonally addressable hydrogel using TMAc chemical switch

Chemical orthogonality is often the key to achieving functional complexity in functional materials.[52-54] To demonstrate the potential impact of this chemistry on preparation of functional materials, we are utilizing both trigger-to-release and trigger-to-reverse processes to synthesize orthogonally addressable hydrogels (Fig 6A). By taking advantage of the distinct reactivity of the same alkene functionality at different reaction stages in "trigger-to-release" and "trigger-to-reverse" processes, chemical orthogonality which is otherwise usually achieved by additively introducing multiple functionalities can be easily accessed by a single functionality. Utilizing the fast reaction kinetics between thiols and an ammonium-based acceptor, hydrogels can be prepared within a minute by mixing two components, a thiol-functionalized 4-arm PEG and a bifunctional ammonium crosslinker, **37**. Formation of hydrogel network was evidenced by the dominant storage modulus (G') over loss modulus (G") (Fig 6B). Though the crosslinking converted the activated TMAc to a deactivated one, the population of alkene is always constant in hydrogel network. In coupling with tetrazole photo-click chemistry, these alkenes can be used as a handle for post-gelation functional engineering. Since the photo-click product offers a fluorescent chromophore, the reaction can be followed by absorption and fluorescence spectroscopy.[55] To test the feasibility of photo-click reactionbetween tetrazole and the deactivated Michael

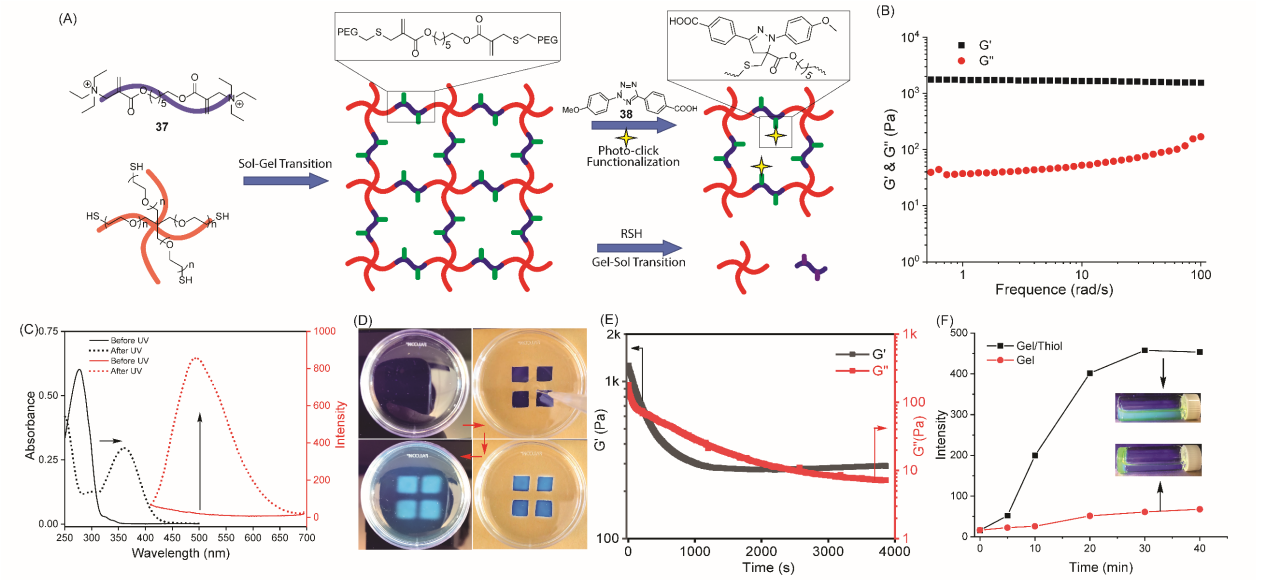

Fig 6. TMAc chemical switch for hydrogel preparation and orthogonal manipulation. (A) Illustration of hydrogel network formation and orthogonal manipulation on post-gelation properties. (B) Frequency sweep of storage modulus (G') and loss modulus (G") of pre-cured hydrogel. (C) Photo-click of tetrazole (**38**) with TMAc (**18)** followed by UV-Vis and fluorescent spectroscopy. (D) Images of photo-patterning hydrogel. Top left: Hydrogel before tetrazole addition; top right: Addition of tetrazole to hydrogel above pattern-masked UV lamp. Bottom right: Hydrogel with tetrazole exposed to UV irradiation for 2 minutes. Bottom left: Patterned hydrogel after removal of unreacted tetrazole. (E) In situ rheological measurement on thiol-triggered hydrogel dissolution. (F) Hydrogel for cargo encapsulation and thiol-triggered release measured by the evolution of fluorescence in the supernatant. Inset: corresponding photographs of hydrogel incubated with and without thiol.

acceptor after crosslinking, photo-reaction of tetrazole (**38**) and corresponding small molecule counterpart, **18** was investigated. Indeed, occurrence of reaction was observed by photoirradiation-induced distinct red-shift and fluorescence generation (Fig 6C). Further, photo-patterning on hydrogels can be conveniently accessed by simply irradiating the pattern-masked hydrogel with **38** for 2 minutes (Fig 6D). These results show that additional functionalities can be conveniently introduced into the hydrogel. Since we have shown that these conjugation reactions can be reversed, chemical reversal in the case of hydrogels should cause them to uncrosslink and dissolve away the gel. Addition of hexaethyleneglycol-monomethyl-ether thiol triggered the dissolution of hydrogel, causing a significant loss in both storage modulus (G', left Y-axis) from ~1400 Pa to ~300 Pa and loss modulus (G", right Y-axis) from ~200 Pa to ~8 Pa (Fig 6E). To further demonstrate the utility of such a process, we encapsulated bovine serum albumin (BSA) within these hydrogels during the formation of the gel. Dissolution of hydrogel caused the release of fluorescently-tagged BSA from the hydrogel incubated with thiol containing phosphate buffer. Increased fluorescence intensity in the liquid phase was observed suggesting entrapped BSA can rapidly diffuse from the gel phase to the liquid phase in the presence of thiol due to gel dissolution. In contrast, the diffusion of BSA from hydrogel to liquid phase was much slower when the gel was intact in the absence of thiol (Fig 6F&S51).

Conclusions

We have developed a simple and versatile triggerable Michael acceptor based molecular switch that concurrently possesses the key attributes of trigger-to-release and trigger-to-reverse reactions. Five different locations in a relatively small molecular volume have been utilized to achieve fine control over the reaction kinetics of both the trigger-to-release and trigger-to-reverse processes. Identification of the structural features that underlie the reaction kinetics, with respect to different nucleophiles, offer the opportunity to be useful in a variety of applications that requires efficient bonding and/or debonding features. We demonstrated this versatility in three different ways. By judiciously choosing the reactivity of the molecule, we have shown that this reaction can be used to either chemoselectively modify cysteines over other nucleophilic residues or indiscriminately label nucleophilic residues on protein surfaces. While the selectivity of the former has implications in site-specific labeling of proteins, the reactivity and reversibility of the latter has implications in proteomics and protein delivery applications. As a second demonstration of the fact that these can be incorporated onto a variety of scaffolds, we have introduced these functionalities as part of macromolecule-drug conjugates, using either a polymer or a DNA aptamer as the macromolecule. These conjugates are then triggered to release the drug molecules with high efficiency at biologically relevant thiol concentrations. Finally, we have utilized the chemistry to achieve reversible hydrogels that can be spatiotemporally addressed using the TMAc functionalities as the reactive handles. Overall, the versatility of the molecular switch, introduced here, is illustrated with the ability to bond

and debond with a variety of functional groups with tunable kinetics, selectivity and reversibility. Because of their tunable features, these reactions hold the promise of impacting a variety of areas ranging from biology to materials science.

Conflicts of interest

The authors declare no competing financial interests.

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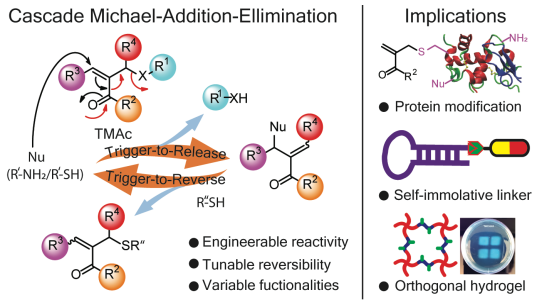
TOC



A triggerable Michael acceptor (TMAc) based chemical switch with programmable reactivity and reversibility for simultaneous coupling and decoupling has been developed for selective protein modification, self-immolative conjugates and orthogonally addressable hydrogel.