Stimuli-promoted *in situ* formation of hydrogels with thiol/thioester containing peptide precursors

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Statement of Authenticity

I, Makafui Yao Folikumah, formally submit my PhD dissertation entitled “Stimuli-promoted in-situ formation of hydrogels with thiol/thioester containing peptide precursors” to the Institute of Chemistry of the Faculty of Mathematics and Natural Sciences at the University of Potsdam in Germany for acquirement of the academic degree of Doctor of Natural Sciences (Dr. rer. nat.) in the Department of Chemistry.

I hereby declare that the work presented in this dissertation is my own original work based on the research carried out at Helmholtz-Zentrum Hereon, Institute of Active Polymers in Teltow, Germany, from March 2017 to May 2022. This dissertation was started under the supervision of Prof. Dr. Andreas Lendlein and finalised under the supervision of Dr. Axel T. Neffe. To the best of my knowledge and belief, this dissertation does not contain any work previously published or written by another person, except where due reference is made in it. No portion of this work has been previously submitted in the support of any other degree to another university or institute. Any contribution made by others to this research is explicitly acknowledged in the dissertation.

Makafui Y. Folikumah

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Abstract

Hydrogels are potential synthetic ECM-like substitutes since they provide functional and structural similarities compared to soft tissues. They can be prepared by crosslinking of macromolecules or by polymerizing suitable precursors. The crosslinks are not necessarily covalent bonds, but could also be formed by physical interactions such as π-π interactions, hydrophobic interactions, or H-bonding. On demand *in situ* forming hydrogels have garnered increased interest especially for biomedical applications over preformed gels due to the relative ease of *in vivo* delivery and filling of cavities. The thiol-Michael addition reaction provides a straightforward and robust strategy for *in situ* gel formation with its fast reaction kinetics and ability to proceed under physiological conditions. The incorporation of a trigger function into a crosslinking system becomes even more interesting since gelling can be controlled with stimulus of choice. The use of small molar mass crosslinker precursors with active groups orthogonal to thiol-Michael reaction type electrophile provides the opportunity to implement an on-demand *in situ* crosslinking without compromising the fast reaction kinetics.

It was postulated that short peptide sequences due to the broad range structural-function relations available with the different constituent amino acids, can be exploited for the realisation of stimuli-promoted *in situ* covalent crosslinking and gelation applications. The advantages of this system over conventional polymer-polymer hydrogel systems are the ability tune and predict material property at the molecular level.

The main aim of this work was to develop a simplified and biologically friendly stimuli-promoted *in situ* crosslinking and hydrogelation system using peptide mimetics as latent crosslinkers. The approach aims at using a single thiodepsipeptide sequence to achieve separate pH- and enzyme-promoted gelation systems with little modification to the thiodepsipeptide sequence. The realization of this aim required the completion of three milestones.

In the first place, after deciding on the thiol-Michael reaction as an effective *in situ* crosslinking strategy, a thiodepsipeptide, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) with expected propensity towards pH-dependent thiol-thioester exchange (TTE) activation, was proposed as a suitable crosslinker precursor for pH-promoted gelation system. Prior to the synthesis of the proposed peptide-mimetic, knowledge of the thiol-Michael reactivity of the would-be activated thiol moiety SH-Leu, which is internally embedded in the thiodepsipeptide was required. In line with *p*Ka requirements for a successful TTE, the reactivity of a more acidic thiol, SH-Phe was also investigated to aid the selection of the best thiol to be incorporated in the thioester bearing peptide based crosslinker precursor. Using 'pseudo' 2D-NMR investigations, it was found that only reactions involving SH-Leu yielded the expected thiol-Michael product, an observation that was attributed to the steric hindrance of the bulkier nature of SH-Phe. The fast reaction rates and complete acrylate/maleimide conversion obtained with SH-Leu at pH 7.2 and higher aided the direct elimination of SH-Phe as a potential thiol for the synthesis of the peptide mimetic.

Based on the initial studies, for the pH-promoted gelation system, the proposed Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH was kept unmodified. The subtle difference in *p*Ka values between SH-Leu (thioester thiol) and the terminal cysteamine thiol from theoretical conditions should be enough to effect a 'pseudo' intramolecular TTE. In polar protic solvents and under basic aqueous conditions, TDP successfully undergoes a 'pseudo' intramolecular TTE reaction to yield an α,ω-dithiol tripeptide, HSLeu-Leu-Gly-NEtSH. The pH dependence of thiolate ion generation by the cysteamine thiol aided the incorporation of the needed stimulus (pH) for the overall success of TTE (activation step) – thiol-Michael addition (crosslinking) strategy.

Secondly, with potential biomedical applications in focus, the susceptibility of TDP, like other thioesters, to intermolecular TTE reaction was probed with a group of thiols of varying thiol *p*Ka values, since biological milieu characteristically contain peptide/protein thiols. L-cysteine,

which is a biologically relevant thiol, and a small molecular weight thiol, methylthioglycolate both with relatively similar thiol *p*Ka, values, led to an increase concentration of the dithiol crosslinker when reacted with TDP. In the presence of acidic thiols (*p*-NTP and 4MBA), a decrease in the dithiol concentration was observed, an observation that can be attributed to the inability of the TTE tetrahedral intermediate to dissociate into exchange products and is in line with *p*Ka requirements for successful TTE reaction. These results additionally makes TDP more attractive and the potentially the first crosslinker precursor for applications in biologically relevant media.

Finally, the ability of TDP to promote pH-sensitive *in situ* gel formation was probed with maleimide functionalized 4-arm polyethylene glycol polymers in tris-buffered media of varying pHs. When a 1:1 thiol: maleimide molar ratio was used, TDP-PEG4MAL hydrogels formed within 3, 12 and 24 hours at pH values of 8.5, 8.0 and 7.5 respectively. However, gelation times of 3, 5 and 30 mins were observed for the same pH trend when the thiol: maleimide molar was increased to 2:1.

A direct correlation of thiol content with *G'* of the gels at each pH could also be drawn by comparing gels with thiol: maleimide ratios of 1:1 to those with 2:1 thiol: maleimide mole ratios. This is supported by the fact that the storage modulus (*G'*) is linearly dependent on the crosslinking density of the polymer. The values of initial *G'* for all gels ranged between (200 – 5000 Pa), which falls in the range of elasticities of certain tissue microenvironments for example brain tissue 200 – 1000 Pa and adipose tissue (2500 – 3500 Pa).

Knowledge so far gained from the study on the ability to design and tune the exchange reaction of thioester containing peptide mimetic will give those working in the field further insight into the development of new sequences tailored towards specific applications.

TTE substrate design using peptide mimetic as presented in this work has revealed interesting new insights considering the state-of-the-art. Using the results obtained as reference, the

strategy provides a possibility to extend the concept to the controlled delivery of active molecules needed for other robust and high yielding crosslinking reactions for biomedical applications. Application for this sequentially coupled functional system could be seen e.g. in the treatment of inflamed tissues associated with urinary tract like bladder infections for which pH levels above 7 were reported. By the inclusion of cell adhesion peptide motifs, the hydrogel network formed at this pH could act as a new support layer for the healing of damage epithelium as shown in interfacial gel formation experiments using TDP and PEG4MAL droplets.

The versatility of the thiodepsipeptide sequence, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-(TDP_o) was extended for the design and synthesis of a MMP-sensitive 4-arm PEG-TDP_o conjugate. The purported cleavage of TDP_o at the Gly-SLeu bond yields active thiol units for subsequent reaction of orthogonal Michael acceptor moieties. One of the advantages of stimuli-promoted *in situ* crosslinking systems using short peptides should be the ease of design of required peptide molecules due to the predictability of peptide functions their sequence structure. Consequently the functionalisation of a 4-arm PEG core with the collagenase active TDP_o sequence yielded an MMP-sensitive 4-arm thiodepsipeptide-PEG conjugate ($PEG4TDP_o$) substrate.

Cleavage studies using thiol flourometric assay in the presence of MMPs -2 and -9 confirmed the susceptibility of $PEG4TDP_o$ towards these enzymes. The resulting time-dependent increase in fluorescence intensity in the presence of thiol assay signifies the successful cleavage of TDP_o at the Gly-SLeu bond as expected. It was observed that the cleavage studies with thiol flourometric assay introduces a sigmoid non-Michaelis-Menten type kinetic profile, hence making it difficult to accurately determine the enzyme cycling parameters, k_{cat} and K_M .

Gelation studies with PEG4MAL at 10 % wt. concentrations revealed faster gelation with MMP-2 than MMP-9 with 28 and 40 min gelation times respectively. Possible contributions

by hydrolytic cleavage of PEG4TDP$_o$ has resulted in the gelation of PEG4MAL blank samples but only after 60 minutes of reaction. From theoretical considerations, the simultaneous gelation reaction would be expected to more negatively impact the enzymatic than hydrolytic cleavage. The exact contributions from hydrolytic cleavage of PEG4TDP$_o$ would however require additional studies.

In summary this new and simplified *in situ* crosslinking system using peptide-based crosslinker precursors with tuneable properties exhibited *in situ* crosslinking gelation kinetics on similar levels with already active dithiols reported. The advantageous on-demand functionality associated with its pH-sensitivity and physiological compatibility makes it a strong candidate worth further research as biomedical applications in general and on-demand material synthesis is concerned.

Results from MMP-promoted gelation system unveils a simple but unexplored approach for *in situ* synthesis of covalently crosslinked soft materials, that could lead to the development of an alternative pathway in addressing cancer metastasis by making use of MMP overexpression as a trigger. This goal has so far not being reach with MMP inhibitors despite the extensive work this regard.

Zusammenfassung

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Hydrogele sind synthetische, potenziell ECM-ähnliche Substituenten, die funktionelle und strukturelle Ähnlichkeiten mit Weichteilgeweben aufweisen. Sie können durch Vernetzung von Makromolekülen oder durch Polymerisation geeigneter Precursoren hergestellt werden. Die Vernetzungen müssen nicht unbedingt aus kovalenten Bindungen bestehen, sondern können auch durch physikalische Wechselwirkungen wie π-π-Wechselwirkungen, hydrophoben Wechselwirkungen oder Wasserstoff-Brückenbindungen entstehen. *In-situ*-Hydrogele, die *on-demand* gebildet werden, haben vor allem für biomedizinische Anwendungen gegenüber vorgefertigten Gelen zunehmend an Interesse gewonnen, da sie relativ einfach *in-vivo* eingebracht und somit Fehlstellen gefüllt werden können. Die Thiol-Michael-Additionsreaktion bietet mit ihrer schnellen Reaktionskinetik und ihrer Fähigkeit, unter physiologischen Bedingungen abzulaufen, eine unkomplizierte und robuste Strategie für die *in-situ*-Gelbildung. Der Einbau einer Triggerfunktion in ein Vernetzungssystem ist besonders interessant, da die Gelierung durch einen gewählten Stimulus gesteuert werden kann. Die Verwendung eines Precursors mit geringer Molmasse und aktiven Gruppen, die orthogonal zu den Elektrophilen des Thiol-Michael-Reaktionstyps sind, bietet die Möglichkeit, eine bedarfsgesteuerte *in-situ*-Vernetzung zu realisieren, ohne die schnelle Reaktionskinetik zu beeinträchtigen.

Es wurde postuliert, dass kurze Peptidsequenzen aufgrund der weitreichenden Struktur-Funktions-Beziehungen, die mit den verschiedenen konstituierenden Aminosäuren zur Verfügung stehen, für die Realisierung von Stimulus-ausgelösten, *in-situ* kovalenten Vernetzungs- und Gelierungsanwendungen genutzt werden können. Die Vorteile dieses Systems gegenüber herkömmlichen Polymer-Polymer-Hydrogelsystemen liegen in der Möglichkeit, die Materialeigenschaften auf molekularer Ebene zu justieren und vorherzusagen.

Das Hauptziel dieser Arbeit war die Entwicklung eines vereinfachten und biologisch-geeigneten, stimulierungsgeförderten *in-situ*-Vernetzungs- und Hydrogelierungssystems unter Verwendung von Peptidmimetika als latente Vernetzer. Der Ansatz zielt darauf ab, eine einzige Thiodepsipeptidsequenz zu verwenden, um getrennte pH- und enzymausgelöste Gelierungssysteme mit geringen Modifikationen der Thiodepsipeptidsequenz zu erreichen. Zur Verwirklichung dieses Ziels, mussten drei Meilensteine erreicht werden.

Nach der Wahl der Thiol-Michael-Reaktion als *in-situ*-Vernetzungsstrategie, musste ein Thiodepsipeptid, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) mit einer zu erwartenden Neigung zu einer pH-abhängigen Thiol-Thioester-Austausch-Aktivierung (TTE), als geeignetem Vernetzer-Precursor für das pH-unterstützte Gelierungssystem designt werden. Vor der Synthese dieses Peptid-Mimetikums war die Untersuchung der Thiol-Michael-Reaktivität der potenziell aktivierten Thiolkomponente SH-Leu erforderlich, die intern in das Thiodepsipeptid eingebettet ist. In Übereinstimmung mit den pKa-Anforderungen für eine erfolgreiche TTE wurde auch die Reaktivität eines saureren Thiols, SH-Phe, untersucht, um die Auswahl des besten Thiols zu ermöglichen, das in den Thioester-tragenden Peptid-basierten Vernetzer-Precursor eingebaut werden sollte. Pseudo-2D-NMR-Untersuchungen zeigten, dass nur Reaktionen mit SH-Leu das erwartete Thiol-Michael-Produkt ergaben, eine Beobachtung, die auf die sterische Hinderung durch die sperrige Natur von SH-Phe zurückzuführen ist. Wegen der schnellen Reaktionsgeschwindigkeiten und der vollständigen Acrylat/Maleimid-Umwandlung, die mit SH-Leu bei einem pH-Wert von 7,2 und höher erzielt wurde, kam SH-Phe als potenzielles Thiol für die Synthese des Peptidmimetikums nicht mehr infrage.

Auf der Grundlage der ersten Studien wurde für das pH-basierte Gelierungssystem das vorgeschlagene Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH unverändert beibehalten. Der geringe Unterschied in den pKa-Werten zwischen SH-Leu (Thioesterthiol) und dem terminalen Cysteaminthiol sollte ausreichen, um eine "pseudo"-intramolekulare TTE zu bewirken. In

polaren protischen Lösungsmitteln und unter basischen wässrigen Bedingungen verläuft die "pseudo"-intramolekulare TTE-Reaktion bei TDP erfolgreich, bei der ein α,ω-Dithiol-Tripeptid, HSLeu-Leu-Gly-NEtSH, entsteht. Die pH-Abhängigkeit der Thiolat-Ionen-Generierung durch das Cysteamin-Thiol trug dazu bei, den notwendigen Stimulus (pH) für den Gesamterfolg der TTE (Aktivierungsschritt) - Thiol-Michael-Addition (Vernetzung) Strategie einzubauen.

Zweitens wurde mit Blick auf potenzielle biomedizinische Anwendungen die Empfindlichkeit von TDP, wie auch anderer Thioester, für die intermolekulare TTE-Reaktion mit einer Gruppe von Thiolen mit unterschiedlichen Thiol-pKa-Werten untersucht, da biologische Milieus typischerweise Peptid-/Proteinthiole enthalten. L-Cystein, ein biologisch relevantes Thiol, und ein Thiol mit geringem Molekulargewicht, Methylthioglykolat, die beide relativ ähnliche Thiol-pKa-Werte besitzen, führten bei der Reaktion mit TDP zu einer erhöhten Konzentration des Dithiol-Vernetzers. In Gegenwart von sauren Thiolen (p-NTP und 4MBA) wurde eine Abnahme der Dithiolkonzentration beobachtet, eine Beobachtung, die auf die Unfähigkeit des tetraedrischen TTE-Zwischenprodukts in Austauschprodukte zu dissoziieren zurückgeführt werden kann und mit den pKa-Anforderungen für eine erfolgreiche TTE-Reaktion in Einklang steht. Diese Ergebnisse machen TDP noch attraktiver und zum potenziell ersten Vernetzer-Precursor für Anwendungen in biologisch relevanten Medien.

Schließlich wurde die Fähigkeit von TDP, die pH-empfindliche *in-situ*-Gelbildung zu fördern, mit Maleimid-funktionalisierten 4-armigen Polyethylenglykolpolymeren in tris-gepufferten Medien mit unterschiedlichen pH-Werten untersucht. Bei einem Molverhältnis von 1:1 Thiol zu Maleimid bildeten sich TDP-PEG4MAL-Hydrogele innerhalb von 3, 12 und 24 Stunden bei pH-Werten von 8,5, 8,0 bzw. 7,5. Bei einer Erhöhung des Molverhältnisses von Thiol zu Maleimid auf 2:1 wurden jedoch Gelierzeiten von 3, 5 und 30 Minuten für denselben pH-Trend beobachtet.

Ein direkter Zusammenhang zwischen dem Thiolgehalt und G' der Gele bei jedem pH-Wert konnte auch durch den Vergleich von Gelen mit einem Thiol/Maleimid-Molverhältnis von 1:1 mit solchen mit einem Thiol/Maleimid-Molverhältnis von 2:1 hergestellt werden. Dies wird durch die Tatsache unterstützt, dass der Speichermodulus (G') linear von der Vernetzungsdichte des Polymers abhängig ist. Die Werte des anfänglichen G′ für alle Gele lagen bei 200 - 5000 Pa, was in den Bereich der Elastizitäten bestimmter Gewebe-Mikroumgebungen fällt, z. B. Gehirngewebe (200 - 1000 Pa) und Fettgewebe (2500 - 3500 Pa).

Die bisher aus der Studie gewonnenen Erkenntnisse über die Möglichkeit, die Austauschreaktion von Thioester-haltigen Peptidmimetika zu entwerfen und abzustimmen, geben weitere Einblicke in die Entwicklung neuer, auf spezifische Anwendungen zugeschnittener Sequenzen.

Das Design von TTE-Substraten unter Verwendung von Peptidmimetika, wie es in dieser Arbeit vorgestellt wurde, hat interessante neue Erkenntnisse im Hinblick auf den Stand der Technik gebracht. Mit den erzielten Ergebnissen als Basis bietet die Strategie die Möglichkeit, das Konzept auf die kontrollierte Freisetzung aktiver Moleküle zu erweitern, die für andere robuste Vernetzungsreaktionen mit hohem Umsatz für biomedizinische Anwendungen benötigt werden. Dieses sequentiell gekoppelte funktionelle System könnte z. B. bei der Behandlung von entzündetem Gewebe im Zusammenhang mit Harnwegsinfektionen wie Blasenentzündungen eingesetzt werden, für die pH-Werte über 7 berichtet wurden. Durch die Einbeziehung von Zelladhäsionspeptidmotiven könnte das bei diesem pH-Wert gebildete Hydrogelnetz als neue Stützschicht für die Heilung von geschädigtem Epithel fungieren, wie in Experimenten zur Bildung von Grenzflächengelen mit TDP- und PEG4MAL-Tropfen gezeigt wurde.

Die Vielseitigkeit der Thiodepsipeptidsequenz Ac-Pro-Leu-Gly-SLeu-Leu-Gly-(TDP_o) wurde um Design und die Synthese eines MMP-empfindlichen 4-armigen PEG-TDP_o-Konjugats erweitert. Die beabsichtigte Spaltung von TDP_o an der Gly-SLeu-Bindung liefert aktive Thiol-Einheiten für die anschließende Reaktion orthogonaler Michael-Akzeptor-Einheiten. Einer der Vorteile stimulierungsgestützter *in-situ*-Vernetzungssysteme unter Verwendung kurzer Peptide dürfte darin liegen, dass sich die erforderlichen Peptidmoleküle aufgrund der Vorhersagbarkeit der Peptidfunktionen und ihrer Sequenzstruktur leicht entwerfen lassen. Die Funktionalisierung eines vierarmigen PEG-Kerns mit der kollagenaseaktiven TDP_o-Sequenz führte zu einem MMP-empfindlichen vierarmigen Thiodepsipeptid-PEG-Konjugat ($PEG4TDP_o$).

Spaltungs-Studien unter Verwendung eines thiolfluorimetrischen Assays in Gegenwart der MMPs -2 und -9 bestätigten die Spaltbarkeit von $PEG4TDP_o$ durch diese Enzyme. Der daraus resultierende zeitabhängige Anstieg der Fluoreszenzintensität in Anwesenheit des Thiol-Assays deutet auf die erfolgreiche Spaltung von TDP_o an der Gly-SLeu-Bindung hin. Es wurde festgestellt, dass die Spaltungsstudien mit dem thiol-fluorimetrischen Assay ein sigmoides, nicht-Michaelis-Menten-artiges kinetisches Profil ergeben, was eine genaue Bestimmung der Enzymzyklusparameter, k_{cat} und K_M, erschwert.

Gelierungsstudien mit PEG4MAL in einer Konzentration von 10 Gew.-% ergaben eine schnellere Gelierung mit MMP-2 als mit MMP-9 mit Gelierungszeiten von 28 bzw. 40 Minuten. Eventuelle Beiträge durch hydrolytische Spaltung von $PEG4TDP_o$ an der Gelierung wurden an PEG4MAL-Blindproben untersucht und führten erst nach 60 Minuten Reaktionszeit zu einer Gelierung. Aus theoretischen Überlegungen heraus wäre zu erwarten, dass sich die gleichzeitige Gelierungsreaktion negativer auf die enzymatische als auf die hydrolytische Spaltung auswirkt. Genaues zum Beitrag der hydrolytischen Spaltung von $PEG4TDP_o$ bedürfte jedoch weiterer Untersuchungen.

Zusammenfassend lässt sich sagen, dass dieses neue *in-situ*-Vernetzungssystem, bei dem Peptid-basierte Vernetzungs-Precursor mit einstellbaren Eigenschaften verwendet werden, eine *in-situ*-Vernetzungs-Gelierungskinetik auf ähnlichem Niveau wie bei bereits berichteten aktiven Dithiole aufweist. Die vorteilhafte *On-Demand*-Funktionalität in Verbindung mit ihrer pH-Sensitivität und physiologischen Verträglichkeit macht sie zu einem interessanten Kandidaten für weitere Forschungen im Bereich biomedizinischer Anwendungen im Allgemeinen und der *On-Demand*-Materialsynthese.

Die Ergebnisse des MMP-geförderten Gelierungssystems weisen einen einfachen, aber unerforschten Ansatz für die *in-situ*-Synthese kovalent vernetzter weicher Materialien, der zur Entwicklung eines alternativen Weges zur Bekämpfung der Krebsmetastasierung führen könnte, indem er die MMP-Überexpression als Auslöser nutzt. Mit MMP-Inhibitoren wurde dieses Ziel trotz umfangreicher Arbeiten in dieser Hinsicht bisher nicht erreicht.

Abbreviations and Symbols

4MBA	4-mercaptobenzoic acid
AChE	Acetylcholinesterase
COSY	Homonuclear Correlation Spectroscopy
CuAAC	Copper catalysed Azide Alkyne Cycloaddition
ECM	Extracellular matrix
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ESI-MS	Electrospray ionisation mass spectrometry
EWG	Electron withdrawing group
FT-IR	Fourier Transform-Infrared Spectroscopy
G'	Storage modulus
G''	Loss modulus
G_e	Equilibrium modulus
GSH	*L*-glutathione
HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HSQC	Heteronuclear Single Quantum Coherence Experiment
LCYS	*L*-cysteine
M_C	Molar mass between crosslinks
MeTGC	Methylthioglycolate
NCL	Native chemical ligation
NMR	Nuclear magnetic resonance spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
PEG4AC	Acrylate functionalized 4-arm polyethylene glycol

PEG4MAL	Maleimide functionalized 4-arm polyethylene glycol
PEG4TDP	TDP functionalized 4 –arm polyethylene glycol
PEG4VS	Vinylsulfone functionalized 4-arm polyethylene glycol
PNIPAM	poly (*N*-isopropylacrylamide)
p-NTP	*Para*-nitrothiophenol
q	Crosslink density
Q_M	Degree of swelling
$S \rightarrow N$	Sulfur to nitrogen acyl transfer
SH-Leu	Thio-leucine ((*S*)-2-mercapto-4-methylpentanoic acid)
SH-Phe	Thio-phenylalanine ((*S*)-2-mercapto-3-phenylpropanoic acid)
SPAAC	Strain-promoted azide alkyne click reaction
TCEP	Tris (2-carboxyethyl) phosphine
TDP	Thiodepsipeptide (Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH)
TDP_o	Thiodepsipeptide (Ac-Pro-Leu-Gly-SLeu-Leu-Gly-)
TEA	Triethanolamine
TOCSY	Total Correlation Spectroscopy
TTE	Thiol-thioester exchange reaction
η^*	Complex viscosity

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1 Chapter 1 - Introduction

Natural and synthetic hydrophilic macromolecules or polymer precursors with their concomitant advantages and disadvantages are usually crosslinked physically or chemically to produce hydrogels for various applications. Hydrogels derived from natural sources and ECM components such as hyaluronic acid,[1, 2] gelatin[3-5] and chitosan[6, 7] are typically associated with being inherently biocompatible and bioactive[8] and hence promote cellular functions. Such hydrogels are however complex in nature and mostly not well defined, which hinders the tuning of their material properties.[9] Purely synthetic or non-natural based hydrogels based on poly (ethylene glycol),[10, 11] poly (vinyl alcohol) poly (2-hydroxy ethyl methacrylate) have therefore become alternatives and are currently employed in a number of applications. The specific application or need of a hydrogel determines the method employed in its synthesis. Until recently, hydrogels for biomedical applications like drug delivery were usually formed outside the body in the presence of target specific drugs/proteins and later administered normally via tedious medical procedures. A recent class of hydrogels that addresses the disadvantages associated with preformed hydrogels are the *in situ* forming or injectable hydrogels. For *in situ* forming gels, polymer solutions are injected into the body in liquid form and converted into a solid hydrogel using physical and chemical crosslinking agents.[12-17] The main criteria for hydrogel classification either preformed or injectable, is the type of crosslinking strategy used. Hydrogels are formed via two main crosslinking strategies, namely physical crosslinking and chemical crosslinking. Whilst physical crosslinking makes use of non-covalent interactions such as π- π interactions,[18] hydrophobic interactions or H-bonding,[19] chemically crosslinked gels use conventional chemical reactions to form chemical net points or covalent bonds.[1, 20] Figure 1 shows the summarized classification of hydrogels according to the crosslinking method, synthesis and applications.

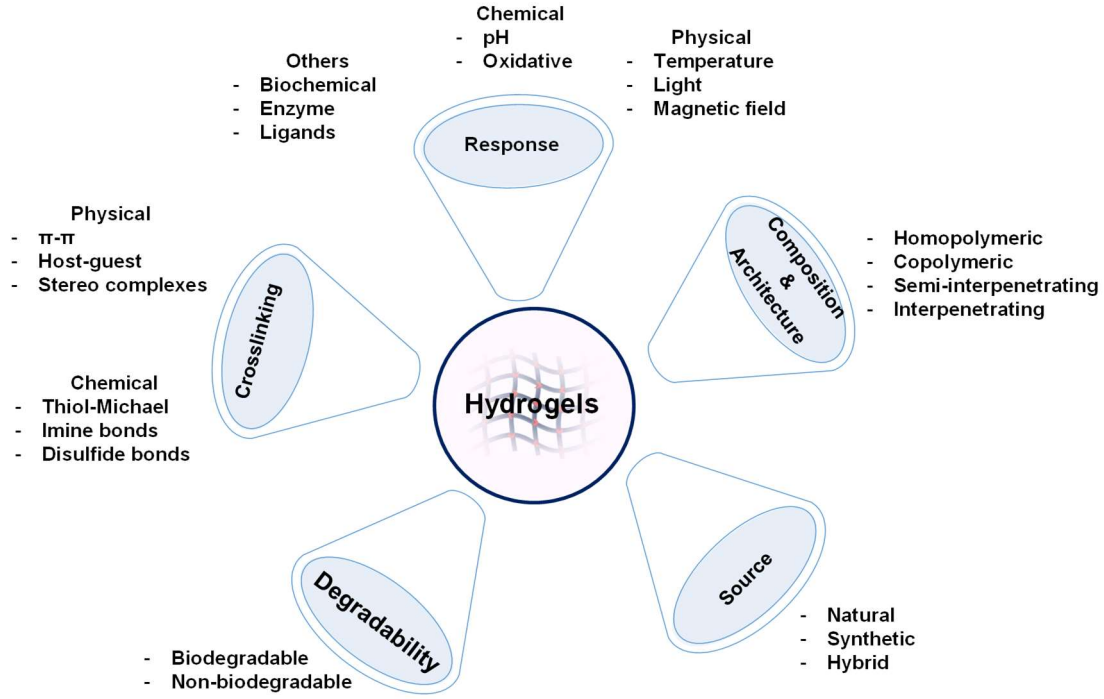


Figure 1: Classification of hydrogels according to principal crosslinking interaction, synthesis and applications

1.1 Physically crosslinked hydrogels

Physical crosslinking as the name implies, makes use of non-covalent (hydrophobic, ionic/charge, π-π, hydrogen bonding) interactions in combination with certain stimuli such as changes in temperature, pH, and ionic strength to crosslink precursors.

Hydrogels formed via hydrophobic interactions are mainly from amphiphilic polymers whose hydrophobic domains aggregate to serve as crosslinking sites in aqueous environments. The lower solubility of the polymer at higher temperatures in aqueous media leads to the aggregation of the hydrophobic domains in order to minimize the surface area in contact with the water.[21] This phenomenon is exploited in the design of temperature sensitive/responsive hydrogels. The gelation temperature- the temperature at which the gel is formed can be tuned by carefully controlling factors such as the concentration and chemical structure of the polymer as well as the length of the hydrophobic block or domain[21]. Triblock co-polymers of (PEO-

PPO-PEO)[22, 23] also known as the pluronics, PNIPAM[24, 25] and PEG-PCL[13, 26, 27] are some of the most extensively studied thermally gelling polymers.

Hydrogels based on supramolecular interactions makes use of specific molecular recognition motifs.[21] Inclusion complexes involving large three-dimensional molecules such as cyclodextrin with natural or synthetic polymers[28, 29] are notable examples.

Hydrogels based on blends of natural polymers such as hyaluronic acid- methylcellulose[15] and gelatin-agar[30] have been prepared via H-bonding interactions. The bonded networks are however susceptible to be weakened easily *in vivo* due to water influx thereby limiting these hydrogels to short time applications such as those requiring rapid release of drugs.[21]

1.2 Covalently crosslinked hydrogels

Albeit the numerous advantages of physical crosslinking strategies in hydrogel synthesis, they are heavily laden with some limitations. One major challenge in physically crosslinked hydrogels is the difficulty to decouple variables such as gelation time, polymer network mesh size, and degradation time.[31] Physically crosslinked hydrogels also exhibit poor tissue dwell times due to dilution followed by degradation/dissipation.[31] Covalent cross-linking on the other hand prevents both dilution and possible diffusion of the of the hydrogel matrix from the site of application.

Aldol and Schiff-base type reactions, Michael addition, Diels-Alder and copper-catalysed or copper-free azide-alkyne click reactions as well as some enzyme-catalysed reactions are some of the commonly used approaches strategies due their selectivity and compatibility especially in biological environments.

These reactions can be carried out with either functionalized orthogonal small molecule-polymer or polymer-polymer systems depending largely on the ease of synthesis. In Schiff-base type crosslinks, which exploit the reactivity of amines and carbonyl moieties

(aldehydes/ketones), small-molecule crosslinkers, are usually employed. Typically, amine-functionalized synthetic or natural polymer precursors are used with aldehydic moieties including but not limited to formaldehyde, glyoxal, glutaraldehyde[32, 33] and genipin.[34-36] Apart from genipin, the inherent cytotoxicity and difficulty in handling these crosslinkers makes them undesirable for the synthesis of hydrogels especially for biomedical applications thereby requiring the need to develop other efficient alternatives.

Polymer-polymer crosslinking requires the use of pre-functionalized polymers thereby avoiding the use of potentially toxic small-molecules although significant modification of these polymers is required to make this approach practical.[21] The versatility and wide scope of such reactions like the CuAAC, Michael addition and Diels-Alder reactions make them highly applicable for polymer-polymer crosslinking. CuAAC,[1, 37, 38] thiol Michael addition[39-43] and Diels-Alder reaction promoted[44] hydrogels and many more have been reported.

1.3 Characterization of hydrogels

The physical structure of hydrogels is commonly characterized with parameters such as the storage modulus (G'), loss modulus (G'') and equilibrium modulus (G_e) which can be measured via standard rheological measurements.[45] Additional parameters for hydrogel characterization include the degree of swelling (Q_M), polymer volume fraction in the swollen state, $v_{2,s}$, the molar mass between crosslinks, M_c and the mesh size of the hydrogel, ξ which reflects the porosity of the gel. These parameters can be used to model the behaviour of both nonionic[46] and ionic[47] hydrogels through equilibrium swelling theory and rubber elasticity theory.[48] The degree of swelling based on the hydrogel mass (Q_M) is determined using Equation 1.

$$Q_M = \frac{W_t - W_o}{W_o} \qquad (1)$$

where W_t is the hydrogel mass after swelling and W_o is the hydrogel mass before swelling. Experimental determination of Q_M enables the calculation of the volume-swelling ratio (Q_V) according to Equation 2:

$$Q_V = 1 + \frac{\rho_p}{\rho_s}(Q_M - 1) \qquad (2)$$

where ρ_p is the density of the dry hydrogel (1.12 g·cm^{-3} for PEG) and ρ_s is the density of the solvent (1 g·cm^{-3} for water). The molar mass between crosslinks (M_c) and crosslink density (q) can be calculated using equations 3 and 4, respectively.

$$\frac{1}{\overline{M_c}} = \frac{2}{\overline{M_n}} - \frac{\frac{\bar{\upsilon}}{v_1}(\ln(1 - v_2) + v_2 + \chi_1 v_2^2)}{v_2^{1/3} - \frac{v_2}{2}} \qquad (3)$$

$$q = \frac{\overline{M_r}}{\overline{M_c}} \qquad (4)$$

where M_n is the number-average molar mass of the polymer, V_1 is the molar volume of the solvent (18 cm^3·mol^{-1} for water), v_2 is the polymer volume fraction in the equilibrium swollen hydrogel (the reciprocal of Q_V), $\bar{v}$ is the specific volume of the polymer (ρ_s / ρ_p), and χ_1 is the polymer-solvent interaction parameter. Thiol-double bond reactions for *in situ*/*in vivo* hydrogel formation

1.4 Thiol-double bond reactions for *in situ*/*in vivo* hydrogel formation

Generally, thiol-ene click chemistry describes the reaction of thiol containing compounds with alkenes with high conversions.[49] Several advantages of the chemistry for biological applications have led to increased interest in thiol-ene chemistry for hydrogel design. The

possibility of thiol-ene reactions to proceed rapidly under mild conditions compatible with cells and other biological molecules; the precise definition and easy characterization of reaction mechanisms and products are some of the advantages.[50] The ease of introducing thiol and "ene" moieties to macromolecular precursors for hydrogel synthesis compared to other biorthogonal functional groups such as strained cyclooctynes[51-53] in strain promoted azide-alkyne click reactions (SPAAC) makes thiol-ene reactions more attractive in biomedicine.

The thiol-ene reaction has also found extensive use in synthesis of hydrogels for tissue regeneration,[54] cell migration[55, 56] and 3D patterning of cell microenvironments.[57] Most of these syntheses were carried out in cell cultures with predominantly viable cell population after the synthesis. The reaction has therefore be applied *in vivo* using *in situ* gellable reactive precursor solutions via subcutaneous[17, 58] or intramuscular injection.[59]

1.5 Thiol-ene Reaction Mechanism

The reaction of thiols with enes proceeds either via the radical method known as the thiol-ene reaction or via the anionic method popularly termed as the thiol-Michael addition, Figure 2.

In the commonly used radical method, an initiator capable of generating radicals that initiate the reaction between thiol and select alkene functional groups. Radical generation can be achieved using a thermal, redox, or photochemical process.[60] Thiyl radicals, which are generated after initiation act as electrophiles and react with the C=C double bond of the alkene, forming a thiol−ene addition product and generate a new thiyl radical. The propagation process is highly efficient, resulting in a reaction.

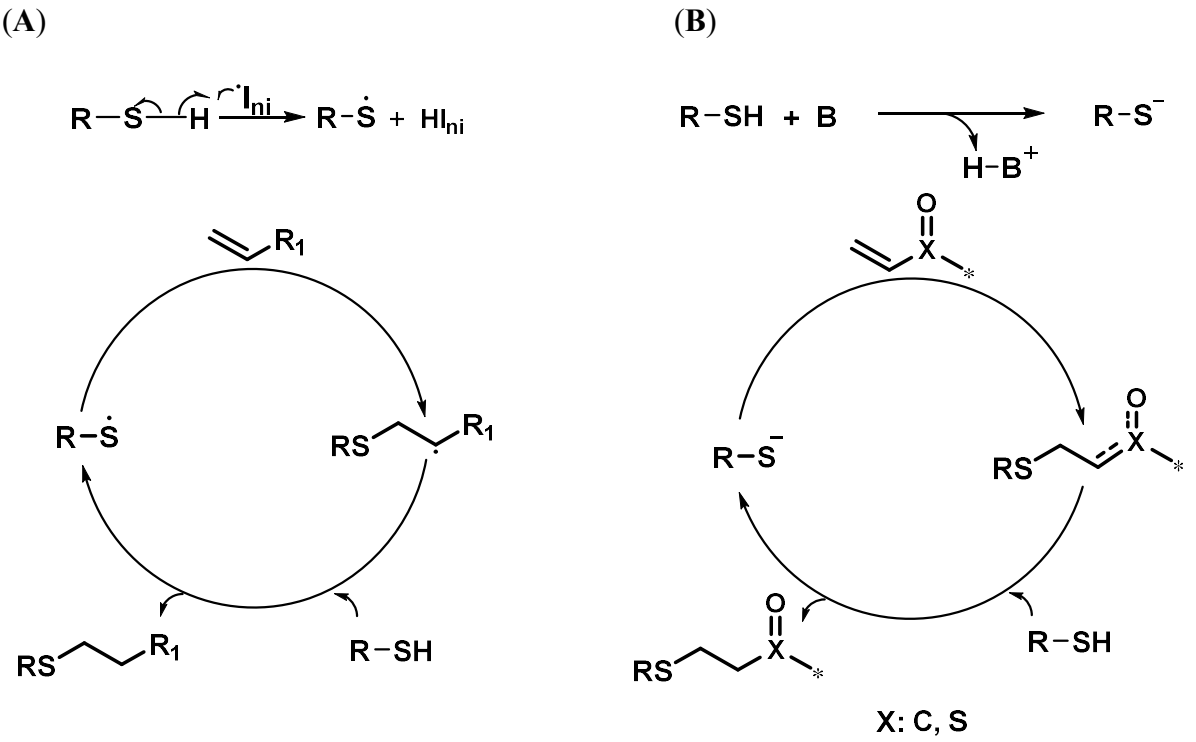


Figure 2: General scheme for A) thiol-ene and B) thio-Michael reaction mechanisms

In the thiol-ene mechanism, a radical initiator generates a thiyl radical that attacks the –ene molecule with spontaneous propagation of the reaction. The thiol-Michael addition mechanism involves the generation a thiolate ion with a base, B. The thiolate ion then attacks the –ene molecule (limited not only to *α,β*-unsaturated carbonyl compounds but also vinyl sulfones). Reaction propagation occurs similarly to the radical method

In thiol-Michael type reactions, a base (B) abstracts a proton from a thiol, forming a thiolate anion that acts as a nucleophile (Figure 2B). The thiolate anion attacks the electrophilic ß-carbon of an alkene conjugated to a carbonyl to form an intermediate, which abstracts a proton from a conjugate acid (BH^+) to generate the thiol-Michael addition product, regenerating the original base. The reaction continues, usually rapidly, until one of the reactive functional groups is consumed. Because of the mechanism, these reactions typically are free of by-products and can occur with no or low amounts of catalyst, making thiol-Michael reactions attractive for injectable hydrogel applications.

The thiol-Michael addition has become arguably a versatile tool in organic chemistry with increasing interest and implementation in hydrogel synthesis[42, 43, 59, 61], surface modification and polymer modification[62]. This versatility is afforded largely by the weak sulphur-hydrogen bond, which enables the thiol-Michael addition to be carried out with a wide range of precursors[63-66].

1.6 Michael Acceptors used for the formation of thiol-ene hydrogels

1.6.1 Thiol-Acrylate Hydrogels

Thiol-Michael reactions with acrylate systems are one of the commonest and have been implemented in the synthesis of degradable hydrogels, surface and particle modification, and block copolymer synthesis.[62, 67, 68] Methacrylates are less reactive in a thiol-Michael addition than the corresponding acrylates,[49] but are known to offer a more hydrolytically stable ester than that of the thiol-acrylate product, and could therefore be useful for applications where prolonged use in aqueous environment is desired.

The of works of Anseth et al.[69] and Hubbell et. al.[70] are some notable examples of the implementation of thiol-acrylate Michael addition in areas of controlled drug delivery, cell encapsulation and degradable hydrogel systems. Formation of robust injectable hydrogels cross-linked via a conjugate Michael addition in aqueous media with mechanical properties closely related to those of soft tissues is also reported.[16]

1.6.2 Thiol-vinyl sulfone hydrogels

Vinyl sulfones are electron-deficient alkenes that react with thiols under slightly basic conditions to give stable β-thiosulfonyl thioether bonds.[49, 54, 71] Vinyl sulfone groups generally are stable for extended periods under aqueous conditions near neutral pH. Vinyl sulfone groups have been introduced on macromers by reaction with free amine and hydroxyl functional

groups under basic pH.[72, 73] Hubbell et. al. have extensively employed vinyl sulfone functionalized multi-arm PEG macromers in hydrogel formation.[54-56, 71, 72]

The mild reaction conditions required for thiol-vinyl sulfone hydrogel formations have led to their applications therapeutic delivery such as delivery of bone morphogenetic protein 2 (BMP-2) for bone regeneration.[54, 74, 75] In the works of Peng et. al.,[75] vinyl sulfone propionic acid was reacted with hydroxyl groups of dextran via esterification in a one-pot synthesis to yield a series of macromers with varying degrees of substitution (DS ≈ 2−12.5). *In vivo* studies were performed in mice where hydrogels loaded with recombinant human bone morphogenetic protein-2 (rhBMP-2) were implanted in muscle pouch. Hydrogels prepared from macromers with higher degrees of substitution (DS = 12.5) exhibited slower degradation and higher ectopic bone formation (318 mg) compared to macromers with lower degrees of substitution (DS = 9, ectopic bone formation = 226 mg). This can be attributed slower degradation rates of these thiol−ene hydrogels leading to sustained release of rhBMP-2 for bone formation.

1.6.3 Thiol-maleimde hydrogels

Maleimides are known are as the most reactive Michael acceptor and the thiol-maleimide addition reaction has been widely implemented in biological systems, primarily due to selectivity of the thiol-maleimide reaction in aqueous environments, the rapid kinetics associated with the reaction, and the stability of the thiol-maleimide product.[49] An added advantage of the thiol-maleimide linkage is its ability to undergo retro-Michael reaction under reducing conditions when aryl thiols[76] are employed and has since proved a useful tool for controlled degradation and release applications.[49, 77] Although maleimide groups under basic aqueous conditions undergo ring hydrolysis yielding maleic acid hence rendering them unreactive with thiols,[76, 78] it still remains the Michael acceptor of choice for applications requiring fast gelling kinetics.[79, 80] Solution pH, temperature, neighbouring functional groups,

and hydroxyl ion concentration affect the rate of ring hydrolysis (k = 500−1600 M^{-1} s^{-1}).[78] Thiol-maleimide reactions have successfully been used to design various cell-compatible hydrogels for tissue engineering and drug delivery applications.[79, 81-83] Semi-interpenetrating thiol-maleimide hydrogels formed *in situ* for controlled release were reported by Fu et al.[84] Thiol and maleimide end-functionalized PEGs were reacted in the presence of gelatin at 37 °C to form a semi-interpenetrating network. A model macromolecule, fluorescein isothiocyanate-labelled dextran (*Mw* ≈ 40 kDa), was encapsulated in the network during hydrogel formation by mixing with the precursor solutions. In the absence of gelatin, 80% of the dextran cargo was released after 1 h, whereas the incorporation of gelatin slowed release of the cargo molecule to 25 h (100% release). The difference in the release profiles was attributed to reduced mesh size as a result of additional physical entanglements caused by gelatin incorporation.

It is important to note despite the numerous works with maleimides that some maleimides act as neurotoxins and so therefore require extreme caution especially in their implementation for biomedical applications.[85]

1.7 Delivery of thiol-ene hydrogels *in vivo*

Thiol-ene hydrogels can be administered *in vivo* by intramuscular[86] or subcutaneous injection[17, 87] of reactive precursor solutions that form a gel *in situ*, application of precursor solutions at desired sites during surgical procedures followed by *in situ* gelation,[88] or surgical implantation of preformed gels.[89] For *in situ* formation, liquid precursor solutions need to polymerize rapidly under physiologically relevant conditions at the site of interest (e.g.< 1 min): if the rate of reaction is too slow, precursor solutions containing therapeutic drugs may diffuse into surrounding tissues, resulting in poor hydrogel formation and potential immunogenic response to unreacted monomers.[12] However, if the rate of reaction is too rapid, premature gel formation may occur within the syringe for thiol-Michael type reactions, resulting in an uneven hydrogel

distribution within the tissue and large network defects that produce undesirable release profiles.

1.8 Thiols and thioesters in Tandem/Sequential Biomaterial Synthesis

Cascade reactions are common in organic chemistry for the construction of natural and complex molecules. These involve the use of a single starting reaction as a trigger for the conversion of a starting material to a product, which then becomes a substrate for the next reaction until termination leads to a stable final product.[90] These reactions are desirable for the synthesis of complex pharmaceutical compounds due to their efficiency and economy in terms of reagent consumption and purification.

The application of such reaction strategies in large molecule and hydrogel synthesis is however not common probably due to the complex reaction conditions present in these systems. In attempts to introduce different functionalities in specific locations and at different times in hydrogels, some researchers have resorted to the strategy of using two or more orthogonal reactions in a sequential fashion with the first reaction to form the cross-linking hydrogel and the second or later reactions to introduce biochemical functionalities.[57] These strategies however do not ensure a spontaneous propagation of one reaction after another but simply provides a platform to run these reactions without interference from the available reaction partners.

The few notable examples however of true coupled/sequential reaction strategy in hydrogel synthesis, namely the native chemical ligation-thiol-Michael coupled reaction and thiol-epoxy reactions, make use of reactions involving thiols or thioesters in the initial step.

Native chemical ligation (NCL) is effectively a coupled/tandem sequential reaction comprised of an initial thiol-thioester exchange reaction followed by a spontaneous *S*→*N* acyl transfer.[91, 92]

This highly chemoselective coupled system relies on the fact that the 2-aminoethanethiol moiety is reactive with a thioester and the transthioesterification is followed by the irreversible formation of a stable amide bond by intramolecular reaction.[12] This implies that, a free thiol or amine group in the middle of a peptide has little effect on the outcome of the NCL reaction. NCL has since proved a useful synthetic method mainly for the synthesis of peptides and proteins[93] and peptide-based block polymers and dendrimers.[94, 95]

Hu et al. applied NCL to form PEG hydrogels *in situ* in an aqueous environment.[96] Mixing solutions of four-armed PEG macromers terminated with thioester and cysteine groups at pH 7~8 resulted in the formation of robust hydrogels rapidly within minutes. These hydrogels remained stable after treatment with excess reducing agents such as tris (2-carboxyethyl)phosphine (TCEP) and 2-mercaptoethanol, implying the polymeric network was crosslinked by amide bonds formed through the NCL mechanism, rather than by intermolecular disulfide bonds between Cys groups. This hydrogel formation strategy was later applied to the encapsulation of extracellular matrix proteins to engineer the microenvironment of human mesenchymal stem cells in 3D culture by Jung and co-workers.[97] In a more recent application of the strategy, cysteine-functionalized hyaluronic acids were crosslinked with multi-armed PEG-thioester macromers to form hydrogels *in situ*, hence making NCL a valuable reaction for biologically relevant *in situ* crosslinking applications.[98]

Su et al. partially functionalized 4-arm PEG-Cys macromers with a maleimide terminated anti-inflammatory peptide using thiol-Michael type reaction.[99] Subsequent mixing of the modified macromers with thioester-terminated four-armed PEGs resulted in the formation of peptide-conjugated NCL hydrogels that promoted the survival of encapsulated pancreatic islet β-cells. The only limitation of this functionalization strategy is that bioactive molecules can only be incorporated at low densities since the majority of Cys groups need to be retained to react with thioester groups for productive crosslinking.

An interesting application of the NCL strategy in the dissolution of thioester-linked hydrogels was demonstrated by Ghobril and co-workers.[100] Dendritic macromers presenting multiple thiol termini were used to react with a PEG crosslinker containing *N*-hydroxysuccinimide active esters to form thioester networks. A small thiol molecule, *L*-cysteine methyl ester, added at high concentrations to the hydrogel, was able to trigger the dissolution of the gel by the NCL mechanism that broke the thioester links in the macromolecular network. This gel dissolution strategy was applied to the development of dissolvable sealant for wound closure,[100, 101] as examples of smart biomaterial design inspired by good understanding of chemical reaction mechanisms.

The thiol-epoxy reaction is another example, which involves a nucleophilic substitution between the thiol/thiolate nucleophile and an electrophilic carbon on the epoxy ring, leading to the ring opening followed by proton transfer to generate a thioether-alcohol product.[102] This substitution- addition reaction sequence leads to an overall efficient synthesis with no organic by-products. Examples of successful application of this coupled reaction system can be found in the polymerization of monomer building blocks,[103] crosslinking to form hydrogels *in situ* gel formation under physiological conditions (pH 7~8, 37 °C) via the crosslinking of poly(*N,N*-dimethylacrylamide-*co*-glycidyl methacrylate)[14] and the functionalization of polymers.[104] The crosslinking and gelation of poly(*N,N*-dimethylacrylamide-*co*-glycidyl methacrylate)[14] was reported to exhibit fast kinetics at 10 – 20% polymer weight composition in aqueous solutions and the resultant hydrogels were shown to be nontoxic to Hela cells. The fast reaction kinetics and relatively high selectivity shown in this study suggest a strong potential of the thiol-epoxy reaction for the development of injectable hydrogel materials.

Research is far advanced in the area of stimuli promoted *in situ* forming hydrogels for biomedical applications, the majority of which have physical netpoints, which are susceptible to the aqueous environments they are intended to be used in. In those examples where the more

water stable covalent linkages are used, a number of challenges makes further development of the underlying principle and successful translation of the designed materials difficult. Of major concern is the complexity of the design of chosen materials as well as the reactions to be employed. For instance, most *in situ* forming gel systems make use of large macromolecules functionalized with orthogonal reacting partners. The most common drawback to this approach is the narrow range of options to tune the properties of the formed gels, which are limited to varying of experimental conditions such as concentration, pH, temperature etc. Further tuning of bulk material properties would require modifications of reaction partners on the molecular level, which often entails laborious synthetic work. Additionally, the large size of these molecules rules out possibilities of fast screening of structures for specific application/function via machine learning/simulation using structural-property relations/models. It is becoming more evident that following the 'keep it simple, stupid (KISS)' principle could hold the key in designing clinically relevant interventions. Short peptide sequences when paired with the appropriate chemistry present not only an alternative but a more robust pathway to the realization of stimuli promoted *in situ* biomaterial synthesis. The robustness of such as a system is afforded not only by the huge number of structure-function relations available with the 20 constituent natural and additional synthetic amino acids, but also the ease of arranging these building blocks in a sequence to suit a specific function. In comparison to large macromolecule hydrogel precursors made up repeating monomeric units, dramatic change in chemical and physical properties can be achieved when amino acids are scrambled even in a short sequence. The short length or small size of these sequences also enable fast screening of function- specific sequences with machine learning or models prior to synthetic work for which automated synthesizers are available.

The structural variability of the peptide in terms different amino acids enables the tuning of properties such as solubility, reactivity in a specific reaction and susceptibility to different stimuli including enzymes by changing their sequence structure.

Following a top-down approach for the design of this *in situ* bioconjugation and crosslinking system, it is necessary to look into the possible means of chemically achieving the target function in biological systems considering the complexity these systems possess as far as their chemical and physical components are concerned.

2 Aims and Strategy

The main aim of this work is to develop a simplified, biologically friendly, stimuli promoted *in situ* crosslinking and hydrogelation system using a peptide mimetic as a latent crosslinker. A peptide based latent crosslinker that can be activated in a prodrug fashion to yield a reactive species separately using pH and selected MMP enzymes as stimuli, will provide a simplified and easily tunable *in situ* gelation system.

A prodrug type approach to obtain a latent crosslinker molecule with on-demand activation was hence chosen. Drawing inspiration from prodrugs, it was decided to use the thiol group as the active moiety that will be masked by an ester, specifically a thioester. In line with the assertion that multi-functionality can be obtained with short peptide sequences by simple modifications to sequence structure, the reference thiodepsipeptide sequence, Ac-Pro-Leu-Gly-SLeu-Leu-Gly was proposed as suitable for the synthesis two separate precursor molecules capable of pH- and enzyme-sensitive activation to yield active crosslinker molecules for *in situ* gelation applications.

Based on the on the chosen active and masking chemical moieties, the thiol-thioester exchange (TTE) reaction, was chosen as the most fitting chemistry due its dependency on pH and could be used as the trigger function. At a critical pH, gel formation via covalent linkages can be

initiated if appropriate orthogonal reaction partners and an efficient crosslinking strategy are employed. For this to work however, hydrolysis of the thiodepsipeptide should be negligible as the TTE reaction is three orders of magnitude faster than the hydrolysis reaction.[105]

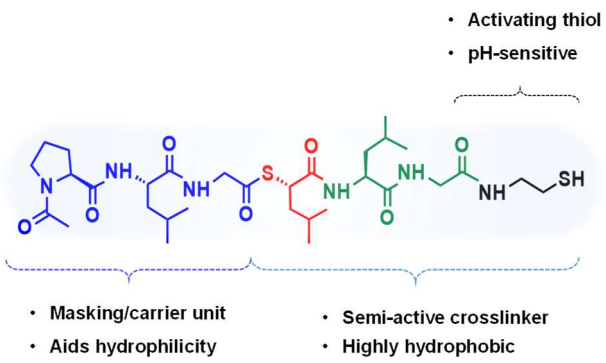
A peptide mimetic based system was selected as it was speculated its suitability as a TTE substrate should be controllable by the sequence of the amino acid residues around the thioester in a particular thiodepsipeptide. In order to realize such a system, two important requirements concerning pH-triggered generation of the crosslinker via TTE and subsequent crosslinking reaction chemistry have to be met.

In the first place, a thiodepsipeptide that is capable of exchange reactions at neutral to basic pHs to yield an *α,ω*-dithiol bearing crosslinker, must contain both, thioester and free thiol moieties. Secondly, the sequence and nature of amino acid residues around the thioester and thiol moieties should permit TTE reactions, which proceed beyond the formation of the initial tetrahedral intermediate to generate the required *α,ω*-dithiol crosslinker.

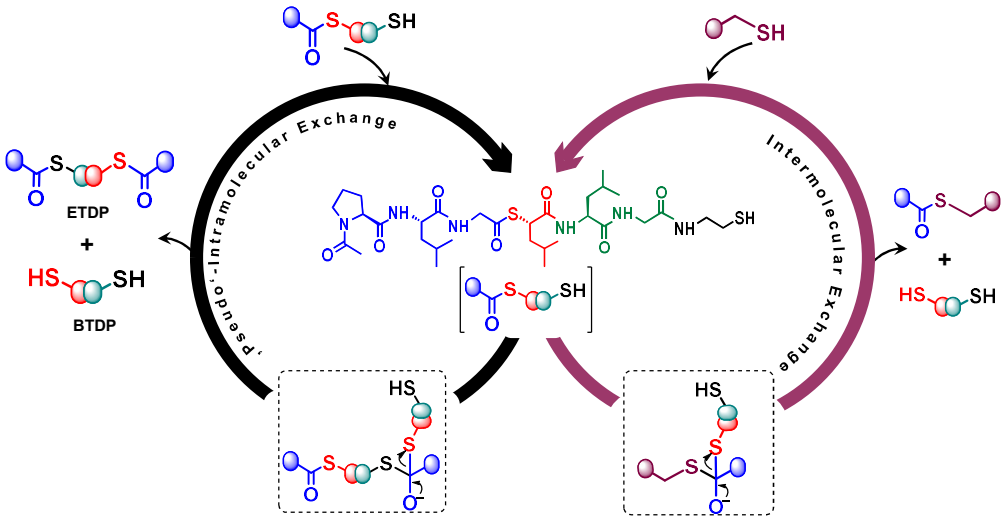
The approach of using peptide mimetic system exploits the application of differences in amino acid type and sequence structure around the thioester and terminal thiol moieties to effect a 'pseudo' intramolecular exchange paying attention to *p*Ka requirements[105, 106] of participating thiol species for a successful TTE reaction. The concept of 'pseudo' intramolecular TTE is used here to refer to the exchange reaction between multi-functional TTE substrate molecules both thioester and thiol functional groups. The formation of the initial tetrahedral intermediate is intermolecular whereas the final acyl exchange is intramolecular.

For the purposes of realising *in situ* crosslinking, the peptide mimetic Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) containing an internal thioester and terminal thiol moiety was designed. Figure 3A shows the chemical structure of the designed peptide detailing the role of sequence blocks to the overall function of TDP as a crosslinker precursor.

(A)



(B)



(C)

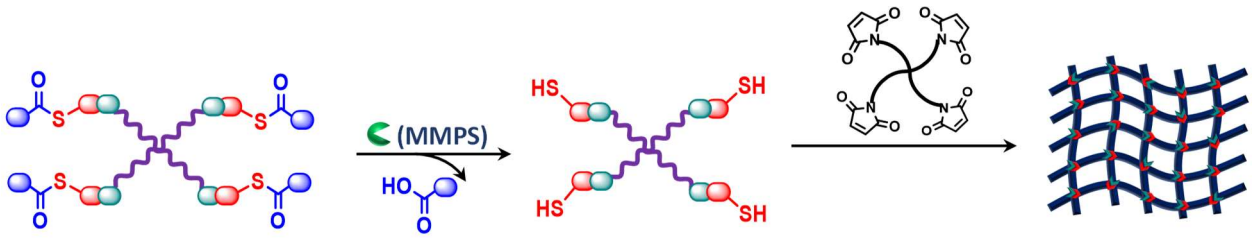


Figure 3: (A) Chemical structure of TDP showing the various sequence blocks and respective functions. (B) Fate of TDP in 'pseudo' intramolecular exchange (self-activation) and intermolecular exchange (activation by external thiols) (C) Cartoon representation of MMP-sensitive PEG-thiodepsipeptide conjugate PEG4TDP$_o$ and mode of gel formation with PEG4MAL

In the absence of additional thiols, TDP undergoes a self-activation mechanism via a 'pseudo' intramolecular exchange reaction assuming thiolate ions are generated by the activating unit as shown in Figure 3B.

The products of this reaction are the active dithiol crosslinker (BTDP) and an extended TDP (ETDP) in equilibrium with TDP. In the presence of external thiols, TDP undergoes either the 'pseudo' intramolecular or an intermolecular exchange or both. The effect of the external thiol on the final reaction outcome however will depend on the relative *p*Ka values of all thiols present.

For enzyme-promoted *in situ* gelation, the reference sequence Ac-Pro-Leu-Gly-SLeu-Leu-Gly (TDP_o) with sensitivity towards MMP cleavage, should be modified with simple synthetic strategy to obtain an MMP-sensitive crosslinker precursor. A multi-arm PEG functionalized with the MMP-active sequence ($PEG4TDP_o$) would be designed, synthesized and probed for its ability to function in MMP-promoted gelation via thiol-Michael reaction with multi-arm maleimide-functionalised PEG. Of critical importance to the design is choice of the PEG core needed to promote the aqueous solubility of $PEG4TDP_o$ without possibly influencing negatively substrate recognition of the attached sequences. Large PEG cores with long chains for example, could effectively shield the hydrophobic peptide sequence from aqueous interaction as a result of their added propensity to entangle than shorter chains. The sustainability of peptide-based approach also requires the use existing standard synthetic protocols to obtain $PEG4TDP_o$ in good purity without employing rigorous purification techniques. A cartoon representation of the structure of $PEG4TDP_o$ showing desired cleavage product after enzymatic hydrolysis and resultant gel formation with PEG4MAL is shown in Figure 3C.

The work was divided into three work packages. The first work package requires the identification of a bio-orthogonal chemistry that can be successfully exploited for bio-

conjugation and crosslinking applications under physiological conditions. Also included in this package is the initial design of the peptide mimetic considering the feasibility of all chemistries needed for its synthesis, activation and final reaction with a macromer orthogonal moiety to yield hydrogels in physiological environments. Prior to the synthesis of the peptide mimetic, proof of concept experiments of the chosen bio-orthogonal reaction using a representative amino acid analogue were performed. These were followed by the synthesis, characterization and performance tests of the peptide mimetic towards stimuli activation. Finally, the pH-promoted and MMP-promoted gelation using the synthesized peptide mimetics with maleimide functionalized 4-arm PEG macromer was studied. To realise the goals of the study the following strategies were adopted:

1. As stated out in the concept, the designed TDP for pH-promoted gelation is expected to undergo a 'pseudo' intramolecular exchange reaction to yield an α-ω-dithiol as a crosslinker which is orthogonal to Michael acceptors in thiol-Michael reaction. The sequence of the peptide should enable transthioesterification reaction of the terminal thiol and the internal thioester at pH close to that in biological systems. Prior to the development of the thiodepsipeptide, model thiol-Michael experiments using thio-leucine (which constitutes the internal thioester) with acrylate and maleimide functionalized PEG oligomers would be performed to probe the feasibility and reaction kinetics.
2. Synthesis of TDP would be accomplished by the modification of Ac-Pro-Leu-Gly-SLeu-Leu-Gly thiodepsipeptide, whose sequence upon careful theoretical consideration could be tailored to undergo a 'pseudo' intramolecular TTE reaction. Substitution of the ethoxy unit with cysteamine yields a terminal thiol bearing thiodepsipeptide with high propensity to engage in 'pseudo' intramolecular exchange reaction. Proof of

'pseudo' intramolecular exchange reactions as well intermolecular exchange reactions would be investigated with model reactions using externally added thiols. Effects of pH on the TDP activation and TDP-PEG4MAL gel formation would be studied using standard buffer solutions with varying pH. If enough active crosslinker molecules are generated from TDP activation, the thiol-maleimide reaction should proceed simultaneously leading to the formation of hydrogels.

3. For enzyme-promoted gelation, synthesis of PEG4TDP$_o$ would be accomplished by the functionalisation of small molecular mass 4-arm PEG-amine (PEG4NH$_2$, 2 kDa) with TDP$_o$ using standard amide coupling reagents. Characterisation of molecular structure as well as initial cleavage kinetic studies with selected MMPs -2 and -9 would be undertaken. A successful cleavage of PEG4TDP$_o$ with the selected enzymes will be followed by *in vitro* MMP-promoted gelation studies

3 Organization of the thesis

Chapter 1 - ‘Introduction’ focuses on the research background prior to the start and during the period of PhD project.

Chapter 2 - ‘Aims and Strategy’ describes motivation aims, formulated hypothesis, and strategies used.

Chapter 3 - ‘Organization of the Thesis’ describes the organizational structure of the thesis.

Chapter 4 - ‘Thiol Michael-type reactions of optically active mercapto-acids in aqueous medium’ – Appendix I. The chapter describes initial experiments as proof-of-concept for the proposed crosslinking chemistry and representative reaction partners.

Chapter 5 - ‘Reaction behaviour of peptide based single thiol-thioesters exchange reaction substrate in the presence of externally added thiols’ – Appendix II. This chapter describes works done as part of the second major milestone, which focused on the synthesis of TDP and probing its suitability as a latent crosslinker capable of self-activating using pH changes as a stimulus.

Chapter 6 - ‘Thiol-thioester exchange reaction in precursor enables pH triggered hydrogel formation’ – Appendix III. The chapter focuses on the first major milestone of the study investigating the suitability of TDP as a latent crosslinker for the pH-promoted synthesis of hydrogels.

Chapter 7 - ‘A 4-arm PEG-thiodepsipeptide Precursor Enables Gelatinase-promoted Hydrogel Formation’ – Appendix IV. The chapter focuses on the final strategy and milestone of the study in relation to the application of PEG4TDP$_o$ as a crosslinker precursor for the MMP-promoted synthesis of hydrogels.

Chapter 8 - ‘Discussion’ presents an overall discussion of all findings of this work.

Chapter 9 - ‘Summary and Outlook’

4 Thiol Michael-type reactions of optically active mercapto-acids in aqueous medium

Summary

This study was carried to ascertain the suitability of thiols to be incorporated in the thiodepsipeptide crosslinker precursor capable of effecting a ‘pseudo’ intramolecular TTE as well thiol-Michael addition reaction. This was necessary because although thiol *p*Ka values are essential in determining the success of a TTE, the results of two proposed structurally different thiols SH-Leu and SH-Phe in thiol-Michael reaction with acrylate and maleimide functionalized PEG oligomers revealed that SH-Leu undergoes successful reaction although SH-Phe has a relatively more acidic thiol. This observation could be explained by steric hindrance of the bulky phenyl unit and the difficulty in solubilizing the highly hydrophobic substrates via the formation of the acid salt. Fast reaction rates and complete acrylate/maleimide conversion were obtained for the less hydrophobic SH-Leu (also used as the acid salt formed *in situ*) at pH 7.2 or higher thereby making SH-Leu the most suitable thiol unit to include in possible thioester containing peptide-based crosslinker precursors.

Own contribution to the publication

- Literature Review
    - Bio-orthogonal and click reactions in biomedical applications
    - Functionalization of natural/synthetic polymers
    - Introduction of sulphur bearing moieties (thiol, thioester) into polymers and peptides
- Study design
    - Material selection (water soluble Michael acceptor functionalized macromers/oligomers, mercapto-acids with markedly different reactivities)
    - Suitability of NMR and HPLC-ESI for studying thiol-Michael reaction kinetics
    - In-situ conversion of hydrophobic mercapto-acids into water soluble analogues
- Experimental work
    - Synthesis and characterization of mercapto-acids, SH-Leu and SH-Phe
    - ^{1}H-NMR characterization of reaction kinetics of mercapto-acids with PEG-acrylate and PEG-maleimide, HPLC-ESI characterization of reaction products
- Analysis and data interpretation
    - Extraction and interpretation of kinetic data from pseudo-2D ^{1}H-NMR spectral data
    - Dependency of structure of mercapto-acid on reactivity (feasibility and kinetics) with Michael acceptor functionalized PEG
    - Characterization and interpretation of reaction progress and products from HPLC-ESI data
- Manuscript preparation
    - Manuscript outline in discussions with the co-authors
    - Writing the first draft of the manuscript

- Manuscript revision and finalisation according to comments of the co-authors

- Publication – Appendix I

M.Y. Folikumah, A.T. Neffe, M. Behl and A. Lendlein: Thiol Michael-Type Reactions of Optically Active Mercapto-Acids in Aqueous Medium. MRS Advances 4, 2515 (2019).

5 Reaction behaviour of peptide based single thiol-thioesters exchange reaction substrate in the presence of externally added thiols

Summary

This work presents a detailed investigation of the ability to predict the outcome of TDP TTE reactions using externally added thiols as the activating stimulus. Results obtained from this study would be a valuable information for the decoupling contributions from external thiols as found in biological environments in the activation of TDP other than the self-activation when only TDP molecules are present.

The presence of external thiols results in two competing reactions namely the 'pseudo' intramolecular reaction of two TDP molecules and the intermolecular reaction of a TDP molecule and an external thiol. Using carefully selected thiols, an overview of the reactivity and ability of the selected thiols to preferentially stabilize the exchangeable acyl unit with the regards to overall structure and *p*Ka of the thiols was obtained. In summary TDP TTE reactions with LCYS (biologically relevant thiol) and MeTGC (small molecular weight thiol) all with comparable thiol *p*Ka values to the attacking cysteamine thiol, resulted in an increase in dithiol crosslinker generated. TDP TTE reaction with GSH (most abundant biological thiol) also with comparable *p*Ka to LCYS, MeTGC and TDP's cysteamine thiol, showed no significance difference in generated dithiol concentration than when only TDP was reacted. This observation could be explained the relatively large size of GSH compared to LCYS and MeTGC considering steric hindrance. However, when TDP was reacted with the acidic thiols,

p-NTP or 4MBA, a significant reduction in the formed dithiol's concentration was observed. Although the acidic thiols have a high probability of attacking the thioester carbonyl, the formed tetrahedral intermediate do not dissociate readily as was evidenced in the prolonged half-life of these species in ESI-MS spectra.

Own contribution to the publication

- Literature Review
    - Overview of thiol-thioester exchange reactions
    - ESI-MS studies of thiol/sulphur compounds (ionisation patterns and challenges)
- Study design
    - Material selection (external thiol compounds considering overall chemical structure, size, *p*Ka value of the thiol functional group)
    - Design of experiments (model thiol-thioester model reactions with external thiols)
    - Design of ESI-MS and NMR experiments to adequately observe changes in the progress of reactions as well as the identification of reactant and product species
- Experimental work
    - Thiol-thioester model reactions of TDP with external thiols
    - Reaction monitoring and reactant/product species investigation with ^{1}H-NMR
    - Investigation of reactant and product species with ESI-MS
- Analysis and data interpretation
    - Identification and elucidation of exchange reaction products from NMR and ESI-MS data
    - Identification of reaction patterns and dependent parameters/conditions
- Manuscript preparation

- Manuscript outline in discussions with the co-authors
- Writing the first draft of the manuscript
- Manuscript revision and finalization according to comments of the co-authors

- Publication – Appendix II

M.Y. Folikumah, M. Behl and A. Lendlein: Reaction behaviour of peptide-based single thiol-thioesters exchange reaction substrate in the presence of externally added thiols. MRS Communications 11, 402 (2021).

6 Thiol-thioester exchange reaction in precursor enables pH triggered hydrogel formation

Summary

This study details the synthesis and detailed characterization of TDP and its ability to undergo TTE reactions controlled by pH. The importance of a carefully planned solution phase peptide coupling synthetic strategy and protecting group chemistry as well as the power of nuclear magnetic resonance spectroscopy in structural elucidation is demonstrated. The consequence of the presence of peptidyl-prolyl bond in a peptide sequence on the ease of NMR data interpretation is also discussed.

Confirmation of 'pseudo' intramolecular TTE reaction by TDP molecules was used in the synthesis of TDP-PEG4MAL hydrogels using pH as stimulus.

When a 1:1 thiol: maleimide molar ratio was used, TDP-PEG4MAL hydrogels formed within 3, 12 and 24 hours for pH values of 8.5, 8.0 and 7.5, respectively and when the thiol: maleimide molar ratio was changed to 2:1, increased gelation times of 3, 5 and 30 mins were observed at the same pH values. A linear dependency of the storage modulus (G') on the crosslinking density of the polymer supported an observed correlation of thiol content on the G' values of the gels.

The similarity in the elasticities TDP-PEG4MAL gels to those of certain tissue microenvironments (brain and adipose tissues) makes TDP-PEG4MAL gels attractive hydrogel substitutes for drug delivery applications in these locations since the mechanical properties can be easily tuned by the variation of reaction components and conditions.

Own contribution to the publication

- Literature Review
    - Crosslinking strategies in hydrogel synthesis
    - Latent chemistries and their application in development of prodrugs
    - Thiol-thioester exchange reaction and design of single TTE substrates
- Study design
    - Design of water soluble peptide based single thiol-thioester reaction substrate
    - Material selection (reference peptide structure and necessary modification to effect pseudo-intramolecular reaction
    - Activation of latent thioester containing peptide to yield bis-thiol crosslinker
- Experimental work
    - Synthesis and detailed characterization of thiodepsipeptide (TDP) with NMR, ESI-MS and FT-IR
    - Investigation of activation of latent TDP via ‘pseudo’-intramolecular exchange reactions with NMR, LC-ESI-MS
    - Investigation of pH-dependent synthesis of PEG hydrogels using TDP in the presence of pH indicator dye bromothymol blue, physical characterisation of form gels (swelling tests and rheological measurements)
    - Demonstrator experiments (pH-dependent interfacial gel formation of PEG-maleimide and TDP droplets upon making contact in silicon oil)

- Analysis and data interpretation
    - Comprehensive structural elucidation of TDP from 1D/2D NMR, ESI-MS and IR data
    - Evidence of 'pseudo-intramolecular' exchange reaction of TDP and its dependence reaction parameters (solvent, pH), identification of exchange products
    - Effect pH on activation of TDP and crosslinking reaction with PEG-4MAL, physical properties (swelling behaviour, rheological behaviour)
- Manuscript preparation
    - Manuscript outline in discussions with the co-authors
    - Writing the first draft of the manuscript
    - Manuscript revision and finalization according to comments of the co-authors
- Publication – Appendix III

M.Y. Folikumah, M. Behl and A. Lendlein: Thiol–thioester Exchange Reactions in Precursors Enable pH-Triggered Hydrogel Formation. Biomacromolecules 22, 1875 (2021).

7 A 4-arm PEG-thiodepsipeptide Precursor Enables Collagenase-promoted Hydrogel Formation

Summary

The design, synthesis and detailed characterisation of thiodepsipeptide functionalised 4-arm PEG (PEG4TDP$_o$) and its MMP-promoted activation to form active crosslinker molecules are reported in this study. A careful balance between the PEG core and thiodepsipeptide molecular size was considered in ensuring water solubility and available of MMP-active thiodepsipeptide ends for recognition by the enzymes.

A sigmoid, non-Michaelis-Menten type kinetics was observed during cleavage studies of PEG4TDPo using MMPs -2 and -9 thereby making it difficult to determine important kinetic parameters, K_M and k_{cat}. Gelation studies of PEG4TDP$_o$ and PEG4MAL resulted in the formation of gels with MMPs -2 and -9 within 28 and 40 minutes respectively.

A potential for the use of PEG4TDP$_o$ –type precursor molecules for MMP-related applications is envisaged.

Own contribution to the publication

- Literature Review
    - Crosslinking strategies in hydrogel synthesis
    - Latent chemistries and their application in development of prodrugs
    - Thiol-thioester exchange reaction and design of single TTE substrates
- Study design
    - Design of water soluble peptide based single thiol-thioester reaction substrate
    - Material selection (reference peptide structure and necessary modification to effect pseudo-intramolecular reaction
    - Activation of latent thioester containing peptide to yield bis-thiol crosslinker
- Experimental work
    - Synthesis and detailed characterization of PEG-thiodepsipeptide conjugate (PEG4TDP$_o$)
    - Investigation of PEG4TDP$_o$ cleavage with MMPs -2 and -9 using fluorescence assay
    - MMP-promoted gel formation *in vitro* PEG4TDP$_o$ and PEG4MAL, characterisation of gelation kinetics and physical properties of formed gels using rheology

- Demonstrator experiments (MMP-promoted gel formation with cell lysates)

- Analysis and data interpretation
  - Comprehensive structural elucidation of PEG4TDP$_o$ from 1D/2D NMR and ESI-MS data
  - Effect enzyme type and concentration on PEG4TDP$_o$ cleavage and crosslinking reaction with PEG4MAL, physical properties (swelling behaviour, rheological behaviour)
- Manuscript preparation
  - Manuscript outline in discussions with the co-authors
  - Writing the first draft of the manuscript
  - Manuscript revision and finalization according to comments of the co-authors
- Publication – Appendix IV

M.Y. Folikumah, A. Neffe: A 4-arm PEG-thiodepsipeptide Precursor Enables Gelatinase-promoted Hydrogel Formation. *Finished Manuscript 2022*

8 Discussion

This chapter collectively discusses the results presented in chapters 4-7 in reference to the current state of the art. First, the intricate considerations taken into account for the design of the peptide mimetic vis-a-vis the creation of simplified stimuli promoted crosslinking and bioconjugation system. The advantages of the selected crosslinking/bioconjugation chemistry (thiol-Michael addition reaction) and the activation method/chemistry of the latent peptide mimetic precursor (thiol-thioester exchange reaction and enzymatic hydrolysis). Furthermore, theoretical assumptions for the successful incorporation and promotion of the self-activation mechanism into the peptide mimetic under chosen stimulus are discussed. Synthesis and detailed structural characterization as well as the reactivity of the peptide mimetics in a thiol-

thioester exchange reaction and enzymatic hydrolysis will be discussed. Finally, the performance of TDP sequence in a coupled activation reaction and gel formation with maleimide functionalized PEG macromer was investigated.

8.1 Design of TDP as latent crosslinker precursor

In designing TDP as a latent crosslinker for the purposes of autonomous bioconjugation and *in situ* gelation applications, a systematic top-down design principle using the intended application chemistry to guide material selection and activating chemistry was employed. In the attempt to incorporate an on/off switch in the crosslinker precursor, the principles used in prodrugs design were combined with the prospects of dynamic covalent linkages in comparison to their supramolecular analogues.[107, 108] Notable examples of dynamic covalent chemistries in stimuli responsive hydrogels include the disulfide, boronate ester, imine, oxime, Diels-Alder and thioester reactions. The majority of these chemistries are however used in polymer systems for self-healing related applications.[109]

Disulfide bonds are dynamic covalent bonds resulting from oxidation of thiols[110] thereby offering reversibility and stimuli responsiveness as exploited in the triggered assembly and disassembly of different polymeric nanostructures, including biodegradable nanogels[111] as well as single-chain nanoparticles.[112] Disulfide bonds are also being increasingly used in self-healing materials either through an exchange reaction. A self-healing hydrogel developed by Chen et. al., by combining disulfide bonds and acylhydrazone bonds, underwent self- healing under basic environments through disulfide exchange reactions.[113] The reported self-healing process was triggered at room temperature without external stimuli and was reversible for multiple cycles. Additionally, the dynamic nature of the acylhydrazone bonds in these gels also aided another self-healing mechanism under acidic conditions. In effect, a dual stimuli-responsive system (pH and chemical) achieved with the authors suggesting the potential of these gels as candidates for organ repair or stimuli responsive drug delivery.

Boronic ester bonds are reversible covalent bonds formed by the complexation between boronic acids and 1,2- or 1,3- diols in aqueous solutions. The dependence of the stability of this bond on the pH of the solution,[114, 115] makes them suitable targets for pH responsive related applications in hydrogels. This was demonstrated by crosslinking polymer-bound phenylboronic acid (PBA) with either poly(vinyl alcohol) or a catechol-functionalized copolymer.[116] The hydrogels demonstrated self-healing at neutral and acidic pH, where the crosslinks reconstituted rapidly after removing an applied strain, restoring the moduli of the original hydrogel. The usefulness of the reversibility of boronate ester bonds was demonstrated in probing the timescale dependent mechanotransduction of fibroblasts using cytocompatible and viscoelastic hydrogels.[117] The viscoelastic properties (storage and loss moduli) of the gels were tuned using the equilibrium kinetics of the dynamic covalent crosslinks.

Kloxin et. al[118] similarly crosslinked statistical copolymers of *N*,*N*-dimethylacrylamide and a pinacol protected ester of 2-acrylamidophenylboronic acid with poly(vinyl alcohol) to form gels with self-healing property. The self-healing property of these gels were demonstrated in relevant cell culture media and could be used in dynamic co-cultures of breast cancer cells and lung fibroblasts. These co-culture hydrogels will enable study of cellular processes such as migration, mechano-transduction, and cell–cell signalling.

Imine bonds (Schiff bases) undergo reversible imine–amine exchange,[119] and this reversible nature has been exploited for self-healing properties in imine crosslinked hydrogels. Using the imine dynamic covalent bond, many complex highly symmetrical molecules and extended structures have been constructed including molecular walkers capable of walking along a track, configurational rotary switches, wholly-organic cages, dynamic catenanes and dynamic rotaxanes, among others.[120]

Maynard and coworkers[121] developed imine- crosslinked poly(ethylene glycol) (PEG) self-healing hydrogels that demonstrated self-healing in less than 10 min. The hydrogels also

demonstrated degradability when treated with pH 5.6 cell culture media because of the pH sensitivity of the imine crosslinks, and were used to encapsulate murine mesenchymal stromal cells (mMSCs) cells to evaluate the controlled release of cells. Similarly, naturally sourced chitosan has been used in preparing dynamic self-healing hydrogels using imine bonds derived from amino-groups along the polymer backbone.[122, 123] Other biomacromolecules such as gelatin and collagen with amine groups have also been used to develop imine crosslinked self-healing hydrogels.[124, 125]

Thiol–thioester bonds undergo reversible exchange reactions in aqueous solution.[100, 105, 126] In the thiol–thioester exchange reaction, a thiolate anion reacts with a thioester to form new thiolate and thioester products.

Adaptable tissue engineering scaffolds for regenerative biology using TTE chemistry was reported by Anseth et.al[127] for which an 8-arm thiolated polyethylene glycol were crosslinked with a thioester-containing divinyl crosslinker via a photo initiated thiol–ene reaction.

The thioester-containing hydrogels in the presence of *L*-cysteine at physiological pH was dissolved as the pK_a of of *L*-cysteine (the attacking thiol) is higher than than that of the leaving thiols in the exchange reaction. Additional experiments involving poly(ethylene glycol) diene crosslinker with thioester groups were used to prove that the breakdown of the hydrogel was solely due to the presence of thioester linkages in the network. While the biological relevance and high efficiency of the exchange reaction is useful for biomedical applications, the reaction efficiency in organic solvents and compatibility with a wide pH range also makes this chemistry suitable for non-biological applications.[106, 128] The dynamic nature of TTE was also successfully applied for the rapid generation and screening of dynamic library of Acetylcholinesterase (AChE) substrates.[129]

Driven by potential *in situ* bioconjugation and crosslinking applications, the water-soluble collagenase substrate, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt[130] with a thio-leucine derived

thioester unit was chosen as a reference sequence. The sequence structure of this peptide fits well into the goal of obtaining *in situ* gelling systems with separate activation mechanisms (pH and enzyme) without requiring significant modification to the sequence.

Amidation of the C-terminal end with a cysteamine unit yielded a TTE amenable thiodepsipeptide, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) bearing a free thiol with higher *p*Ka than that of thioester thio-leucine. The utility of the thiol–thioester exchange (TTE) as the "gold standard" of dynamic covalent linkages,[131] was clearly demonstrated by the TDP's ability to self- activate and exist in a dynamic equilibrium with formed products, BTDP and ETDP.

The subtle difference in *p*Ka values between the attacking thiol (cysteamine moiety) and thioester thiol aided 'pseudo' intramolecular TTE of TDP to yield an active -Leu-Leu-Gly- bearing dithiol crosslinker. Additionally, the relative position of thioester carbonyl ensured that TDP TTE resulted exclusively in a split and one-time extension of the reactant. This observation is in contrast with similar works which employed single molecule TTE substrates. Wang et. al.,[132] in adhering to *p*Ka requirements,[106] designed cysteine and glycine derived single molecule TTE monomers bearing terminal thiophenol thioester and alkyl-thiol units. TTE of these substrates resulted in the polymerization of the monomers to obtain cysteine and glycine containing polythioesters. The use of thiophenol (highly acidic thiol) to drive the TTE equilibrium however leaves this system with application limitation due to the strong thiol odour left in the polymers from the liberated thiophenol.

A simplified representation of the importance of the combination of thiol/thioester type and sequence structure in tailoring or adapting the peptide to a specific application is shown in Figure 4. Thiodepsipeptides bearing attacking thiols with higher *p*Ka value than the thioester thiol readily undergo 'pseudo' intramolecular TTE to yield products whose fate is determined by the relative position of the thioester carbonyl in the sequence.

Thiodepsipeptides, which do not undergo the intramolecular TTE due to the reversal of relative *p*Ka values of participating thiol species, can still react in an intermolecular TTE in presence of an external thiol with pKa_{ext} if pKa_{ext} > *p*Ka of departing thiol (thioester thiol). The external thiol effectively serves as an activating stimulus.

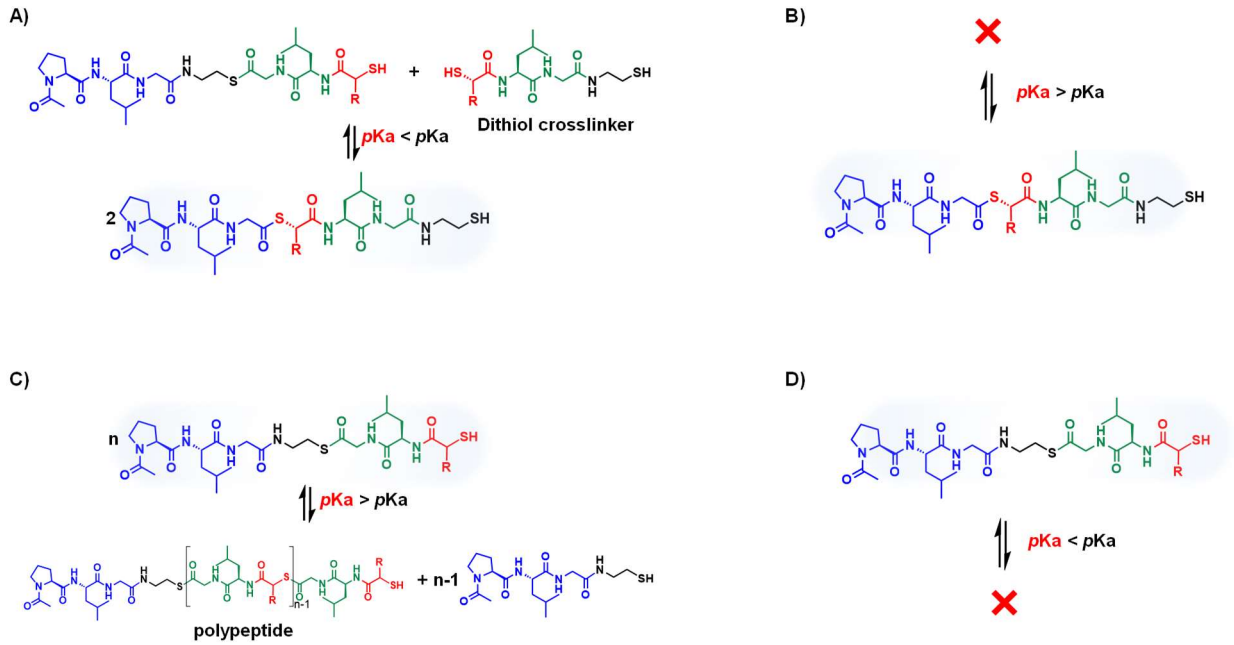


Figure 4: Scheme showing the effect of sequence structure on fate of TTE of single thiol/thioester containing peptide in TTE reaction.

TTE is favoured when the *p*Ka of the attacking thiol is greater than that of departing thiol (thioester thiol) (A) peptide sequence similar to TDP, higher *p*Ka value of attacking thiol than departing thiol promotes TTE and crosslinker activation (B) peptide sequence similar to TDP, lower *p*Ka value of attacking thiol than departing thiol hinders TTE and crosslinker activation (C) peptide sequence with respect to thioester carbonyl position switched, higher *p*Ka value of attacking thiol promotes TTE and an insertion type polymerization occurs (D) same peptide sequence in C but lower *p*Ka value of attacking thiol hinders TTE reaction.

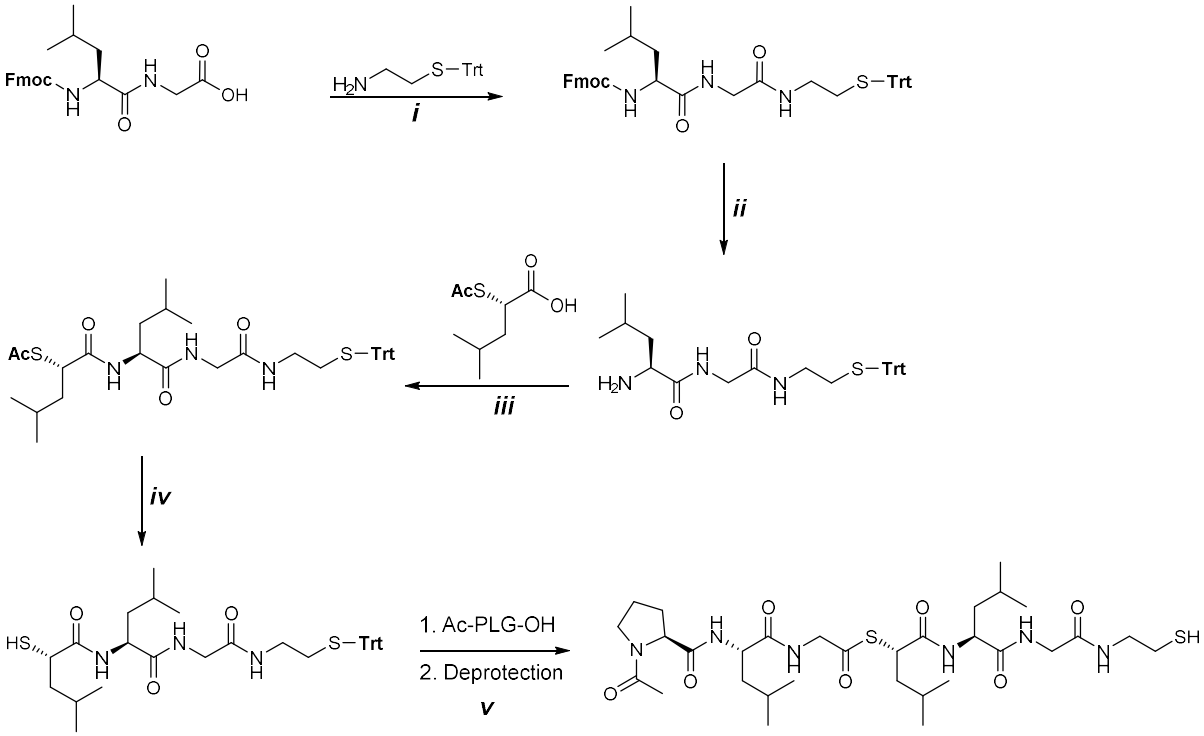
8.2 Synthesis and characterization of TDP

The synthesis of TDP was accomplished a multi-step solution phase peptide coupling as depicted in Scheme 1 by employing HOBt/EDC and the uronium based coupling agent, HATU where appropriate. While the synthesis of Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt and similar thioester containing peptides is already documented in literature,[130, 133] the synthesis of TDP (Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH) poses some challenges. The main constraint was the need to introduce the terminal thiol without promoting a possible TTE reaction at any stage prior to obtaining the desired compound. Careful considerations were therefore made in the choice of protecting groups to be used at all stages of the synthesis. Secondly, the solution phase synthesis and purification of nearly neutral and short chain peptides can be very problematic, due to the similarity in polarity of the peptides and unreacted reactants, intermediates and side products. Apart from the final TDP where HPLC purification might be required, the goal was to obtain all intermediates in high yields with less rigorous purification procedures. Finally, to prevent TDP from possible formation of disulfide bonds and 'premature' intramolecular TTE reaction, the final thiol deprotection and purification conditions should provide permit the stability of the thiols.

In order to address all the challenges, a trityl-protected cysteamine was chosen as the most suitable thiol-bearing unit for the synthesis. Amidation of the C-terminal end of Fmoc-LG-OH was preferred over possible esterification due to the enhanced stability of amide bonds towards hydrolysis over a corresponding ester. This ensures a higher probability of maintaining the complete TDP molecule in aqueous media without losing the thiol unit to hydrolysis. The use of trityl protection for the thiol on the cysteamine had a dual purpose in ensuring the success of the synthesis. On one hand, the trityl group should remain unaffected during the deprotection of Fmoc and acetyl groups in steps ***ii*** and ***iv*** respectively. Secondly, the hydrophobicity of all intermediates prior to the final trityl deprotection was increased to aid purification of the

intermediate ***i*** by simple precipitation in 10 % methanol and the rest by flash column chromatography on gram scales.

Finally, by maintaining a slightly acidic conditions with ($BF_3{\cdot}Et_2O$, Et_3SiH, HFIP) trityl-deprotection cocktail[134] and H_2O/ACN (0.1 % TFA) HPLC mobile phase, TDP could be obtained with active thiols.



Scheme 1: Synthetic route for TDP, step ***v*** combines final coupling and trityl deprotection steps but are not carried out separately and not as a one pot synthesis

8.3 Consequence of Prolyl-peptide Bond Isomerization on NMR Characterization of TDP

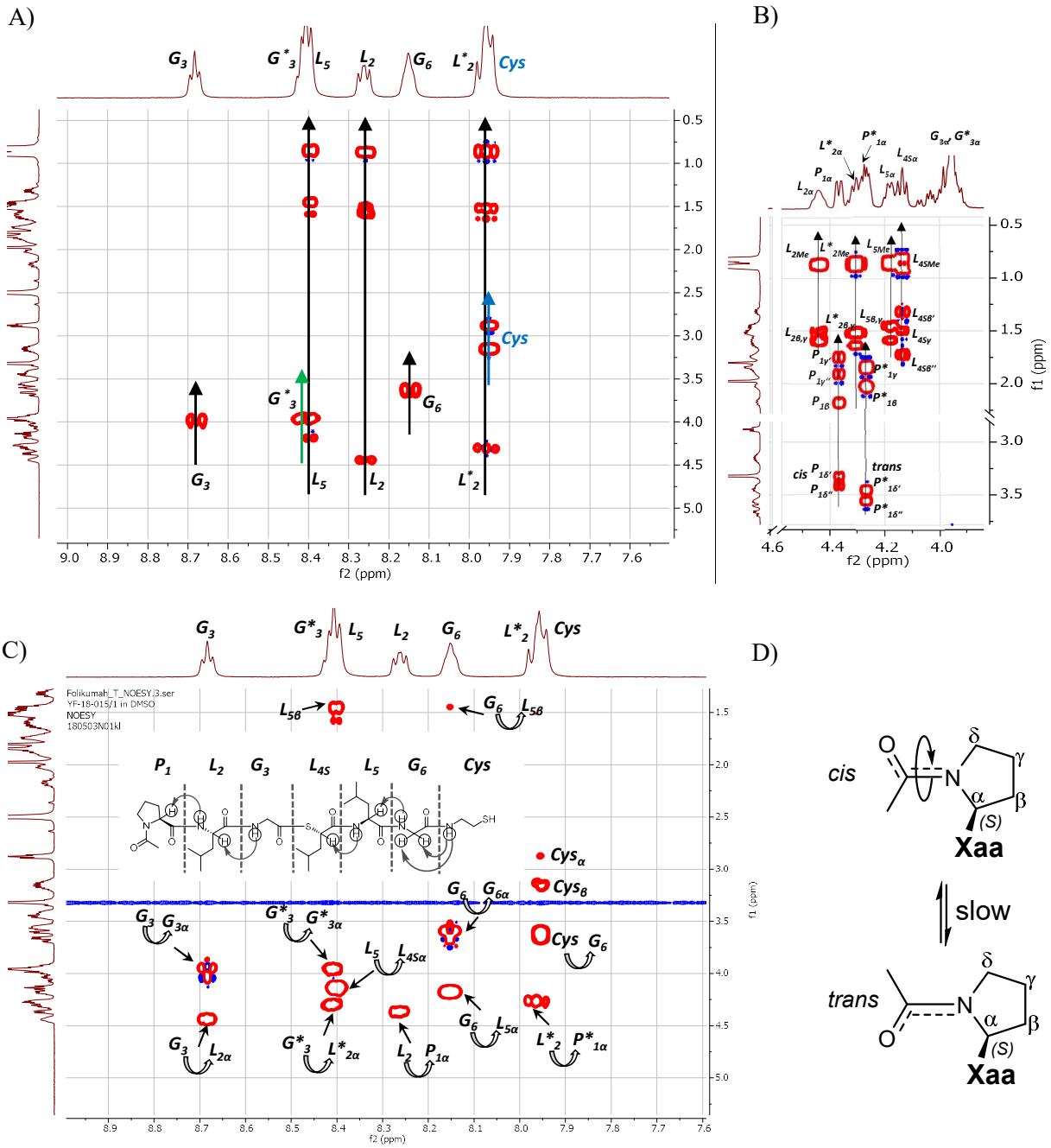


Figure 5: A) Amide region of TOCSY spectrum of TDP showing two sets of resonances for G_3 and L_2 B) α- H region TOCSY spectrum of TDP with spin assignments C) NOESY spectrum of amide region of TDP with NOE assignments. D) Peptidyl-prolyl *cis-tans* isomerization

The synthesis of TDP despite the carefully laid out synthetic route was repeated multiple times because although molecular mass from ESI-MS measurements were consistent with expected mass, results from initial NMR experiments casted doubt on the correct or expected structure. ^{1}H- NMR spectrum of TDP in DMSO-d_6 lacked the active thiol on the cysteamine and depicted the presence of more than the five expected amide proton signals. Because of the large number of protons per residue and the resulting resonance overlap, even small peptides cannot be fully characterized from one-dimensional spectra and require two-dimensional analyses.

In fact, from the total correlation spectroscopy (TOCSY), more spin systems than the five expected for TDP with respect to the available amide groups were observed in the amide region (Figure 5A). A careful examination of TOCSY spectrum reveals the presence of two pair each of spin systems belonging to amino acid residues, G_3 and L_2.

Although a possible overlap of thiol proton signal with the rest of the spectrum could be the reason for its absence, the thiol signal could not be located in 2D-NMR experiments (COSY, TOCSY, HSQC and NOESY). Studies[135, 136] have reported the propensity of aqueous DMSO to oxidize thiols resulting in disulfide formation. Although this phenomenon possibly explains the absence of thiol proton signals, it does not account for the occurrence of the observed two sets of amide resonances for L_2 and G_3. Assuming quantitative disulfide formation, not more than the expected 5 spins systems should be observed for TDP either as free thiol or disulfide form in a TOCSY experiment. Since TOCSY experiments rely on scalar coupling, possible formation of secondary structures if any from the elongation of the molecule via disulfide formation should have also yielded the expected 5 spin systems in the TOCSY and not the observed 7 spin systems. The observed differences in chemical shifts of L_2, G_3 amide and acyl-prolyl methyl resonances with the concomitant presence of two sets of signals for each was an indication of possible presence of different isomers of TDP. This observation could be attributed largely to the acetylation of the prolyl residue resulting in a *cis-trans* isomerization

of the prolyl-peptide bond. Of the 20 commonly gene-coded amino acids, proline stems out as unique due to the fact that unlike the rest of amino acids which exist predominantly as the *trans* form, both the *cis* and *trans* isomers of the Xaa-Pro (prolyl-peptide) bond can be significantly populated.[137] An equilibrium mixture of the *cis* and *trans* conformations is reported for prolyl-peptide bonds in peptides and denatured proteins, while in the native, functional state of proteins, most prolyl peptide bonds are exclusively in the *trans* conformation with a small number in the *cis* conformation.[138]

Cis-trans isomerization of prolyl peptide bonds involves a shift of the nitrogen lone pair of electrons to an sp^3 orbital resulting in a partial double bond character of the C−N bond with a slow rotation around the C−N bond.[137, 139, 140] The equilibrium structures arising from this slow rotation can therefore be conveniently studied with a number of analytic techniques including NMR.

From TOCSY and NOESY experiments of TDP, proline (P_1) and amino acids (L_2, G_3) proximal to the prolyl residue exhibited *cis* or *trans* forms. By combining the expected local magnetic environment of all prolyl proton resonances due to the rotation of the prolyl C-N partial double bond and the observed α-proton spins of the TOCSY spectrum (Figure 5B), the *cis* and *trans* isomers of TDP were identified with the notations *P, L,G and P*, L*, G** respectively. The relative position of the methyl or C=O of the prolyl-acetyl unit should alter the local magnetic environment of surrounding atoms and therefore direct shifts in chemical changes shift changes of the α, β and δ protons of P_1 as well as the α and amide protons of L_2 and G_3. Consequently, in line with expectations, the prolyl-acetyl $C^{\alpha}H$ of the *trans* isomer are shifted downfield relative to the chemical shifts of the *cis* isomer while P_1 $C^{\alpha}H$ and L_2 $C^{\alpha}H$ are shifted upfield. Integration of *cis* and *trans* isomer resonances gives an approximate 40% *cis* to 60% *trans* isomer proportions. Research on structural influences on *cis/trans* ratios in oligopeptides[141-143] suggest the local substitution around the proline residue as an important

determinant of the fraction of *cis* isomers in solution. Another factor of probably greater impact than the local substitution effect on the *cis/trans* ratio is the propensity of structure formation. Although detailed study of the structural influences on *cis/trans* ratio of TDP isomers was out of scope of this study, two additional but related peptide sequences were briefly studied to rule out the presence of Ac-PLG-OH as a hydrolysis product, giving rise to the observed extra set of resonances. NMR spectra of the tripeptide Ac-Pro-Leu-Gly-OH also shows same phenomenon as observed in TDP but with an almost equal proportion of *cis* and *trans* isomers. When the tripeptide, Ac-Pro-Leu-Gly-OH is extended with (*S*)-2-mercapto-4-methylpentanoic acid, the resultant Ac-Pro-Leu-Gly-SLeu-OH exhibits 40% *cis* to 60% *trans* isomer proportions as observed for TDP. Interestingly, in deuterated acetonitrile (CD_3CN), not only did TDP exist in its free thiol form but also exhibits only one set of resonances thereby simplifying the assignment of signals. This observation is however in contrast with studies on the effect of solvent on the conformational equilibrium of prolyl-peptide bond. Rittner et al[144] used both theoretical and NMR data to confirm that the conformational equilibrium of Ac-Pro-NHMe is affected by the solvent change as a function of the solvation variation of the solvents. However, the population of *trans* isomers in $CDCl_3$ (86.7%) is only about 10 % greater than in more polar solvents CD_3CN, DMSO-d_6 and D_2O with 75.5%, 68.6 and 75.8% *trans* isomer populations respectively.[144]

8.4 Evaluating the role of TDP in TDP-PEG4MAL hydrogel formation

8.4.1 Preparation of TDP-PEG4MAL hydrogels

Cytotoxicity is an important concern for the preparation of materials for biomedical applications. In this sense, concerns about the material's characteristics in the complex biological environment consisting of various chemical and physical parameters must be into taken into consideration. Of specific importance in the design of *in situ*

crosslinking/bioconjugation systems is the selection of the chemistry with particular attention to its robustness (proceed efficiently in the presence of other functional groups found in the biological milieu), ease of application and synthesis of required substrates. The application of *in situ* forming gels administered intramuscular or subcutaneously[17, 86] requires the use of reactive precursor solutions that gel rapidly under physiologically relevant conditions at the applied sites. This prevents the diffusion of precursor solutions and therapeutic drugs in case of drug delivery applications into surrounding tissues, which might cause potential immunogenic reactions. On the other hand, too rapid reaction rates, which are typical for thiol-Michael type reactions, could result in difficulties in the delivery of the reactive precursors, uneven hydrogel distribution and large network defects. Not many examples of latent crosslinking in material chemistry making use of small molecular weight latent crosslinkers combined with macromers are found in literature. A case can be made for uncured epoxy resins, which are mixed with curing agents known as latent initiators, to promote curing reactions under appropriate conditions e.g. light and temperature to form three-dimensional network structure.[145-147] These systems however differ significantly from our approach which, instead of further hardening a partially crosslinked material, uses pH changes as stimulus to initiate the crosslinking of pristine PEG4MAL macromer with a crosslinker precursor. The approaches involving dynamic covalent bonds for stimuli responsive material synthesis[123, 148, 149] makes use of reactants, which do not entirely fit the description of latent crosslinkers since these are already in their active forms.

Designing tandem/sequential reaction strategy with thiol-thioester exchange reaction (activation of latent crosslinker precursor) coupled to the final crosslinking thiol-Michael reaction serves two purposes. The TTE – thiol-Michael addition tandem reaction will not only ensure a controlled gelation but also provide the possibility to obtain an on-demand gelation system when suitable conditions are met.

Since the fate of the initiation TTE reaction is determined partly by the concentration of available thiolate ions, a pH-promoted gelation was realized. Analogous to this coupled reaction system for *in situ* gelation is the NCL-thiol-Michael addition reaction. Although this system is chemoselective, it presents some level of rigidity as far as the substrates for the generation of active thiol is concerned. Unlike our proposed TTE-thiol-Michael reaction strategy, which involves 2 reactions, the NCL-thiol-Michael system proceeds via 3 reactions in order to realize successful gelation. This is because the activation process in the NCL strategy to generate an active thiol explicitly demands the presence a 2-aminoethanethiol moiety in the precursor as a requirement for the *S*→*N* acyl transfer through a 5-membered ring transition state.[150]

The use of a peptide mimetic in the design of self-activating single TTE substrate also confers a tuning function that can be exploited to control the activation and overall gelation kinetics by varying the type and sequence of the amino acid residues around the thioester moiety. In contrast to designed thiodepsipeptide, standalone TTE reaction with single *α,ω*-thioester/thiol bearing substrates explicitly yields polythioester polymers with accompanying application limitations.[132]

8.4.2 Gelation kinetics and physical properties

Fast reaction rates for the 1, 4-addition of a thiol to the *β* position of an *α, β*-unsaturated carbonyl and therefore explains why it is a widely investigated approach for injectable hydrogel preparation. The success of this crosslink under physiological conditions makes it particularly interesting for *in vivo* applications.[82] Acrylates, vinyl sulfones and maleimides are the most reported *α,β*-unsaturated electrophiles that have been used in conjunction with thiols for the preparation of injectable hydrogels.[151-153] Although acrylates are the most commonly used

Michael acceptors for thiol-Michael reactions, the reaction rates offered by vinyl sulfones[56, 72, 154, 155] and maleimides[156, 157] are superior.

Our choice of maleimide functionalized 4-arm PEG macromers for gel formation is an attempt to maintain a fast gelling system since an initial step of crosslinker activation is involved and is principally the rate-determining step. From the gelation times, dependence of TTE, and thiol-Michael reactions, hence the dependence of the crosslinking reaction on the pH of the reaction medium can be observed. This is in line with the fact that both reactions required *a priori* generation of thiolate ions for the initial nucleophilic attack on the thioester carbonyl carbon[105] followed by a subsequent addition reaction to the maleimide units attached to the PEG assuming a base-catalysed addition mechanism.[49] The thiol-maleimide reaction is reliant on the thiol *p*Ka, because the thiol/thiolate equilibrium is modulated by buffer pH, in agreement with the Henderson-Hasselbalch theory.[158, 159] Since only one buffer was employed, the effect of the conjugate base on gelation kinetics could not be accessed, but changing the solution pH should have an effect on the speed of gelation. The choice of pHs between 6 and 9 is in line with the range of pH for physiological reactions. As expected, more basic pHs increased the reaction speed, while acidic pHs slowed the reaction. In comparing the conjugate base strength however, citrate buffer with a weaker conjugate base has been reported to decrease gelation rate of thiol-maleimide type gels relative to PBS buffer with a stronger conjugate base.[160]

When the TDP content was increased, faster gelation times were observed. The use of buffered media for both TDP activation and TDP-PEG4MAL gel formation excludes the potential toxicity issues associated with organic bases such as the commonly used TEA in thiol-Michael reactions.[64, 82, 161] Gelation times of 30 min, 5 min and 3 min were observed at pHs 7.5, 8.0 and 8.5 respectively when a thiol: maleimide molar ratio of 2:1 assuming complete conversion of TDP was used. Alteration thiol-ene ratio is one of the commonest methods of tuning thiol-ene hydrogels.[162-164] For systems where dithiol crosslinkers were directly employed, faster gelation

times between seconds to 1 min were recorded even for 1:1 thiol: maleimide molar ratio for similar PEG4MAL macromers.[79]

García and co-workers[161] compared the advantages of vinyl sulfones and maleimides over acrylates in preparing functional PEG hydrogels using thiol-containing adhesive peptide (GRGDSPC) with acrylate, vinyl sulfone or maleimide functionalized 4 –arm PEG using TEA as an organic base. In the presence of 4 mM triethanolamine (TEA), gelation kinetics depended considerably on the choice of Michael acceptor, ranging from 1–5 min for PEG4MAL, 30–60 min for PEG4VS, and 60 min for PEG4AC. Furthermore, the maleimide-based hydrogels required two orders of magnitude less TEA to gel, a significant attribute considering the known cytotoxic effects of TEA on endothelial cells, cells in the ovarian follicles, and pancreatic islet cells.[79]

Gels formed were analysed by equilibrium swelling in water and by rheometry. The latter was performed on swollen gels to obtain storage and loss moduli (G' and G''). The data obtained for all gels are characterized by G' exhibiting a plateau in the frequency range studied and by a G'' that is 1 to 3 orders (depending on the preparation conditions) of magnitude smaller than G'. An increase of G'' at higher frequencies was observed for gels with no correlation on the pH nor thiol: maleimide molar ratio. Since G' was constant at frequencies below 1 Hz for all gels, regardless of preparation state and thiol: maleimide molar ratio, G' in the constant frequency range was taken as equal to G_e, the equilibrium modulus of the network. The final network properties were slightly dependent on the pH of cross-linking. Although differences in swelling were not significant, rheological measurements revealed that networks formed at pH 8.0 had the highest equilibrium moduli. Although values of G_e for gels formed at pH 8.5 were lower than for those formed at pH 8.0 for a thiol: maleimide molar ratio of 1:1 molar ratio, the opposite was observed when the thiol: maleimide molar ratio was increased to 2:1.

TTE reaction rate increases with pH, and possible disulfide formation as thiol content increases could be reason for lower G_e.

The decrease in G_e at lower pH might be explained by both insufficient active crosslinker production due to slow reaction rate at lower pH resulting in a lower crosslinking efficiency.

The combination of pH and thiol: maleimide ratio gel preparation conditions provided gels with G_e values that ranged between 200 – 5000 Pa. Additional parameters for tuning the physical properties of TDP gels would include polymer weight concentration and variation of thiol-Michael acceptor[72] and variation of buffer systems[160] thereby providing more control over the tunability of these gels. The potential of these gels for biomedical related applications stem from the fact their G_e values compare favourably with the elasticities of certain tissue microenvironments for example brain tissue 200 – 1000 Pa and adipose tissue 2500 – 3500 Pa.[165]

8.5 MMP-promoted gelation using PEG-thiodepsipeptide conjugates

8.5.1 Design, synthesis and characterisation of 4-arm PEG-thiodepsipeptide

From the initial argument, use of peptide sequences provides a more robust alternative pathway for stimuli-promoted *in situ* gelation and biomolecule bioconjugation because:

1. simple modifications in the sequence structure results in dramatic changes in property even on the macro scale
2. property changes can also be achieved in short peptide sequences for which structure-property relationships can be determined beforehand with simulation studies unlike traditional polymers due to size reasons, hence aiding fast screening of potential sequences for particular function or application and
3. the ease of synthesis automation of peptides makes the development of peptide-based materials for biomedical applications less laborious.

The applicability of this assertion as demonstrated for the pH-promoted gelation of TDP in coupled TTE- thiol-Michael reaction was subsequently extended for MMP-cleavage-thiol-Michael gelation. The self-activation property of TDP in the pH-promoted gelation application is switched in favour of its MMP-sensitivity. By design, removal of the cysteamine unit deactivates TDP self-activation. The susceptibility of TDP sequence towards MMP cleavage of the thioester bond was exploited for the design of an MMP-sensitive crosslinker precursor by the functionalization of 4-arm PEGNH_2 with the peptide sequence. A 4-arm PEG core 2 kDa was chosen to aid the solubility of the conjugate without affecting enzyme's access to peptide sequence, which might result in the case of long polymer chains. It was assumed that long polymer chains could promote the possible formation of micelles with the peptide moieties being embedded in the micelle core.

Simplified solution phase peptide coupling strategies with minimal purification procedures were enough to obtain PEG4TDP$_o$ in good yield and purity. Successful cleavage of a minimum of two peptide molecules on the PEG-peptide conjugates creates a thiol-bearing crosslinker for a thiol-Michael addition gelation. In an initial design of the multi-arm TDP for MMP-promoted gelation, 1,1,1-tris(aminomethyl)ethane (TAME) core was functionalised with the TDP sequence. The resulting increase in the hydrophobic nature of the formedt molecule however required the use of high concentration of surfactant molecules to solubilize, thereby stressing the effect of minute modifications in peptide structure on the properties of the resultant peptide. The effect of such minute modifications on the molecular level were barely visible during structural elucidation especially when results of NMR characterisation of TDP, 3-arm TDP on TAME core and PEG4TDP$_o$ were compared. Identification and confirmation of molecular structure of PEG4TDP required less characterisation time due to the homology of the signals.

8.5.2 PEG4TDP$_o$ enzymatic cleavage and gel formation

To gain insight into the susceptibility of PEG4TDP$_o$ towards MMP hydrolysis, cleavage studies with MMPs -2 and -9 were conducted using a highly sensitive flourometric thiol assay kit. The propensity of vertebrate gelatinases to also hydrolyse thioesters and the reported k_{cat}/K_m ratio in excess of 10^4 $M^{-1}s^{-1}$[1130] are key to the use of Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt as an important assay molecule for MMPs -2 and -9 activity. The initial attempt of functionalising TAME with the TDP sequence was to obtain a 3-arm crosslinker precursor with increased probability of generating a crosslinker with at least two active arms for crosslinking. An added consideration on the use of TAME was to limit the size of the resultant molecule as well as maintain its lipophilicity which is mostly required by MMPs.[166]

The thiodepsipeptide functionalised TAME substrate was hydrolysed by MMPs -2 and -9 at concentrations (0.25 to 8 μM) as evident from flourometric kinetic measurements. Due to the limited solubility of this substrate at higher concentrations, its use for MMP-promoted gelation studies could not be realised. PEG4TDP$_o$, despite its larger molecular mass, addresses the solubility challenges with the introduction of a small PEG core. Hydrolysis of PEG4TDP$_o$ with MMPs -2 and -9 was also studied with flourometric assay similar to the TDP functionalised TAME.

Data from kinetic measurements when fitted with the usual Michaelis-Menten fit produced large deviations thereby making it impossible to extract the cleavage kinetic parameters. Consequently, the extraction of kinetic parameters via the Lineweaver-Burk plot was not possible. In a typical enzyme-promoted gel formation, a gelling mixture comprising PEG4MAL, PEG4TDP$_o$ and enzymes in 100 μL tris buffer was monitored for gel formation during incubation at 37 °C. Table 1 shows results from gel formation in the presence of varying concentrations of MMPs -2 and -9 where t_{onset} and t_{gel}, represent times for onset of gelation and final hardening of gelling mixtures respectively. The observed gel formation signifies cleavage

of the thioester bond of the thiodepsipeptide sequence between G_3 and thio-leucine derivative, L_{4S}. The resulting active thiols react fast with maleimide bearing PEG chains to form crosslinks. Both t_{onset} and t_{gel}, were lower for reactions involving MMP-2 than MMP-9 indicating a possible higher sensitivity of the peptide sequence towards cleavage by MMP-2. Cleavage of *G-L* bond is reported across several MMPs albeit with varying specificities depending on the MMP used. Experimental evidence of preference of MMP-2 towards cleavage of *G-L* bond for PLGLAG sequence is in line with the observed faster gelation with MMP-2 over MMP-9.[167] Although the bonds cleaved in these studies and ours are amide and thioester respectively, the direct comparison stems from the structural similarity of leucine and thio-leucine at the P_1'position of the substrates. MMPs however cleave a broad range of peptide and protein bonds not only limited to the *G-L* bond.[168] For substrates containing the *G-L* bond, enzyme specificity depends on not only the primary structure (type of amino acid at prime and non-prime positions) but also on the length of the peptide. [130, 167, 169] Subsequently scrambled sequences of PLGLAG with *G-L* bond were neither MMP-2 nor MMP-9 active.[167]

Fast gelling maleimide functionalized PEG macromers with thiol-based crosslinkers[158, 160] have been reported as ideal for *in situ* applications,[17, 87] .However, the lack of control over the structural homogeneity of these gels and difficulty in handling make them less unattractive. The combination of gel forming precursors with fast reacting orthogonal moieties but with an intermediate orthogonal activation step for one of those moieties[170] promises a regain of that control. Gelation times between 3 and 30 minutes depending on pH and thiol: maleimide molar ratio variations were obtained with the maleimide-thiol coupling system, which included an intermediate thiol activation via thiol-thioester exchange reaction.

The gelation rates are observed for the MMP-promoted PEG4TDP$_o$ –PEG4MAL system are about two times more those observed for MMP-assisted supramolecular hydrogelation of amphiphilic peptide precursors.[171] Moreover, this two-step/cascade reaction system provides a

more controlled gel formation. Depending on the specific application, the gelation times and the final physical properties of the gels formed can be further tuned using variations in polymer weight concentrations, thiol: maleimide molar ratio and use of different molecular weight maleimide functionalized polymer .

The gelation of enzyme-free control experiments although after extended incubation times of 60 mins indicated possible non-enzymatic hydrolysis of PEG4TDP$_o$. as reported by Weingarten et. al.[130] observed not more than 25 % hydrolytic contribution to the total observed hydrolysis in the first 5 minutes of Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt cleavage using vertebrate collagenases between pH 6.5 and 7. Non-enzymatic hydrolysis in PEG4TDP$_o$ cleavage studies using flourometric assay were limited to 30 % even at 8 μM substrate concentration and after 45 minutes of reaction time. At increased substrates concentrations and for extended reactions times, the non-enzymatic contribution is expected to be significant, hence the observed gelation seen in the control experiments after 60 minutes.

MMP-catalysed hydrogelation systems driving by the physical assembly of amphiphilic peptide derivatives,[171] present promising and potential alternative avenue for the synthesis of soft biomaterials in addressing cancer metastasis. However, the physical nature of the gels and relatively slow crosslinking rates remain a challenge. The use of thiodepsipeptide based crosslinker precursor not only addresses these issues but could provide a basis for the development of superior approach especially if all aspects of structure-function relationship, substrate recognition and gelation kinetics are fully understood.

9 Summary and outlook

A novel approach to realising a pH and MMP-promoted on demand *in situ* gelation and bioconjugation system based on coupled activation– crosslinking (thiol-Michael reaction) using peptide-based latent crosslinker precursors and maleimide functionalized 4-arm PEG

macromers is reported. The importance of applying a top-down approach in the design of such a system and in this case, firstly considering the final potential application with all related factors, then followed by analysing the means and ease of achieving the targeted functions is clearly depicted in how the design of the respective pH and MMP activatable molecule fulfilled their desired functions. Using targeted function to structure design approach also enabled the incorporation of stimuli (pH and enzyme) activation into the design TDP. It also became apparent how the numerous possibilities of directing and tuning the function of a particular TDP can be achieved by exploiting its sequence structure. The tunability of the structure-function relationship afforded by primary and higher order structure peptide sequences was exploited for the design of two separate stimuli activatable gelation systems. The versatility of peptide-based precursor molecules for the design and synthesis of *in situ* stimuli-promoted soft materials is demonstrated in how easily a single peptide sequence structure with little modification can be adapted for different functions.

First with biomedical applications in mind and keeping to the 'KISS' principle, a bioconjugation and crosslinking system was designed that takes into account the state-of-the-art by not only advancing the current strategies but also opening new and interesting possibilities in the field. TDP was designed as a moderately sized (ca. 700 $gmol^{-1}$) peptide with capabilities closely related to prodrugs by (1) behaving as a latent/inactive form of the active crosslinker moiety achieved by the esterification of the internal thiol with the tripeptide, Ac-PLG-OH (2) possessing a carrier function due to the improvement of water solubility conferred on the highly hydrophobic active dithiol-peptide also by the tripeptide, Ac-PLG-OH and (3) possessing an on/off switch and self-trigger function by virtue of pH sensitivity and the terminal cysteamine moiety. The propensity of the designed TDP to possess all these mentioned attributes required careful theoretical evaluation of the various aspects from applicable chemistry, material selection and synthesis. The synthesis of the designed TDP was

achieved by standard solution phase carboxylic acid-amine/thiol coupling and protecting group strategies. During synthetic steps where both amino and thiol groups were simultaneously present, protecting groups that could be cleaved under diametrically opposed pH conditions were used.

Detailed structural elucidation of the synthesized TDP using NMR, ESI-MS and FT-IR confirmed the validity of the expected peptide structure. The correct structure of the TDP was only confirmed when polar aprotic solvent such as acetonitrile were used in the structural evaluation. In polar protic solvents such as DMSO, mixture of structures to *cis* and *trans* isomers of TDP, the disulfide as well as possible 'pseudo' intramolecular TTE reaction products were observed. This signified the role of the terminal cysteamine unit (self-trigger component) and thiolate ions in initiating this intramolecular TTE given the high chance of thiolate ion generation from thiols in polar protic solvents. The consequence of the presence of peptidyl-prolyl residues in peptides on the ease of NMR characterization was seen in TDP and related tri -and tetra-peptides. A number of factors vis-à-vis the local substitution around the proline residue, peptide length and solvent significantly affects the *cis-trans* isomerization of peptidyl-prolyl bond. Prior knowledge of these factors is therefore a useful tool in studies using containing peptides/protons since the challenges presented for example by the complexities in analytical data might lead to unwarranted doubts in obtained results. In probing the external activation of TDP with a group of thiols, two of the most prominent thiols found in biological systems LCYS and GSH competed favourably in an intermolecular exchange reaction against TDP's 'pseudo' intramolecular reaction. This resulted in an overall increased concentration of the exchange product of interest, the dithiol crosslinker. Although, the *p*Ka values of thiols on LCYS, and GSH are relatively similar, results suggested intermolecular exchange of TDP with LCYS and GSH were favoured over the 'pseudo' intramolecular exchange of TDP molecules only. This observation could be largely attributed to the relative smaller sizes of LCYS and

GSH to TDP considering steric factors and hence their ability to preferentially attack the thioester carbonyl. However, TTE reactions with acidic thiols (*p*-NTP and 4MBA) showed an opposite trend with a significant reduction in the concentration of desired dithiol crosslinker. This observation can be explained by the inability of formed thiol-thioester tetrahedral intermediate to dissociate into exchange products and is in line in *p*Ka requirements for successful TTE reaction. This study also highlighted the usefulness of the complementarity of high resolution ESI-MS and NMR as powerful tools for monitoring and elucidation of chemical structures in complex chemical reaction mixtures.

For application purposes, TDP was successfully used for pH promoted gel formation maleimide functionalized 4-arm PEG in tris-buffered medium. Gelation kinetics, hydrogel equilibrium swelling and mechanical properties (η^*, G', G'') were found to have direct correlation with the pH of the reaction medium. The observed TDP-PEG4MAL gelation time of 3 min with 10 wt % 20 kDa PEG4MAL at pH 8.5 affords a system fast enough to enable controlled and consistent gel formation. Significant increase in the gelation can be achieved if the thiol: maleimide molar is increased.

The multi-functionality of the thiodepsipeptide sequence was shown in the synthesis of the MMP-activatable PEG4TDP$_o$ crosslinker precursor. The ease of using a single core peptide sequence to realise different functionality by modifying one end of the sequence was demonstrated. By design, removal of cysteamine end group on TDP and functionalization of a 4-arm PEGNH_2 with the core sequence, Ac-Pro-Leu-Gly-SLeu-Leu-Gly- yielded a 4-arm thiodepsipeptide PEG conjugate, PEG4TDP$_o$. Synthesis was accomplished using standard solution phase peptide coupling strategy with the uronium based coupling agent, HATU to obtain intermediates and final product in good to excellent yields without complicated purification procedures. Consequently, characterization of PEG4TDP$_o$ structure using detailed NMR and ESI-MS studies was made easy due the structural similarity of TDP and PEG4TDP$_o$,

thereby allowing in most instances direct comparison of results. Similar *cis-trans* isomerization of the thiodepsipeptide moiety in TDP was observed for PEG4TDP$_o$ with 40 % *cis* to 60 % *trans* isomer distribution.

PEG4TDP$_o$ was active with MMPs -2 and -9 in cleavage studies conducted with flourometric assay. Determination of the enzyme cycling parameters, k_{cat} and K_m was however not possible due the non-Michaelis-Menten nature of cleavage kinetics. This sigmoid type kinetics, which was also observed for the blanks, could most likely be due to interference from the flourometric assay molecules. Additional work would be required to accurately characterize PEG4TDP$_o$ cleavage kinetics.

Successful MMP-promoted gelation of 4-arm maleimide functionalised PEG (PEG4MAL, 20 kDa) with PEG4TDP using MMPs -2 and -9 was confirmed. Average gelation time using 10 % w/v polymer solutions was about 30 minutes with possible room to tune through the variation of polymer molecular mass, polymer weight concentration and polymer: PEG4TDP$_o$ molar ratios.

Depending on the design of the precursors, this concept could be extended to the controlled delivery of active molecules needed for other robust and high yielding crosslinking reactions for biomedical applications. Generally, this sequentially coupled functional system could be seen also in the transport and on-demand release of biologically active yet hydrophobic molecules as intermediate substrates required for further biotransformation or reaction processes.

Application for this sequentially coupled functional system could be seen e.g. in the treatment of inflamed tissues associated with urinary tract like bladder infections for which pH levels above 7 were reported.[172] By the inclusion of cell adhesion peptide motifs, the hydrogel network formed at this pH could act as a new support layer for the healing of damage epithelium as shown in demonstration experiments.

Results from MMP-promoted gelation system unveils a simple but unexplored approach for *in situ* synthesis of covalently crosslinked soft materials that could lead to the development of an alternative pathway in addressing cancer metastasis by making use of MMP overexpression as a trigger. This goal has so far not being reached with MMP inhibitors despite the extensive work this regard.

10 References

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11 Appendix I - Thiol Michael-type reactions of optically active mercapto-acids in aqueous medium

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Thiol Michael-Type Reactions of Optically Active Mercapto-Acids in Aqueous Medium

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Abstract

Defined chemical reactions in a physiological environment are a prerequisite for the in situ synthesis of implant materials potentially serving as matrix for drug delivery systems, tissue fillers or surgical glues. 'Click' reactions like thiol Michael-type reactions have been successfully employed as bioorthogonal reaction. However, due to the individual stereo- electronic and physical properties of specific substrates, an exact understanding their chemical reactivity is required if they are to be used for in-situ biomaterial synthesis. The chiral (S)-2-mercapto-carboxylic acid analogues of L-phenylalanine (SH-Phe) and L-leucine (SH-Leu) which are subunits of certain collagenase sensitive synthetic peptides, were explored for their potential for in-situ biomaterial formation via the thiol Michael-type reaction.

In model reactions were investigated the kinetics, the specificity and influence of stereochemistry of this reaction. We could show that only reactions involving SH-Leu yielded the expected thiol-Michael product. The inability of SH-Phe to react was attributed to the steric hindrance of the bulky phenyl group. In aqueous media, successful

reaction using SH- Leu is thought to proceed via the sodium salt formed in-situ by the addition of NaOH solution, which was intented to aid the solubility of the mercapto-acid in water. Fast reaction rates and complete acrylate/maleimide conversion were only realized at pH 7.2 or higher suggesting the possible use of SH-Leu under physiological conditions for thiol Michael-type reactions. This method of in-situ formed alkali salts could be used as a fast approach to screen mercapto- acids for thio Michael-type reactions without the synthesis of their corresponding esters.

INTRODUCTION

Generally, the term "thiol−Michael click chemistry" describes the reaction of thiol containing compounds with α,β conjugated carbonyls with high conversion rates [1]. The capability of thiol Michael-type reactions to proceed rapidly at conditions compatible tissue and the facile characterization of reaction mechanisms and products are some of the advantages. [2, 3] Compared to other biorthogonal functional groups such as strained cyclooctynes [4-6] in strain promoted azide-alkyne click reactions (SPAAC), macromolecular precursors can be quite effectively functionalized with thiol and Michael-type acceptors, which makes thiol Michael-type reactions attractive orthogonal reactions for biomedical application. The versatility of thiol Michael-type reactions is afforded largely by the weak sulfur-hydrogen bond, which enables the Michael-thiol addition to be carried out under a wide variety of conditions suitable for many reaction partners. The versatility, scope and application of this reaction have been concisely reviewed in the literature [1, 7].

The 'Click' nature of this reaction however does not guarantee a successful formation of thiol-Michael addition product with every thiol/mercaptan. The thiol Michael-type reaction rate is largely affected by the pKa of the thiol as well as its molecular structure regardless of the reaction pathway. Depending on the chemical nature of the catalyst used, the thiol Michael-type reaction proceeds via a base- or nucleophile catalyzed reaction pathway [8]. In a base catalyzed reaction, a common base abstracts a proton from the thiol to generate a thiolate anion, along with a conjugate acid [9-11]. The thiolate anion, which is generally a strong nucleophile, initiates the addition of the anion across the electron-deficient beta-carbon of the ene to yield an intermediate carbon-centered anion which, being a strong base, abstracts a hydrogen from the conjugate acid to yield the thioether as a product. The presence of protic species in comparable concentrations as the catalyst such as a strong acid is therefore a drawback in this pathway.

The suitability of the different thiols (aromatic thiols, thioacetates, thiopropionates and aliphatic thiols) with respect to their reactivity towards various α,β conjugated carbonyl systems was extensively investigated, however, in organic media and the mercapto-carboxylic acids often used are in the form of their corresponding esters [8-11] requiring additional, laborious synthetic steps.

In this work, we explored the thiol Michael-type reactions of (*S*)-2-mercapto- carboxylic acid analogues of L-phenylalanine (SH-Phe) and L-leucine (SH-Leu) with 2- (2-ethoxyethoxy) ethyl acrylate (PEG-AC) and methoxy polyethylene glycol maleimide (PEG-MAL) as Michael acceptors in both organic and aqueous media. It is hypothesized that, by neutralizing these acids with a suitable base to pHs just slightly above neutral value, the thiol-Michael-type addition can be effected by making use of excess base as the catalyst

for thiol deprotonation. Although structurally similar, the electronic differences introduced by the isopropyl and phenyl units in SH-Leu and SH-Phe is furthermore expected to influence the overall reactivity. The chiral thio-analogues of hydrophobic amino acids are of interest as subunits of peptido-mimetic thioesters [12] and their potential for bioorthogonal reactions via thiol Michael-type reaction particularly under aqueous conditions.

Here, we have circumvented the interference of the protic carboxylic acid functionality and the need to synthesize the esters of the mercapto-acids by using NaOH pH-conditioned solutions. Above neutral pH, a slight excess of hydroxide ions remain to act as a catalyst for the Michael addition reaction using acrylate and maleimide based Michael acceptors chosen specifically for their differences in reactivity.

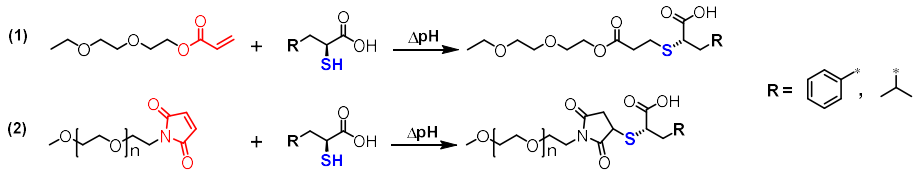
EXPERIMENTAL DETAILS

Materials

SH-Leu and SH-Phe were synthesized according to methods described in ref. [13, 14] using D-leucine and D-phenylalanine from Iris Biotech (Marktredwitz, Germany). Diethylene glycol ethyl ether acrylate (PEG-AC), methoxy polyethylene glycol maleimide (PEG-MAL), acetonitrile, deuterated solvents water (D_2O), chloroform ($CDCl_3$) and dimethyl sulfoxide (DMSO-d_6), triethylamine (TEA), trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Munich, Germany). All chemicals were used as received.

Methods

Apart from the synthesis of the mercapto-acids, all thiol Michael-type reactions were performed at room temperature and under normal atmospheric conditions without special precautions. A detailed procedure for thiol Michael-type reactions according to the reaction equations depicted in Scheme 1 is described below.



Scheme 1: General reaction scheme of PEG-based Michael acceptors with mercapto-acids

Characterisation

Pseudo-2D ^{1}H-NMR spectroscopy was used to determine the substrate conversion. NMR spectra (^{1}H and ^{13}C) were recorded on a DRX 500 Avance spectrometer (Bruker, Rheinstetten, Germany) at 25 °C in deuterated NMR solvents. Reaction monitoring spectra were acquired in arrayed mode using the Bruker zg2d pulse program (pseudo-2D) with 20 experiments recorded in 4 minute intervals. Each experiment/spectrum was acquired with 8 scans and a 15 s relaxation delay. Unless otherwise noted, duration between addition of reagents and completion of the first experiment was approximately 8 minutes. All chemical shifts are reported in ppm ( ) relative to tetramethylsilane (TMS), referenced to the chemical shifts of residual solvent resonances (^{1}H and ^{13}C). Reported errors on conversions were calculated assuming a ±5% accuracy of signal integrals.

ESI-MS was carried out using Bruker Impact II quadrupole/time-of-flight (QqTOF) mass spectrometer equipped with an atmospheric pressure ionization source operating in the nebulizer assisted

electrospray mode and was used in negative/positive ion mode.

Reversed-phase High Performance Liquid Chromatography (RP-HPLC) was carried out on a Dionex Ultimate 3000 system (Dionex Softron GmbH, Germering, Germany) equipped with a LiChrospher® 100 RP-18 (5 µm) column operating at 25 °C, using water/acetonitrile w/ 0.1% TFA gradient. Signals were monitored at 220 nm and 254 nm.

Triethylamine (TEA) catalyzed reactions of diethylene glycol ethyl ether acrylate (PEG-AC) and SH-Leu in DMSO and D_2O

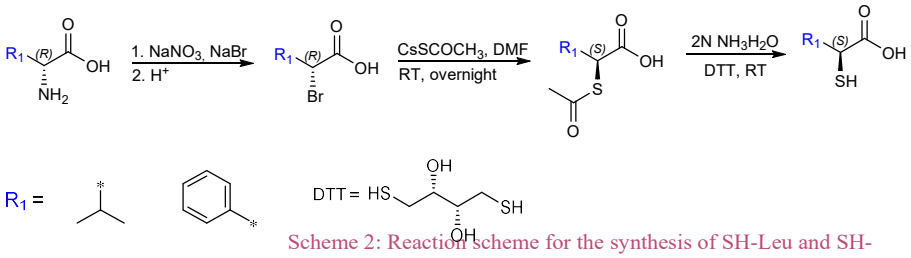
300 µL each of 0.53 M stock solutions of both reagents PEG-AC and SH-Leu in DMSO/D_2O was pipetted into the NMR tube and different mole equivalents (0.1, 0.5 and 1.0) of TEA was added before the experiments were started. Timings for blanks without the TEA catalyst were carried out right after the mixing of the two reagents.

Reactions of PEG-AC and methoxy polyethylene glycol maleimide (PEG-MAL) with SH-Leu at different pHs in D_2O

0.53 M stock solutions of SH-Leu of different pHs were prepared by first mixing weighed amount of SH-Leu into 1 mL D_2O. The pH was then adjusted with NaOH solution and additional D_2O to obtain the required concentration. 300 µL of these solutions were then pipetted, mixed with 300 µL 0.53 M PEG-AC and PEG-MAL solutions and ^{1}H-NMR spectra taken afterwards.

RESULTS AND DISCUSSION

The syntheses of the mercapto-acids, SH-Leu and SH-Phe were accomplished as shown in Scheme 2. (*R*)-α-amino acids were converted to their corresponding (*R*)-α- bromo acids via a diazotization reaction with retention of configuration followed by substitution with cesium thioacetate (via reaction of $CsCO_3$ and thioacetic acid) with inversion of configuration [14]. The acetyl group was removed with aqueous ammonia to give the thiols.



Scheme 2: Reaction scheme for the synthesis of SH-Leu and SH-Phe

^{1}H-NMR monitored kinetic experiments

TEA catalyzed reactions were conducted using deuterated DMSO and D_2O separately. Prior to obtaining spectra in arrayed mode, 1D spectra of all reactions were obtained in order to determine parameters needed to set up the pseudo-2D experiments. This helped to reduce the preparation times by skipping the wobbling procedure for the pseudo-2D experiments. The first spectrum/experiment for all reactions was however

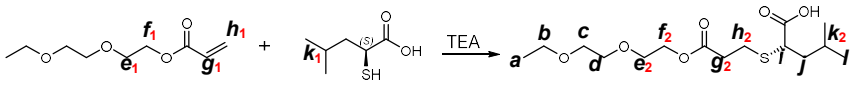
completed after approximately 8 minutes since shimming was carried out on each reaction. For clarity of presentation, conversions are reported for the first, 10th and last spectra recorded (hence 8 min, 44 min and 84 min respectively after mixing reagents) as seen in Table 1.

Reactions were monitored with respect to the acrylate vinyl protons g_1 and h_1, which were compared with the corresponding product protons g_2 and h_2 as seen in Figure 1. Final conversions were however calculated with signals from methylene (-CH_2) protons f_1 from the reactant at δ = 4.22 ppm compared with the product methylene protons f_2 at δ = 4.13 ppm. Similar shifts in signals can be seen for methylene and methanetriyl protons $e_1$and k_1 respectively after product formation to corresponding e_2 and k_2.

Table 1: Results of Michael-thiol reactions using PEG-AC and SH-Leu in DMSO and D2O *

Code**	TEA (µL)	pH	Conversion (%)		
			8 mins	44 mins	84 mins
DMSO-1	0	ND	NR	NR	NR
DMSO-2	2.3 (0.1 eq)	ND	60±3	79±4	84±4
DMSO-3	11.2 (0.5 eq)	ND	82±4	87±4	87±4
DMSO-4	22.3 (1.0 eq)	ND	81±4	82±4	82±4
D_2O -1	0	2.5	NR	NR	NR
D_2O -2	2.3 (0.1 eq)	3.8	NR	NR	NR
D_2O -3	11.2 (0.5 eq)	6.0	21±1	27±1	32±2
D_2O -4	22.3 (1.0 eq)	8.1	76±4	77±4	78±4

*0.53 M stock solutions of PEG-AC and SH-Leu in DMSO-d_6 or D_2O were used
** DMSO-x: DMSO was used as solvent, D_2O-x: D_2O was used as solvent
ND: Not determined
NR: No reaction product



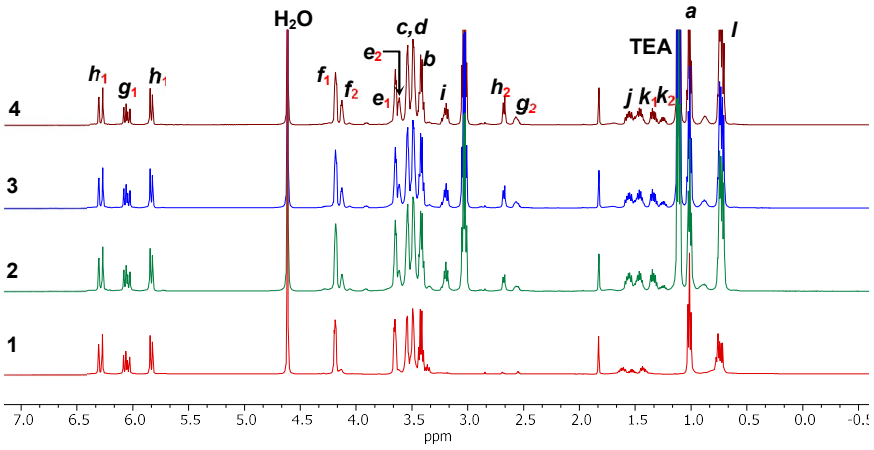


Figure 1: Overlay of ^{1}H-NMR spectra of PEG-AC and SH-Leu reactions in D_2O. (1) (blank without TEA) after 84 mins, 0% acrylate conversion, (entries 2-4 including 0.5 eq TEA, after (2) 8 min 21% ± 1 % acrylate conversion, (3) 44 min 27% ± 1% acrylate conversion, and (4) 84 mins 32% ± 2% acrylate conversion.

Results clearly show a strong dependence of the reaction on the amount of catalyst and solvent employed. Although amount of TEA used is well above catalytic values [9], this was necessitated due the presence of carboxylic acid which is expected to undergo deprotonation and salt formation before the deprotonation of the weaker thiol to generate thiolate ions. Chan et. al [9] reported the inability of the tertiary amine, TEA to catalyze thiol Michael-type reaction when the primary hexanethiol was used. The fast reaction kinetics observed using the primary hexylamine as catalyst in the same study however led to the suggestion of the presence of hybrid reaction pathway other than base-catalyzed process since TEA has more basic character than hexylamine. It can however be argued that the lower pKa of SH-Leu (assuming structural similarity to cysteine) could facilitate an improved thiolate anion formation as compared to hexanethiol. Nonetheless, considering the competing reaction of the stronger carboxylic acid with TEA, our observed acrylate conversion at lower TEA amounts could also point to the presence such alternative reaction pathways apart from a base-catalyzed mechanism or a combination of a number of possible mechanisms as noted by Northrop et. al. [15] The low conversions in D_2O compared to DMSO could be due to the poor solubility of the mercapto-acid (oily) in D_2O producing a two-phase system and possible reduced generation of thiolate ions or ion pairs required for the addition reaction. Thiol Michael-type reactions of SH-Phe with PEG-AC and the more reactive maleimide-based Michael acceptor, PEG-MAL did not result in the expected Michael addition product in both solvents (DMSO and D_2O) and at all TEA concentrations. Being the more hydrophobic of the two mercapto-acids, SH-Phe is additionally insoluble hence making its reactivity in D_2O more challenging. However, the lack of reactivity in DMSO could only point to differences in steric properties between SH-Phe and SH-Leu. If the TEA catalyzed reaction is via a hybrid system like the thiolate ion/Et_3NH^+ ion pair as pointed out by Northrop et. al., steric restrictions by the highly substituted TEA and SH-Phe makes the formation and stability of SH-Phe thiolate/Et_3NH^+ highly unlikely. Similar steric effects could also play a role in the inability of TEA to deprotonate SH-Phe for the generation of required thiolate anions.

Reactions of PEG-AC and PEG-MAL with SH-Leu at different pHs in D_2O

Based on the lack of reactivity for SH-Phe even in DMSO the subsequent experiments were conducted in D_2O at varied pH conditions with SH-Leu only. Stock solutions of SH-Leu in D_2O were carefully adjusted the required pH values using NaOH solution. The previously cloudy emulsion of D_2O and SH-Leu at acidic pHs turned completely colorless at pHs closer to and above neutral. Because of their enhanced acidity, carboxylic acids react with bases to form ionic salts. The salts have pronounced ionic character and are usually soluble in water when the bases used are alkali metal hydroxides [16] or simple amines. These pH adjusted SH-Leu solution was reacted with PEG-AC and PEG-MAL, and ^{1}H-NMR spectra were taken as previously explained. Apart from the reaction of PEG-AC with SH-Leu at pH 7.2, which proceeded very slowly with only (38 ± 2)% conversion after 84 minutes, all other reactions produced quantitative conversions after 8 min and it is possible that very fast kinetics could be involved and complete conversions reached even in seconds.

Quantitative conversion was observed after 8 min for reactions at pH > 7.2 and was detected by the complete disappearance of the acrylate vinyl proton signals with a concomitant appearance of the corresponding methylene signals of the product. Another distinguishing feature is the complete transition of reactant peak at δ=4.22 ppm to the product peak at δ=4.13 ppm. It is worth noting that although thiol proton signals were observed before as a doublet in $CDCl_3$ and DMSO-d_6, this signal could not be found in D_2O as expected due the probable rapid exchange of thiol protons with D_2O hence, thiol conversion could not be followed.

Table 2: Results of Michael-thiol reactions using PEG-MAL and SH-Leu in D2O at different pH values

Code	pH	Conversion (%)		
		8 min	44 min	84 min
PEG-AC-1	7.2	8.3±0.4	25±1	38±2
PEG-AC-2	7.5	85 ±4	95±5	97±5
PEG-AC-3	8.0	Qnt	Qnt	Qnt
PEG-AC-4	9.0	Qnt	Qnt	Qnt
PEG-MAL-1	7.2	Qnt	Qnt	Qnt
PEG-MAL-2	8.0	Qnt	Qnt	Qnt
PEG-MAL-3	9.0	Qnt	Qnt	Qnt

PEG-AC-x: Reactions with PEG-AC PEG-MAL-x: Reactions with PEG-MAL
Qnt: Quantitative yield

Table 2 summarizes the results of PEG-MAL reactions with different pH-adjusted solutions of SH-Leu in D_2O. The highly activated maleimide group undergoes complete conversion as seen in figure 2 even with 7.2 pH-adjusted SH-Leu compared to the acrylate substrate with lower reactivity. This was expected since the maleimide functional group is the most reactive Michael-type acceptor [9-11, 15, 17, 18]. The ^{1}H-NMR spectrum PEG-MAL/SH-Leu reaction product shows a complete disappearance of the maleimide protons but the formed succinimide proton peaks expected at δ=2.5 - 3.0 ppm were not observed. These peaks could be further shifted downfield and overlap with signals from the methoxy –CH_3 protons or signals from PEG polymer backbone. ^{13}C- NMR was used to additionally confirm product formation.

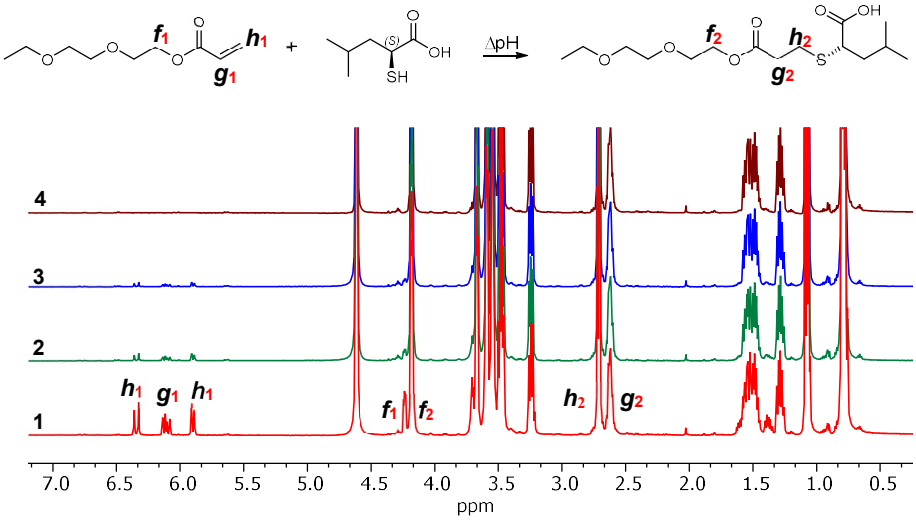


Figure 2: Overlay of ^{1}H-NMR spectra of PEG-AC and pH adjusted SH-Leu reactions in D_2O. (1-3)- Entry PEG-AC-1 (1) after 8 mins, 8.3% ± 0.4% conversion (2) after 44 mins, 25% ± 1% conversion and (3) after 84 mins, and 38% ± 2% conversion compared with entry PEG-AC-2 after 8 mins, quantitative conversion (4).

Electrospray Ionization Mass Spectrometry

ESI-TOF MS was run in both negative and positive ion modes to confirm product formation. In the negative ion mode, prominent signals observed correspond to the $[M-H]^-$ for SH-Leu, $[M-H^+]^-$ and $[2M-2H^++Na^+]^-$ ions whilst $[M+Na^+]^+$, $[M-HCO^-]^+$ and $[2M-H^++2Na^+]^+$ were observed as the main peaks in the positive ion mode for thiol- Michael-type addition product. Although ions for diethylene glycol ethyl ether acrylate were not observed, very strong signals of ions for the thio-carboxylic acid were observed (Figure 3). The supposed thio-leucine peaks (147.0490 Da) could also belong to double- charged ions from the product molecule with loss of water, $[M-2H^+-H_2O]^{2-}$. In the absence of a unique ionization/fragmentation pattern, the occurrence of the thio-leucyl ion peak could only be seen in case of incomplete conversion of the reaction partners. This observation however throws some doubt on the results obtained from NMR studies and therefore required additional characterization of the reaction mixture. When ESI-MS of PEG-AC and SH-Leu reaction mixture was rerun in the positive ion mode, mass spectra as in seen figure 3 produced two major peaks corresponding to the pseudo- molecular ion/dimers carrying sodium ions at 359 and 717 m/z respectively and a peak corresponding to possible loss of $[^+O{\equiv}COH]$ ions from the parent molecule. Signals belonging to the thio-leucyl ion or adducts thereof were not observed.

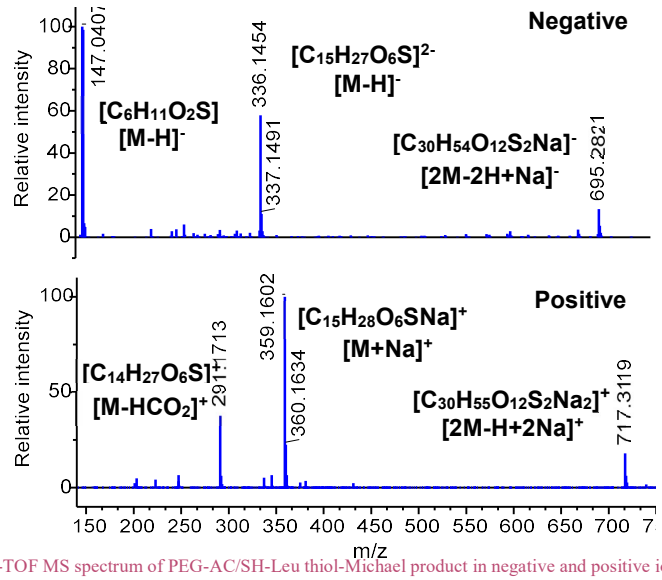


Figure 3: ESI-TOF MS spectrum of PEG-AC/SH-Leu thiol-Michael product in negative and positive ion modes. Peak at m/z 147.0407 corresponds to SH-Leu with loss of a proton

Figure 4 shows the ESI-MS spectrum of PEG-MAL/SH-Leu reaction mixture in the negative ion mode. The thiol Michael-type addition product upon complete reaction of equimolar mixture of PEG-MAL and SH-Leu, is expected to yield a mass shift of 148.06 Da. The ESI-MS spectrum of the commercially obtained PEG-MAL and PEG-MAL/SH-Leu product shows one main m/z distribution corresponding to $[M+OH^-]^-$ and $[M-HCO^+]^-$ respectively. Common to all the main series is the separation of peaks by ca. 44 atomic units of the PEG repeating unit. The observed mass shift when the different ionization patterns are considered is 148.03.

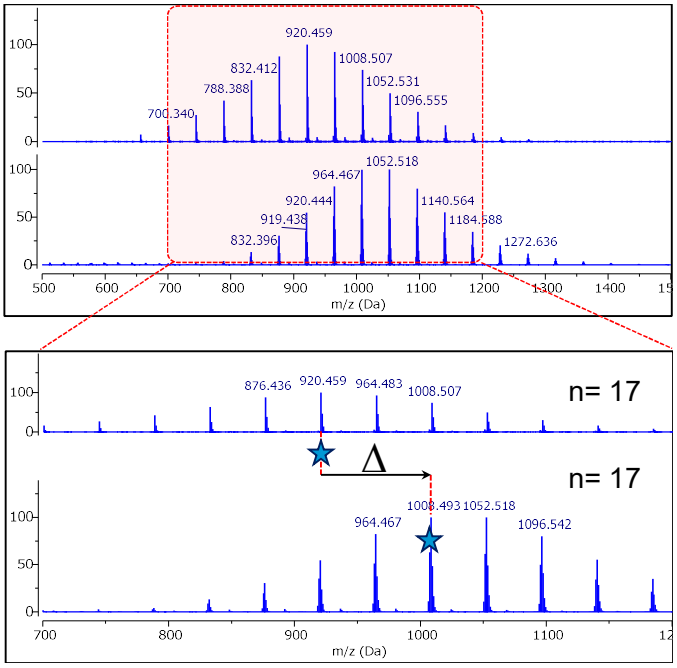


Figure 4: (A) Overlay of ESI-TOF MS spectra of PEG-MAL 750 and thiol-Michael addition product (B) Zoomed 700 – 1200 Da region showing change in mass for n = 17 PEO units

Reversed-phase High Performance Liquid Chromatography (RP-HPLC)

Additional RP-HPLC analysis of the reaction mixture PEG-Ac and SH-Leu was required to confirm the complete conversion of reaction partners as seen in both ^{1}H- and ^{13}C NMR studies. This was necessitated due to the presence of strong thio-leucyl ion peaks in ESI-MS of the PEG-AC/SH-Leu reaction mixture when analyzed in the negative ion mode. RP-HPLC with an analytical C18-column was hence carried out on the starting materials and the reaction mixture. Results (Figure 5 A) of the RP-HPLC using same water/acetonitrile w/ 0.1% TFA gradient and conditions clearly show all substances with different retention times. Peaks corresponding to both starting materials are completely absent in the reaction mixture chromatogram indicating the complete conversion for an equimolar mixture of PEG-AC and SH-Leu. ESI-MS of the collected product fraction shows an identical mass spectrum compared to the sample without HPLC analysis (Figure 5B).

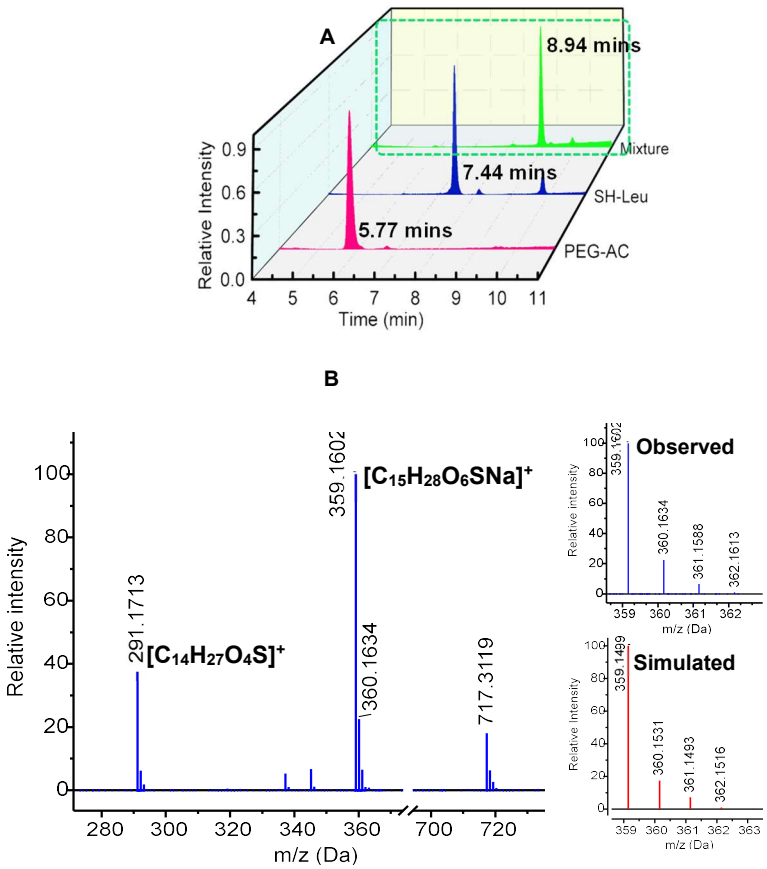


Figure 5: (A) 3D overlay of RP-HPLC chromatograms of diethylene glycol ethyl ether acrylate, thio-leucine (pH 7.5) and reaction mixture (B) ESI-MS of HPLC product fraction/peak with simulated and observed isotopic patterns compared

CONCLUSION

By studying the thiol Michael-type reaction of the chiral (S)-2-mercapto- carboxylic acid analogues of L-phenylalanine (SH-Phe) and L-leucine (SH-Leu) as subunits of certain collagenase sensitive synthetic peptides in aqueous solution, we could show that SH-Leu is a potential candidate substrate for the in-situ biomaterial formation via 'Click' reaction. The formation of the thiol Michael-type addition product was always preceded by carboxylic acid salt formation when catalysts were added. Whereas TEA catalyzed reactions might have proceeded via combination of base-catalyzed and hybrid ion pair reaction pathways, the base-catalyzed reaction pathway was at play for reactions involving NaOH. At optimum conditions (pH $\geq$ 7.2), the generation of the required thiolate ions and subsequent addition reaction is highly favored and could proceed to complete conversions in seconds, which can be explained by a base-catalyzed mechanism. The unsuccessful reaction of SH-Phe under all reaction conditions even in organic medium could only be attributed to the electronic and steric differences introduced by the bulky phenyl group compared to the isopropyl group for SH-Leu. Results of SH-Leu have demonstrated the possibility of conducting thiol Michael-type reactions on mercapto-acids without first converting them to esters in aqueous medium assuming their carboxylate salts have enhanced solubility. The use of in-situ formed alkali salts of such mercapto-acids eliminates the added work involved in the synthesis of their corresponding esters before

carrying out thiol Michael-type reactions.

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12 Appendix II - Reaction behaviour of peptide based single thiol-thioesters exchange reaction substrate in the presence of externally added thiols

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Research Letter

Reaction behaviour of peptide-based single thiol-thioesters exchange reaction substrate in the presence of externally added thiols

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Abstract

Identification of patterns in chemical reaction pathways aids in the effective design of molecules for specific applications. Here, we report on model reactions with a water-soluble single thiol-thioester exchange (TTE) reaction substrate, which was designed taking in view biological and medical applications. This substrate consists of the thio-depsipeptide, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) and does not yield foul-smelling thiol exchange products when compared with aromatic thiol containing single TTE substrates. TDP generates an **α**,**ω**-dithiol crosslinker in situ in a 'pseudo intramolecular' TTE. Competitive intermolecular TTE of TDP with externally added "basic" thiols increased the crosslinker concentration whilst "acidic" thiols decreased its concentration. TDP could potentially enable in situ bioconjugation and crosslinking applications.

Introduction

Dynamic covalent systems are inherently more robust than their supramolecular analogues, and their reaction dynam- ics, which often include an on/off switchability, are generally responsive to external stimuli such as temperature and pH.[1,2] These systems are easily reproducible, straightforward to perform, and have a broad applicability as well as tolerance towards other functional groups and reaction conditions especially with respect to solvents. The thiol–thioester exchange (TTE) is such a reaction, with rapid reaction kinetics in biochemical systems and being considered the "gold standard" of this subset of dynamic covalent linkages.[3] In some systems, the high efficiency of the reaction is evidenced by the often stoichiometric (1: 1 thiol: thioester) interchange of functionality at low concentrations and in the presence of multiple functional groups at room temperature and in aqueous environments.[4–6] The application of TTE in material chemistry particularly in the synthesis of polythioester-based hydrogels require the use of reaction partners in high concentrations, extremely basic catalysts and the presentation of both thioester and thiol functionalities in separate and mostly hydrophobic molecules.[7–9] In the few examples where both functionalities were present in a single molecule, the requirement of highly specific ring sizes close to the thioester to drive the equilibrium in the desired direction, limits their use in aqueous or biological environments. Single TTE reaction substrates function by a 'pseudo intramolecular exchange' where two exact molecules react with each other. These substrates are normally designed by the incorporation of thioesters derived from more acidic thiols (lower pKa) to facilitate easy exchange with thiols of higher pKa on the same molecule. Therefore, the exchangeable acyl group is preferentially accommodated by the less acidic thiol in TTE reactions of substrates, which exhibit a substantial difference in the acidity of thioester and attacking thiols. For this purpose, thioesters derived from aromatic thiols are commonly employed. But this presents challenges for their potential use in biological systems because of limited solubility in aqueous media, toxicity and liberation of foul-smelling thiols in the exchange reactions.[8] Our approach towards thiol-based compounds aims at a latent crosslinker, which can form polymer networks in biological systems when exposed to a suitable stimulus. Our concept of a biologically derived and relevant peptide mimetic integrates both functionalities required for a 'pseudo' intramolecular exchange reaction in one substrate without incorporating aromatic acidic thiols.

Hence the thio-peptide mimetic shall comprise a thioester unit and a free thiol moiety with a sequence that can promote exchange reactions at neutral to slightly basic pHs to yield a thio-peptide mimetic with α,ω-free thiols, which are suitable for a crosslinking reaction with multi-arm macromolecular precursors bearing orthogonal thiol functionalities.

Designing a peptide-based TTE substrate for aqueous environments requires careful balancing of the peptide sequence structure to facilitate solubility and successful exchange of thioester acyl group. We selected the water-soluble collagenase

substrate, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt[10] with a thio-leucine derived thioester unit as a reference compound. Amidation of the C-terminal end with a cysteamine unit yielded a TTE amenable thio-depsipeptide, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) bearing a free thiol with higher expected pKa than that of thioester thio-leucine.

A higher pKa value of the attacking thiolate ion than that of the displaced or departing has been noted as a requirement for the breakdown of the tetrahedral intermediate to form exchanged products.[11] In line with pKa requirements[12] for TTE reactions and from steric considerations, the Ac-Pro-Leu- Gly- acyl unit should preferentially rest on the cysteamine thiol assuming a lower pKa value than that of the thioester derived thiol. In the TTE, a mixture of products including a rearranged thio-depsipeptide (RTDP) and α,ω-free thiol bearing -Leu–Leu- Gly- unit (BTDP) orthogonal to Michael acceptor functionalized multi-arm macromolecules should be generated.

The combination of the sensory capability of sulfhydryl chemistry towards pH changes and the dynamic nature of TTE reaction make single TTE substrates ideal candidates for the on-demand synthesis of active compounds. Acetylcholinesterase (AChE) in conjunction with TTE was exploited for the rapid generation and screening of dynamic library of substrates in a process, which resulted in the amplification of the best substrate.[6]

The importance of TTE reactions in biomaterial synthesis was recently demonstrated using TDP as latent, pH-sensitive crosslinker to form a hydrogel with multi-arm polyethylene glycol maleimide in a process analogous to the activity of prodrugs.[13] The report explained how this sequentially coupled functional system could be beneficial in the treatment of inflamed tissues associated with urinary tract like bladder infections for which pH levels above 7[14] were reported. The applicability of our designed TDP is however not limited to pH-sensitive crosslinking reactions, also a potential system for the rapid identification of thiols by dynamic combinatorial screening as shown in Scheme 1 can be thought of.

To effectively exploit the entire application spectrum of the designed TDP, knowledge of the fate of exchange prod- ucts of TDP in the presence of thiols other than the TDP thiol (herein referred to as external thiols) is required. A detailed investigation on the predictability of TDP TTE reactions in the presence of external thiols would be a valuable reference point for the application of the TDP substrate in biological systems, where other (external) thiol might be part of the physiological environment.

In model reactions, we probe the exchange of TDP in the presence and absence of a set of thiols according to Scheme 1. The presence of external thiols results in two competing reactions namely the 'pseudo' intramolecular reaction of two TDP molecules and the intermolecular reaction of a TDP molecule and an external thiol. The thiols were carefully selected to give an overview of their reactivity and ability to preferentially stabilize the exchangeable acyl unit with the regards to overall structure and pKa of the thiols. TDP TTE reactions with two of the most abundant thiols in biological systems, l-cysteine (LCYS), and L-glutathione (LGLU) together with a small molecular weight thiol, methylthioglycolate (MeTGC) were studied. Although these thiols have similar acidities compared to TDP, their small size relative to TDP should the outcome of the overall TTE reaction. Para-nitrothiophenol (*p*-NTP) and 4-mercaptobenzoic acid (4MBA) were selected as acidic thiols to compare the effect of pKa of the attacking thiols on the exchange reaction.

Reaction mixtures were analysed with ESI–MS, which was run in both ion modes as it was found in preliminary measurements that the reaction products ionize preferentially only in one of the ion modes. These were complemented with ^{1}H and 2D-NMR experiments (COSY and multiplicity edited HSQC) where necessary.

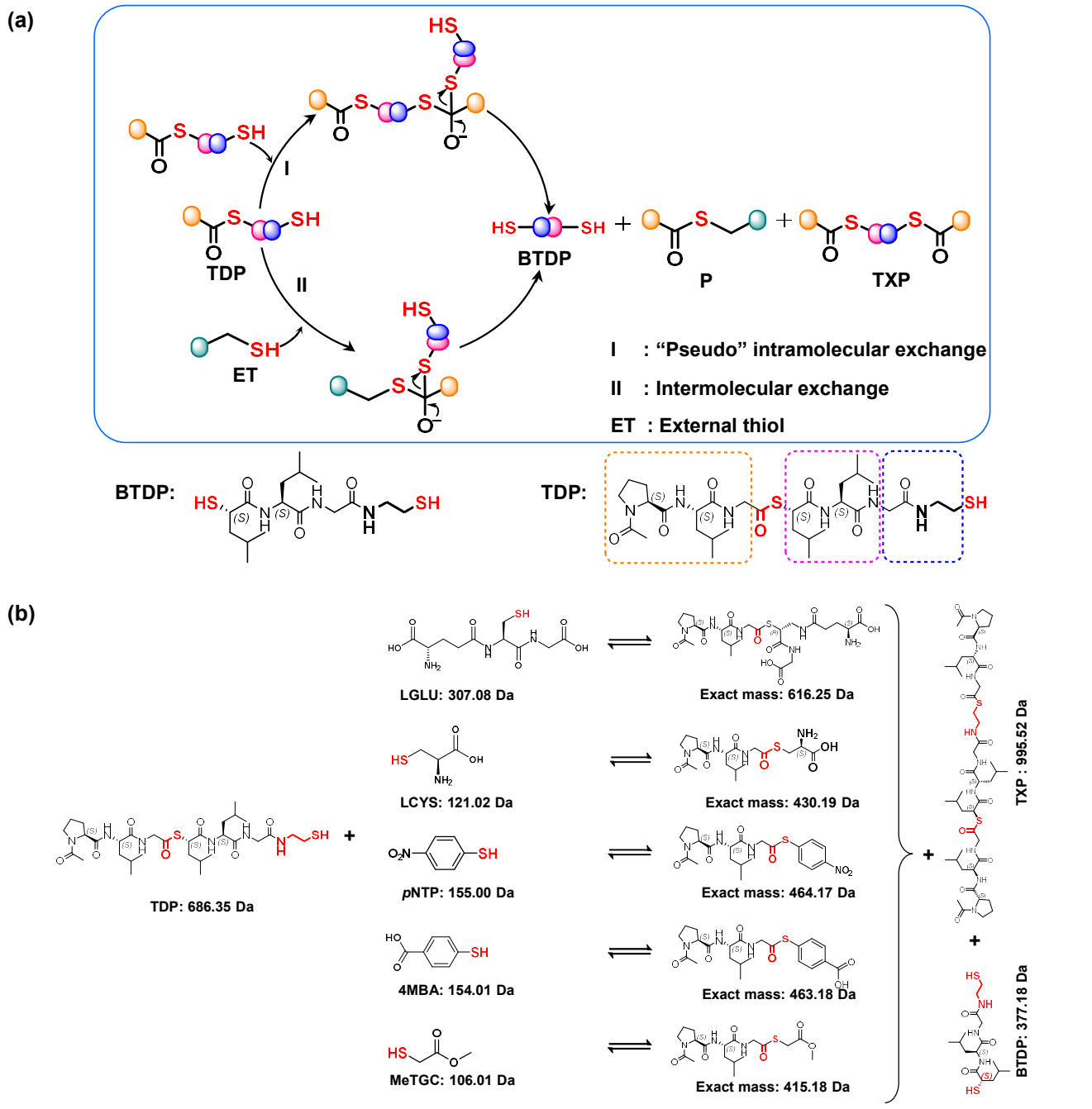
Materials and methods

l-cysteine (97%), l-glutathione (98%), 4-mercaptobenzoic acid (99%), methylthioglycolate (95%) and MeCN-d_3 (99.8%) and D_2O (99.0%) were purchased from Aldrich Chemicals (Darmstadt, Germany) and were used as received. Para-nitrothiophenol (99%) and deuterated dimethyl sulfoxide, DMSO-d_6 (99.8%) were purchased from Alfa-Aeser (Kandel, Germany) and VWR Chemicals (Darmstadt, Germany), respectively. MeOH-d_4 (99.8%) was purchased from Carl Roth (Karlsruhe, Germany). TDP was synthesized according to the method described in Ref. 13. In brief, the multi-step synthesis was accomplished by the initial coupling of Fmoc-Leu-Gly-OH with 2-(tritylthio)ethanamine to yield Fmoc-Leu-Gly-NEtS- Trt (**1**). Deprotection of the Fmoc from **1** yielded the free amine H_2N-Leu-Gly-NEtS-Trt (**2**), which was reacted with 2-(acetylsulfanyl)-4-methylpentanoic acid (acetylated thioleucine) to obtain AcSLeu-Leu-Gly-NEtS-Trt (**3**). Compound 3 was deacetylated to produce HSLeu-Leu-Gly-NEtS-Trt (**4**). Reaction of intermediate **4** with Ac-Pro-Leu-Gly-OH yielded Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtS-Trt (**5**). Detritylation and high performance liquid chromatography of **5** yielded TDP as colourless fluffy material after freeze drying.

Electrospray ionization mass spectrometry (ESI–MS)

ESI–MS (direct injection) spectra were obtained on a Bruker Impact II quadrupole/time-of-flight (QqTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an atmospheric pressure ionization source operating in the nebulizer assisted electrospray mode. ESI–MS (direct injection) spectra were obtained in positive/negative ion mode by direct injection of samples into the system using a syringe pump (Cole-Parmer, Vernon Hills, IL), which was operated at a flow rate of 180 μL·h^{-1}. Internal calibration of the system was carried out using standard sodium formate mixture.

TTE reactions of TDP were carried out using 1 mM stock solutions of TDP and thiols in milli-Q water or ESI-grade MeOH where specified. 300 μL of 1 mM TDP solution and



Scheme 1. (a) TDP–TDP "pseudo" intramolecular TTE reaction in the absence of external thiols (I). In the presence of external thiols, a competing TDP-ET intermolecular TTE reaction (II) as well as (I) can occur resulting in the generation of mixed exchange products BTDP, P, and TXP. The relative concentration of exchange products depends on the relative acidities of the TDP and the external thiols. (b) TDP TTE reactions with external thiols (LGLU, LCYS, *p*NTP, 4MBA and MeTGC).

300 µL of 1 mM solution of each commercial thiol were reacted for 30 min. The reaction mixture was diluted 20-fold to obtain a final concentration of 0.05 mM (with respect to each reactant) before the mixture was injected into the mass spectrometer for mass analysis.

All data were processed with the software Bruker Com- pass Data Analysis 4.3 (Bruker Daltonics, Bremen, Ger- many) and Mestrenova 12.0 (Mestrelab Research, S.L., Santiago de Compostela, Spain).

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H-NMR (500 and 700 MHz) and ^{13}C-NMR (101 MHz) were recorded in D_2O, in MeCN-d_3 (internal standard: 1.94 ppm, ^{1}H; 118.3 ppm, ^{13}C), in DMSO-d_6 (internal standard: 2.50 ppm, ^{1}H; 39.52 ppm, ^{13}C), in MeOD-d_3 (internal standard: 3.31 ppm, ^{1}H; 49.15 ppm, ^{13}C), on a Bruker Avance-500 MHz or

Ascend-700 MHz spectrometer. Chemical shifts (δ) were reported as parts per million (ppm). Atoms are numbered with respect to all major connecting points on each molecule. The labels therefore refer to all the different atoms at each point.

Results and discussion Electrospray ionization mass spectrometry

Although the synthesized TDP was thoroughly characterized and identified with detailed NMR studies in polar aprotic sol- vents like ACN-d_3 and DMSO-d_6 as previously reported,[13] in polar protic solvents a mixture of structures were observed when NMR spectra of TDP were obtained. This observation is however expected for systems with no additional catalyst to promote the TTE reaction. To comprehensively understand the nature and fate of exchange products of TDP, additional TTE reactions in which a set of model thiols was mixed with TDP in equimolar quantities was carried out. Since the thioester unit is constant, it was speculated that by careful selection of the model thiols (pKa and structure considered), the TTE equilibrium position and kinetics should be influenced.

To a large extent, all TTE reaction of TDP yielded the expected results. TDP undergoes a "pseudo" intramolecular TTE in which the thiol of the cysteamine unit with anticipated high pKa attacks the thioester of another TDP molecule resulting in BTDP and TXP as the exchange products in the equilibrium reaction (Fig. 1).

When TDP was mixed with the selected thiols, the fate of exchange products was found to be dependent on the relative acidities of the TDP thiol and the external thiol. As an example, in the presence of l-cysteine, TDP yields an additional product peak, which is visible in the negative ion mode at m/z = 429.1819 Da as an intense peak and as a weak signal at m/z = 453.1791 Da in the positive ion mode (Fig. 2).

A mass peak at m/z = 1115.5263 Da was initially assigned to the **[P_{21} + BTDP]** ion cluster as an evidence of a subsequent S → N acyl transfer of **P_1**. **P_{21}** would have been formed by TTE of **P_2**, as a product of the S → N acyl transfer of **P_1**. Mechanistic studies on TTE reactions however suggests the presence

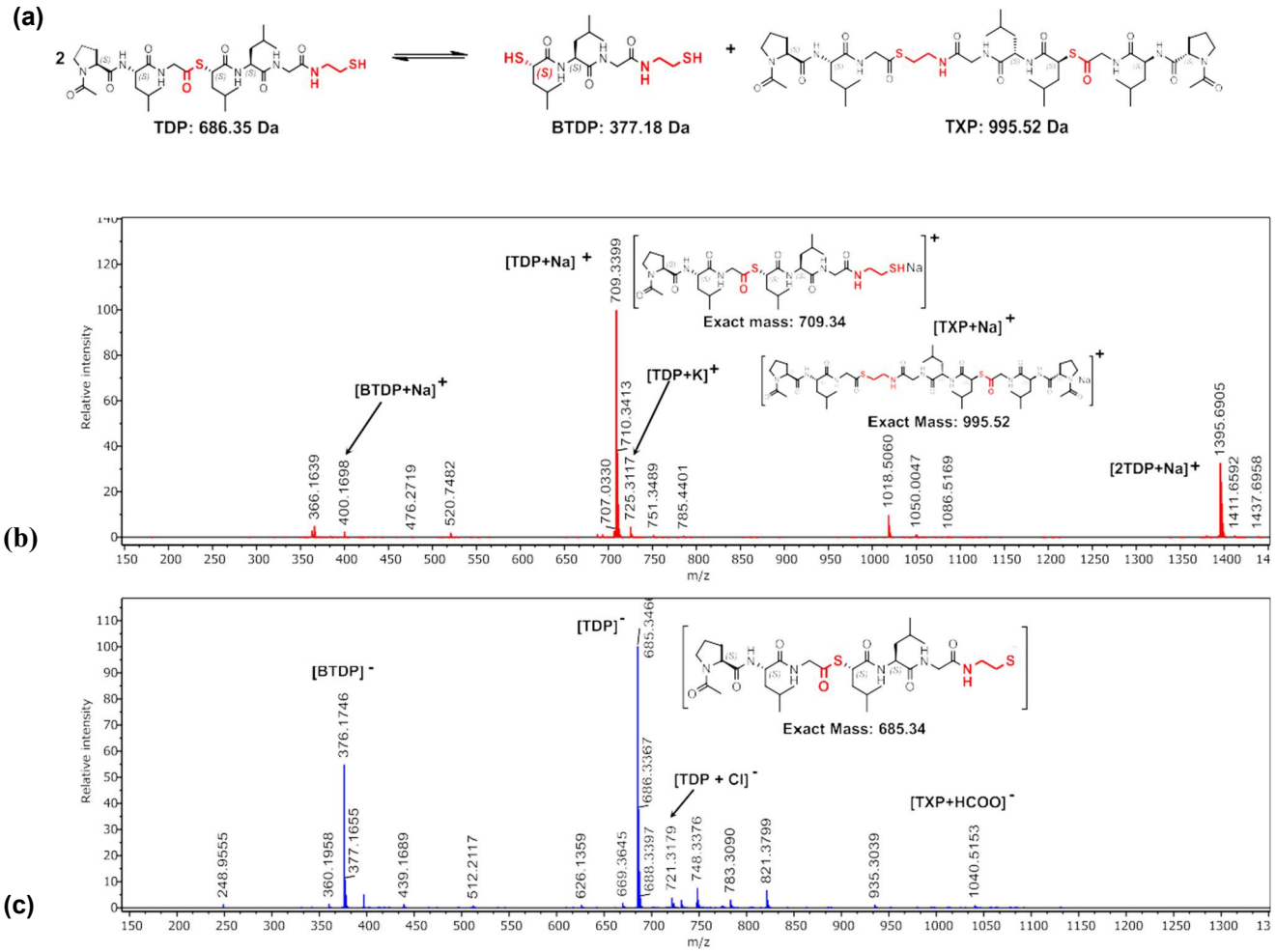


Figure 1. (a) TTE of TDP only. The scheme details TDP in equilibrium with BTDP and TXP as TTE products. Recorded mass spectra in positive ion mode (b) and negative ion mode (c) show reactant and product peaks with assignments.

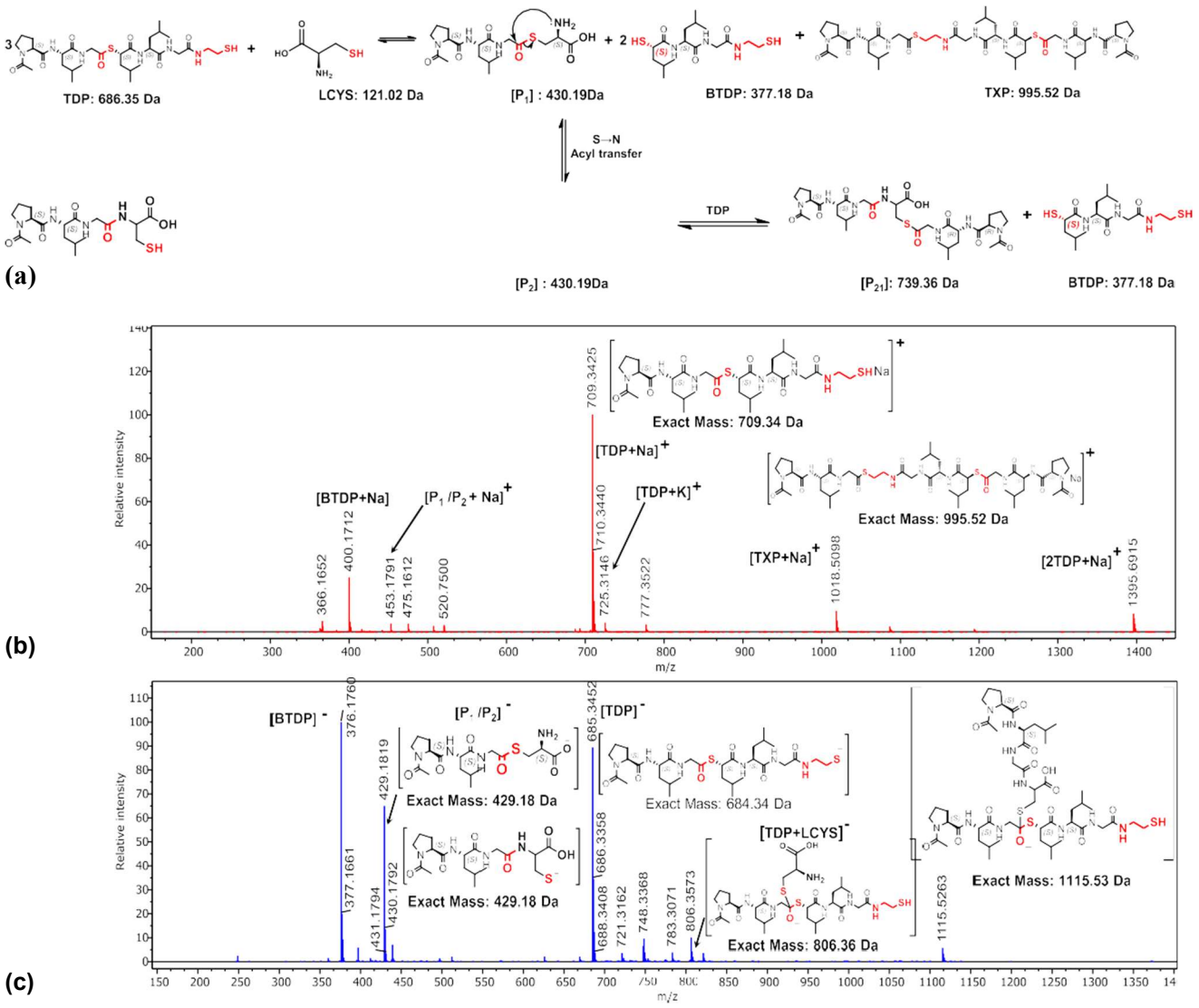


Figure 2. (a) TTE reaction of ʟ-cysteine (LCYS) with TDP. The scheme details the reaction partners with potential S→N acyl transfer of the formed exchanged product. Recorded mass spectra in positive ion mode (b) and negative ion mode (c) show reactant and product peaks with assignments.

of two reaction steps leading the final exchange product with the first step being with the formation of a negatively charged tetrahedral intermediate. The mass peaks at m/z = 806.3573 Da and 1115.5263 Da were therefore assigned to the tetrahedral intermediates of TDP with cysteine and the S→N acyl transfer product, $\mathbf{P_{21}}$ in line with the findings from these mechanistic studies. This assumption is confirmed not only by the observed masses, but also by the fact that these ions were only observed in the negative ion mode since the intermediates are always negatively charged. The ability to detect such intermediates could be also be explained by the relative pKa values of the incoming and departing thiols as the important parameter determining the half-life of the intermediates.

In the presence of l-glutathione, the expected TTE product peak was not observed in both ion modes. However, a mass peak with m/z = 992.4252 Da corresponding to the LGLU-TDP intermediate was present (Figure S1, Supplementary Information). The lack of a mass peak for the expected TTE product would have been accompanied by the corresponding lack of a BTDP ion peak if the only source of BTDP would be the breakdown of the LGLU-TDP tetrahedral intermediate. The presence of a relatively intense BTDP peak therefore implies the existence a competing TTE reaction by TDP molecules resulting in intermediates, which break down faster compared to LGLU-TDP.

Unlike LCYS and LGLU, the product peak of the reaction of methylthioglycolate (MeTGC) and TDP (MeTGC-TDP) was only found in the positive ion mode at m/z = 438.1676 Da (Figure S2, Supplementary Information). Signals corresponding to BTDP, TDP, and the expected TTE product, all with sodium counter ion were observed although the tetrahedral intermediate MeTGC-TDP was not found. The absence of MeTGC-TDP intermediate could be explained by a potential fast breakdown, resulting in the expected TTE products and BTDP.

The signal intensity of BTDP relative to that of TDP was found to be higher for the MeTGC-TDP reaction compared to the signals obtained when TDP was mixed with LGLU, LCYS, 4MBA and *p*NTP. Since the pKa of the thiols of MeTGC, LGLU, and LCYS are comparatively similar; the observed shift towards product formation could be explained by the small size and reduced steric hindrance of the MeTGC compared to rest of the thiols.

Reaction of 4-mercaptobenzoic acid (4MBA) providing a thiol group of pKa = 5.9 was expected to form 4MBA-TDP tetrahedral intermediate with faster kinetics than LCYS, LGLU and MeTGC. The breakdown of the intermediate is slow because of the low pKa value of MBA compared to the departing thiol from TDP. The negatively charged intermediate is hence still visible at m/z = 839.3223 Da (Figure S3, Supple- mentary Information).

The intensity of BTDP ions compared to those observed in the reaction of LGLU and TDP is low. Similarly, as it was observed for the LGLU-TDP reaction, the competing TDP–TDP TTE reaction resulted in BTDP mass peak with lower intensity than observed for the LGLU-TDP reaction. This is due to the low pKa value of 4MBA compared to the pKa of TDP, which significantly reduces the likelihood of TDP–TDP intermediate formation since the 4MBA-TDP would be preferentially formed. Like 4MBA, para-nitrothiophenol (*p*NTP) with thiol pKa of 4.6 also yielded a mass peak corresponding to the *p*NTP-TDP intermediate at m/z = 840.3467 Da only in the negative ion mode (Figure S4, Supplementary Information).

Nuclear magnetic resonance spectroscopy

ESI–MS is known to operate via non-destructive ionization of analytes being studied and is a more sensitive analytical technique compared to NMR. There is however some loss of information due to the fact that analytes are in direct interaction with the instrument leading to certain undesired modifications. For simple to moderately complicated systems, NMR serves an additional or complementary tool to obtain valuable information of analytes in their pristine condition. ^{1}H-NMR was therefore used to study the TDP TTE reaction mixtures in similar experiments as performed for ESI–MS study. For each thiol, NMR spectra were collected for pristine thiol, TDP only solution and for the TDP- external thiol mixture in deuterium oxide or methanol-d_4 depending on the solubility of the selected thiol. Methanol-d_4 was used for reactions involving 4MBA and *p*NTP due to their limited solubility in D_2O.

For each solvent, the corresponding TDP only spectrum was obtained to enable a true comparison. Spectra for TDP only solution were taken after 30 min of standing whilst spectra for TDP-external mixtures were taken after 30 min of mixing and repeated after 12 h. Resonances for methylene protons on the cysteamine unit of the TDP and BTDP are labelled $\mathbf{TD_1}$ and $\mathbf{TD_2}$ whilst the peaks denoted with $\mathbf{TX_1}$ and $\mathbf{TX_2}$ are resonances associated with the methylene protons on the cysteamine unit TXP (Fig. 3a).

Figure 3(b) presents an overlay of ^{1}H-NMR spectra of LCYS, TDP only (after 30 min), TDP-LCYS (after 30 min) and TDP-LCYS (after 12 h) in D_2O. The general trend observed in this series of spectra compare favourably with observations from the ESI–MS studies. In this regard, the acidity of the added thiol determines if TDP–TDP exchange products are formed preferentially over TDP-external thiol products or vice versa. As an example, NMR spectra TDP only solution shows the occurrence of $\mathbf{TX_2}$ resonance downfield of $\mathbf{TD_2}$ and overlapping signals of $\mathbf{TX_1}$ and $\mathbf{TD_1}$ at δ = 3.26–3.39 ppm.

The newly formed and observed $\mathbf{TX_2}$ signal at higher ppm value compared to $\mathbf{TD_2}$ is an indication of a successful TDP–TDP exchange reaction to form TXP. Since the electronic environments of $\mathbf{TD_1}$ and $\mathbf{TX_1}$ remain relatively unchanged, these signals occurred at the same ppm value as a multiplet.

In the presence of LCYS however, the exchangeable acyl group preferentially rests with cysteine leading to the absence of $\mathbf{TX_2}$ and concomitant emergence of new cysteine β-proton resonance, **P** at δ = 2.86 ppm. The loss of the broad $\mathbf{TD_1/TX_1}$ overlapping peak seen for the TDP only spectrum was accompanied by the presence of an intense $\mathbf{TD_1}$ signal contributed largely by BTDP from LCYS-TDP reaction. This result thus signifies that TDP-LCYS TTE was more favourable than that of TDP–TDP. Based on the assumption that cysteine and TDP thiols do have comparable pKa values, the observed preference of TDP for LCYS could be attributed to the smaller size and hence less steric hindrance of the cysteine molecule compared to TDP. Similar size effect on the fate of the exchange product was observed for TDP-MeTGC (Figure S2, Supplementary Information) although MeTGC has a more acidic thiol (pKa 7.8) compared to cysteine (pKa 8.3).

Due to the limited solubility of the more acidic thiols, 4MBA and *p*NTP in D_2O, methanol-d_4 was used as solvent. The effect of solvent on the TDP–TDP reaction was directly evident in the TTE kinetics when the ^{1}H-NMR spectra of TDP in D_2O and methanol-d_4 after 30 min of solution preparation are compared (Figure S6A, Supplementary Information). The $\mathbf{TX_2}$ resonance from TXP was observed in D_2O after 30 min of solution preparation but after 12 h in methanol-d_4 although this could be faster. In polar aprotic solvents such as acetonitrile, TDP required the addition of an organic base, diisopropylethylamine to effect the exchange reaction (Figure S6B, Supplementary Information).

This observation should be largely anticipated considering the polarity of the two solvents and in line with reported findings[12] assuming thiolate ions are the main reactive species as would be in a base catalysed reaction.

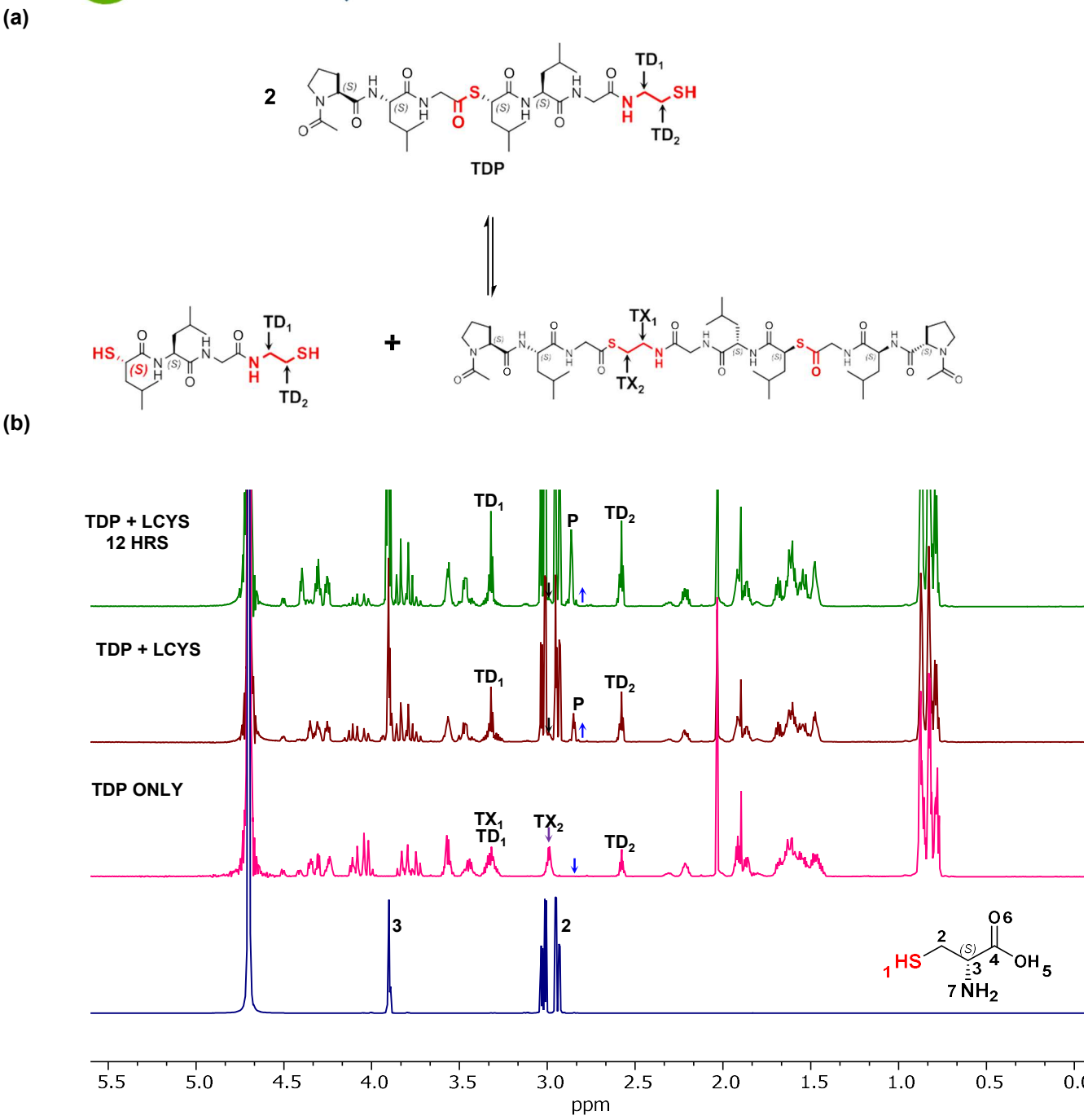


Figure 3. (a) TDP–TDP reaction scheme and labelling of relevant protons. Cysteamine protons TD_1 and TD_2 are assumed to have similar resonances for TDP and BTDP. (b) Overlay of NMR spectra from bottom of LCYS, TDP only (after 30 min), TDP-LCYS (after 30 min) and TDP-LCYS (after 12 h) in D_2O.

When TDP was mixed with L-glutathione, **TX_2** resonance was not observed after 30 min of solution although a weak signal was present after 12 h (Figure S7, Supplementary Information). As was previously noted in ESI–MS studies TDP-LGLU reaction was assumed to be arrested at the initial reaction step due to the presence of a relatively intensive peak identified as the tetrahedral intermediate. If the highly intense BTDP mass peak observed for TDP-LGLU reaction was largely contributed by a competing TDP–TDP reaction, then a corresponding strong **TX_2** resonance should be observed in the ^{1}H-NMR spectrum. Since thiolate ions play significant role in TTE, these opposing findings could be attributed to the increased acid effect of the two glutathione carboxylic acid groups

considering the final LGLU concentration used for NMR and ESI–MS studies.

Results of TTE of TDP with the more acidic thiol 4MBA (pKa 5.9) compare favourably with ESI–MS studies. In summary, due to the slow breakdown of their tetrahedral intermediates, the increased build-up of these negatively charged species could lead to the generation of TDP thiolate ions resulting in a competing TDP–TDP reaction and the presence of $\mathbf{TX_2}$ signal after 30 min (Figure S8, Supplementary Information). Similar results were obtained for *p*NTP with a pKa value of 4.6 (Figure S9, Supplementary Information).

Conclusions

The thio-depsipeptide Ac-Pro-Leu-Gly-SLeu-Leu-Gly- NEtSH (TDP) is capable to undergo a pH sensitive pseudo intramolecular exchange reaction producing α, ω-dithiol, which can then serve as a latent crosslinker or precursor. Since the thio-depsipeptide could be a candidate compound to be used for potential application in biological systems where thiol molecules are already present, an in-depth knowledge of its reactivity in the presence of some biologically relevant and organic thiols should provide valuable information for the future design of these peptide mimetics. In model TTE reactions, the reactions of TDP with l-cysteine and L-glutathione, which are two of the most abundant thiols in biological systems, methylthioglycolate, para-nitrothiophe- nol and 4-mercaptobenzoic acid were analysed with ESI–MS and ^{1}H-NMR. The model reactions of TDP with this set of external thiols confirmed the ability of TDP to undergo TTE reactions with results reflecting the role of pKa of thiols in the fate of exchange products. Small-sized aliphatic thiols, MeTGC and LCYS (pKas 7.8 and 8.3, respectively) promoted increased production of useful α,ω- dithiol (BTDP) whilst aromatic and acidic thiols, 4MBA and *p*NTP (pKas 5.9 and 4.6, respectively) hindered BTDP production.

Thiol-thioester exchange remains a significant reaction to several biological processes and a widely used dynamic covalent chemistry in material chemistry. Whereas the designed TDP finds potential use in bioconjugation and crosslinking reactions in biological systems, the exchange reaction results present those working in the field insight into the development of new sequences with tuneable exchange kinetics and equilibria not only for material synthesis but also in the areas of peptide mimetic probes for thiol identification and sequestration.

Acknowledgments

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Data availability

Data will be made available on reasonable request.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Open Access

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1557/s43579-021-00041-z.

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Supplementary Information

Reaction behaviour of peptide based single thiol-thioesters exchange reaction substrate in the presence of externally added thiols

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Content

ESI-MS spectra of the TDP-LGLU reaction

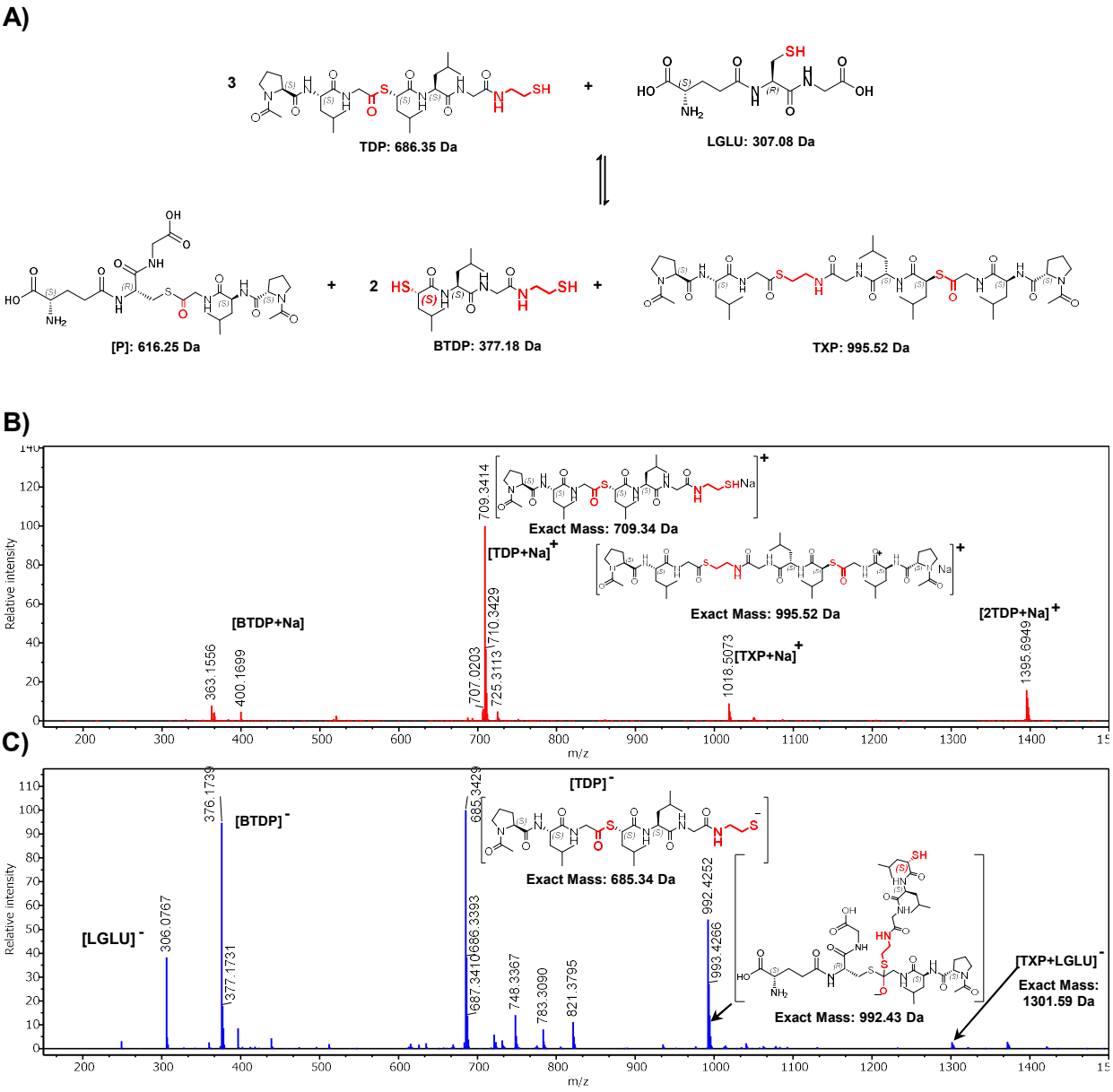

Figure S1. Overlay of ESI-MS spectra of TTE reactions of TDP with a commercial thiol, L-glutathione. (A) Reaction scheme, (B) ESI-MS spectrum in positive ion mode, (C) ESI-MS spectrum in negative ion mode. The signal at 992.4252 Da in the negative ion mode corresponds to the tetrahedral intermediate in the first step of the TTE reaction.

ESI-MS spectra of the TDP-MeTGC reaction

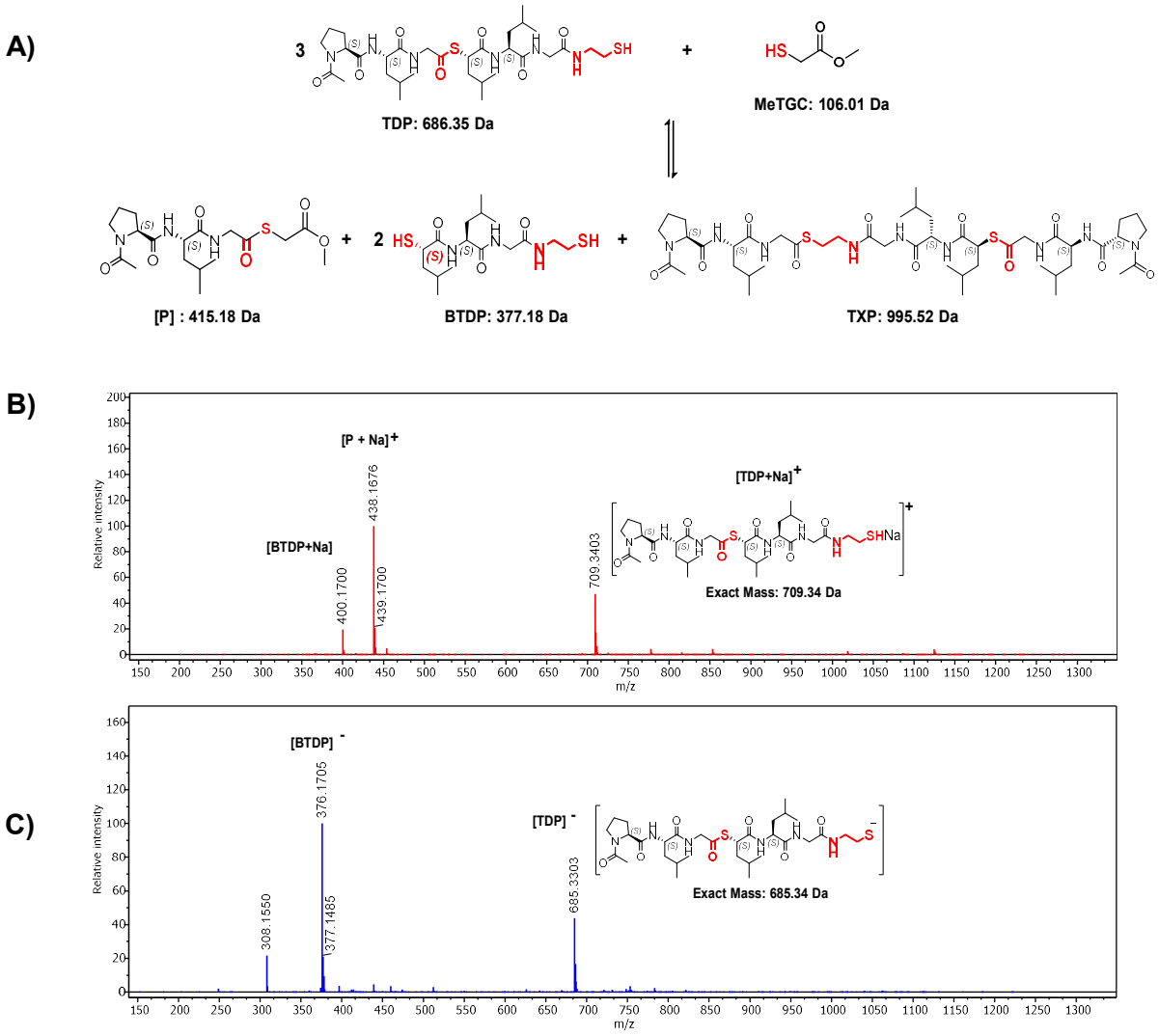

Figure S2. Overlay of ESI-MS spectra of TTE reactions of TDP with methylthioglycolate (MeTGC). (A) Reaction scheme, (B) ESI-MS spectrum in positive ion mode, (C) ESI-MS spectrum in negative ion mode.

ESI-MS spectra of the TDP-4MBA reaction

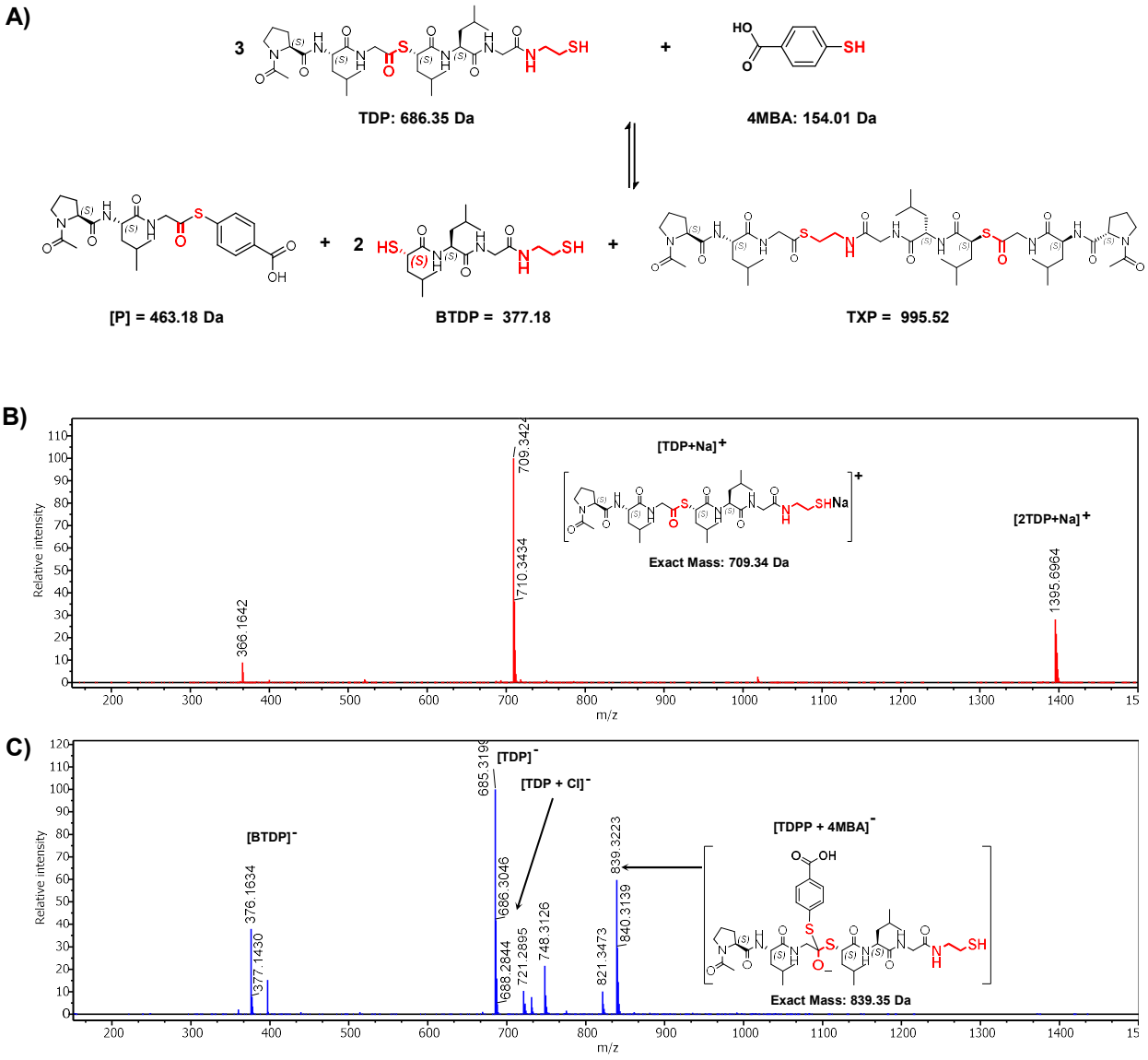

Figure S3. Overlay of ESI-MS spectra of TTE reactions of TDP with 4-mercaptobenzoic acid. (A) Reaction scheme, (B) ESI-MS spectrum in positive ion mode, (C) ESI-MS spectrum in negative ion mode. The signal at 992.4252 Da in the negative ion mode corresponds to the expected negatively charged tetrahedral intermediate in the first step of the TTE reaction.

ESI-MS spectra of the TDP-*p*NTP reaction

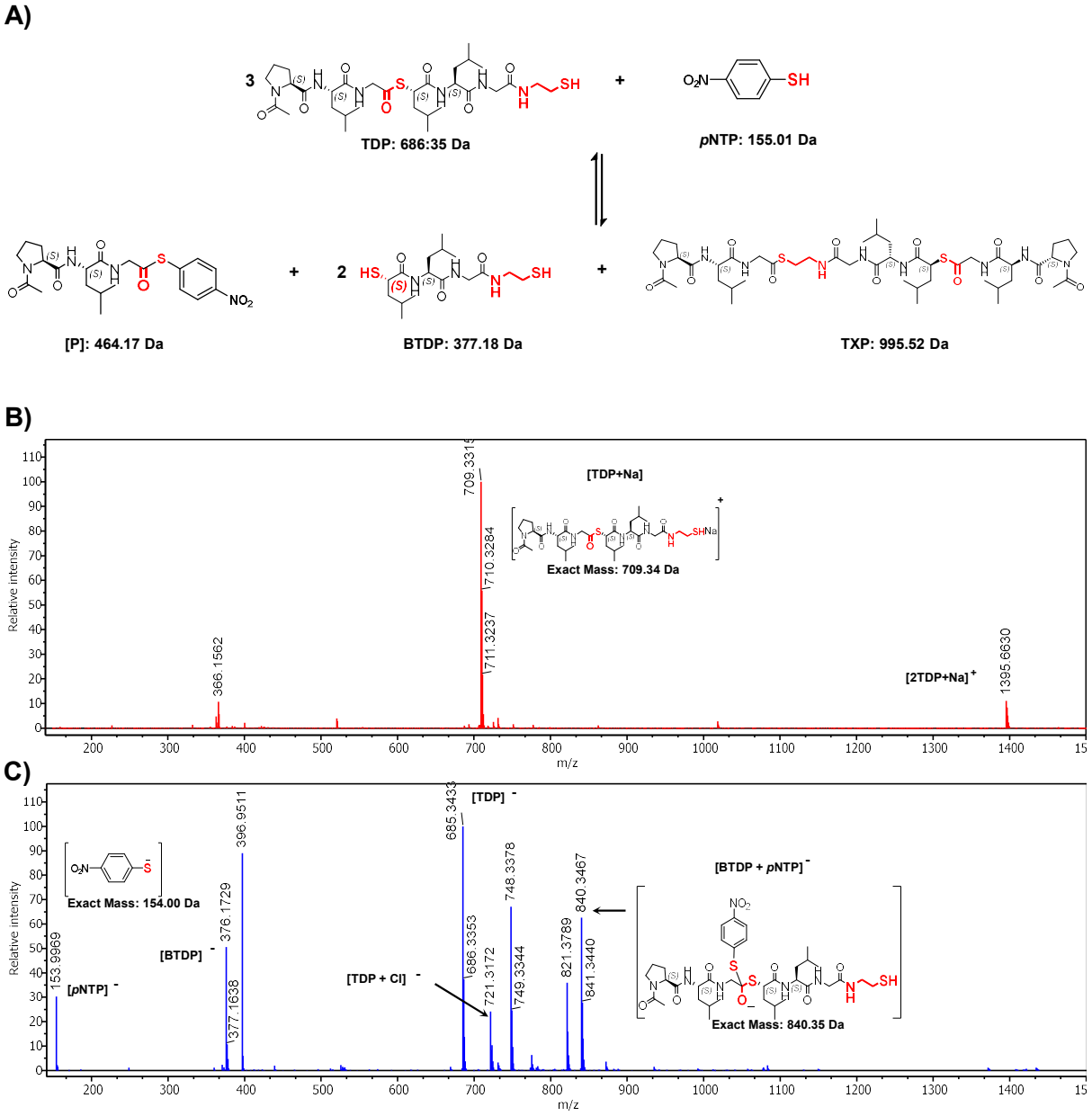

Figure S4. Overlay of ESI-MS spectra (red: positive ion mode, blue: negative ion mode) of TTE reactions of TDP with a commercial thiol, para-nitrothiophenol. (A) Reaction scheme, (B) ESI-MS spectrum in positive mode, (C) ESI-MS spectrum in negative mode. The signal at 840.3467 Da in the negative ion mode corresponds to the tetrahedral intermediate in the first step of the TTE reaction.

^{1}H-NMR: Thiol-thioester reaction of TDP with MeTGC

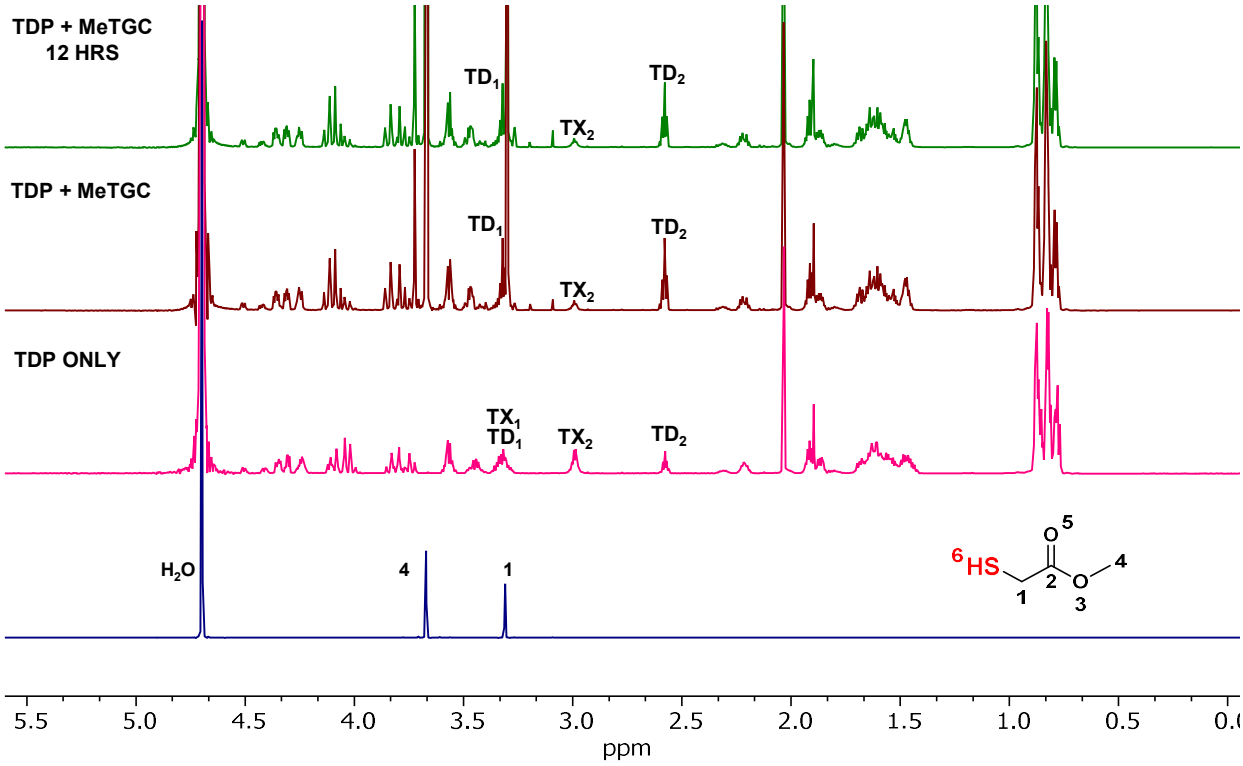

Figure S5. Overlay of ^{1}H-NMR spectra from bottom of MeTGC, TDP only (after 30 minutes), TDP-MeTGC (after 30 minutes) and TDP- MeTGC (after 12 hours) in methanol-d_4

^{1}H-NMR: Solvent effect on thiol-thioester reaction of TDP

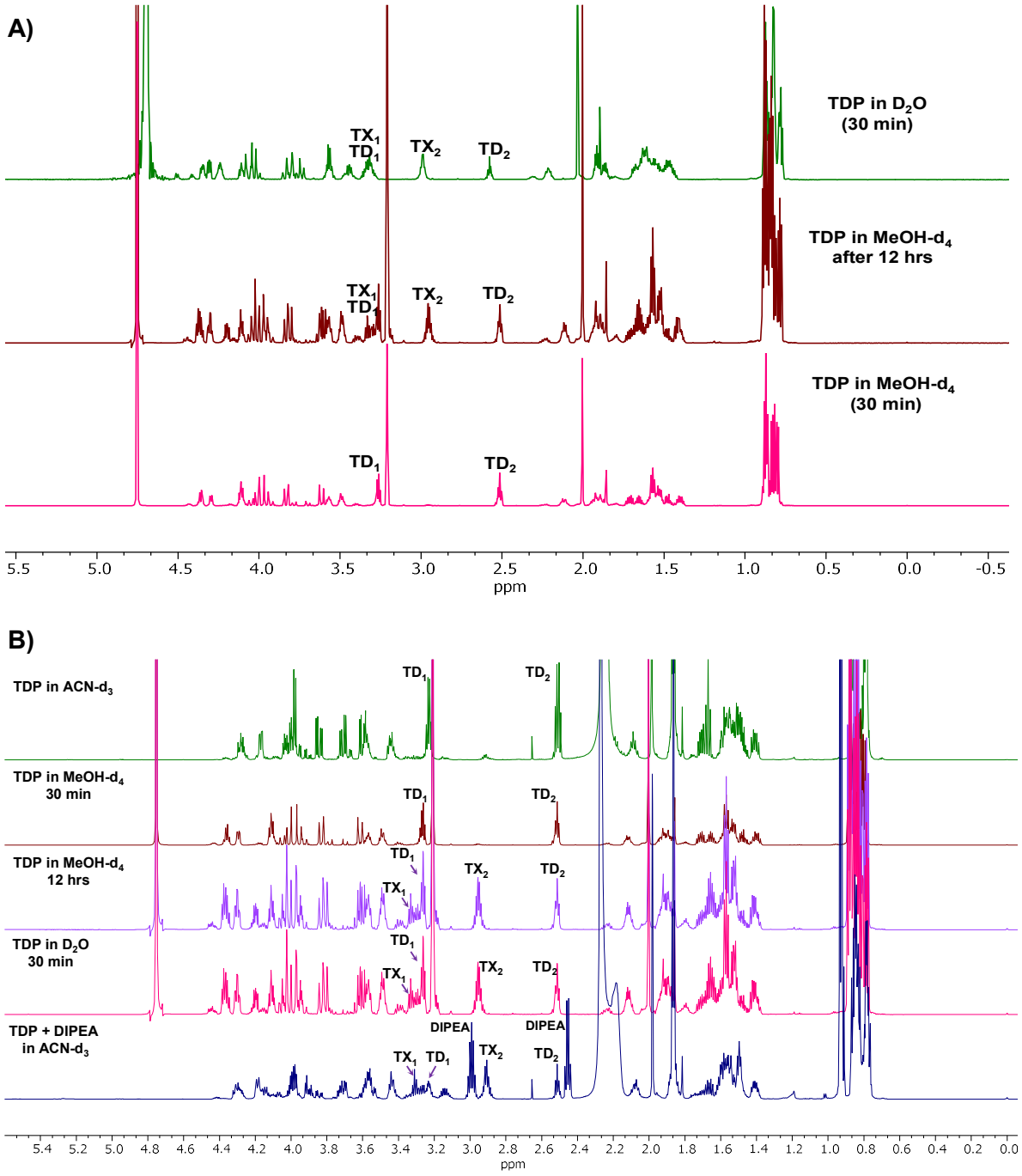

Figure S6. (A) Overlay of ^{1}H-NMR spectra from bottom of TDP in methanol-d_4 (after 30 minutes), TDP in methanol-d_4 (after 12 hours) and TDP in D_2O (after 30 minutes) (B) Comparison of solvent effect on TTE of TDP. Polar protic solvents (D_2O and MeOH-d_4) aided TTE of TDP although TTE in D_2O had faster kinetics. ACN-d_3 which is polar aprotic however required addition of the organic base, diisopropylethylamine (DIPEA) to effect the TTE.

^{1}H-NMR: Thiol-thioester reaction of TDP with LGLU

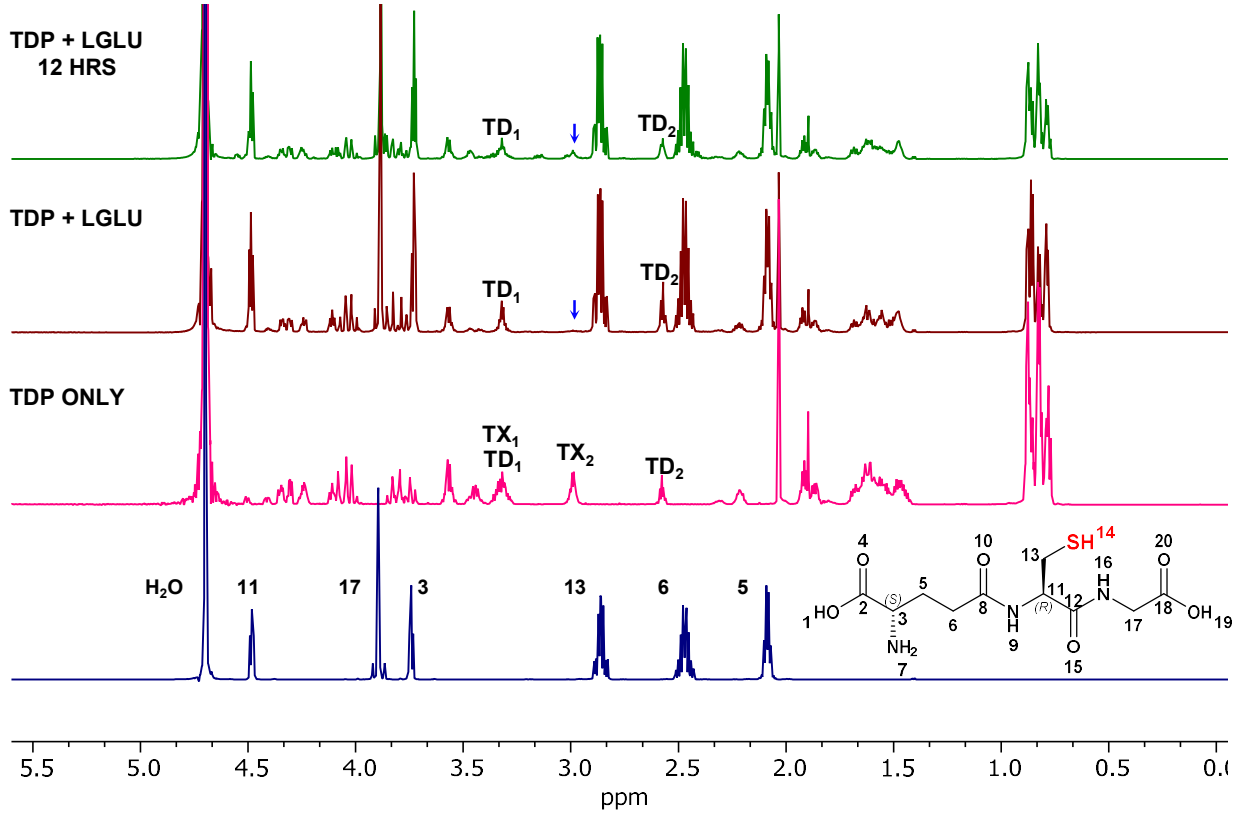

Figure S7. Overlay of ^{1}H-NMR spectra from bottom of LGLU, TDP only (after 30 minutes), TDP-LGLU (after 30 minutes) and TDP-LGLU (after 12 hours) in D_2O

^{1}H-NMR: Thiol-thioester reaction of TDP with 4MBA

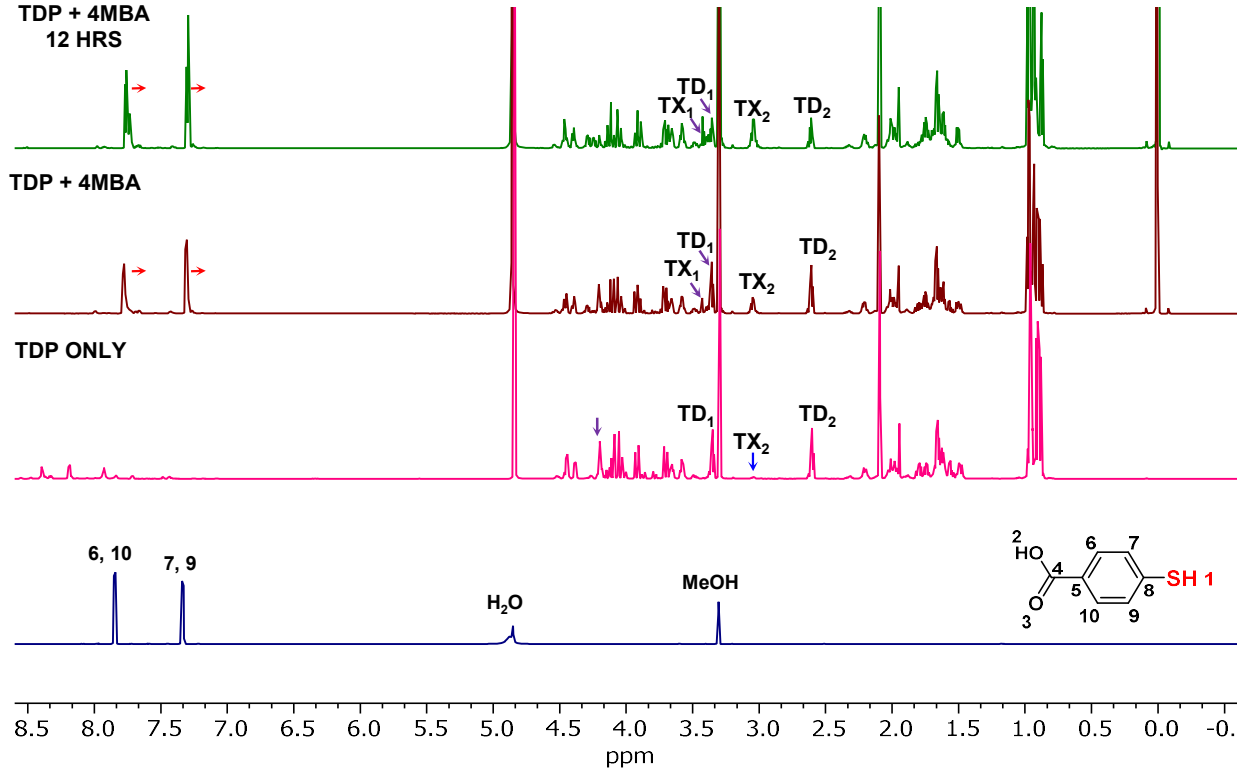

Figure S8. Overlay of NMR spectra from bottom of 4MBA, TDP only (after 30 minutes), TDP-4MBA (after 30 minutes) and TDP-4MBA (after 12 hours) in methanol-d_4

^{1}H-NMR: Thiol-thioester reaction of TDP with *p*NTP

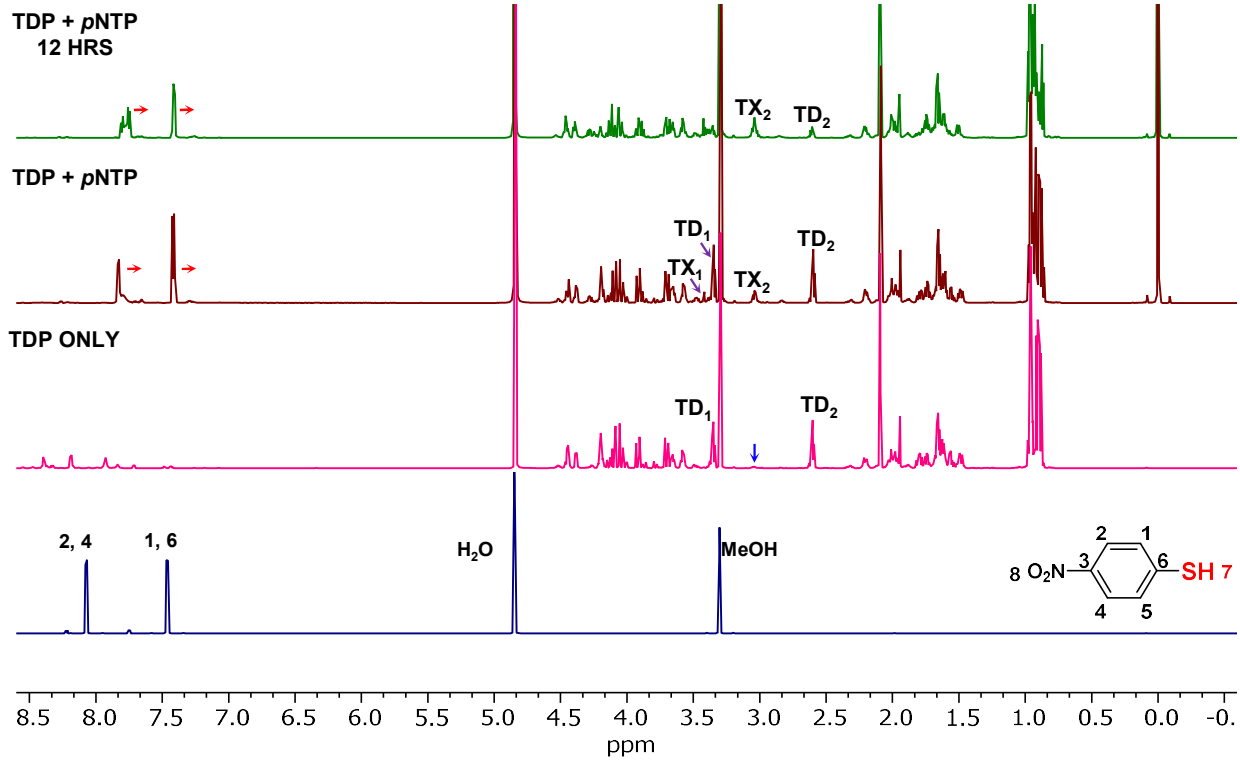

Figure S9. Overlay of ^{1}H-NMR spectra from bottom of *p*NTP, TDP only (after 30 minutes), TDP- *p*NTP (after 30 minutes) and TDP- *p*NTP (after 12 hours) in methanol-d_4

Comparison Multiplicity-edited HSQC spectrum of TDP in ACN-d_3 and D_2O

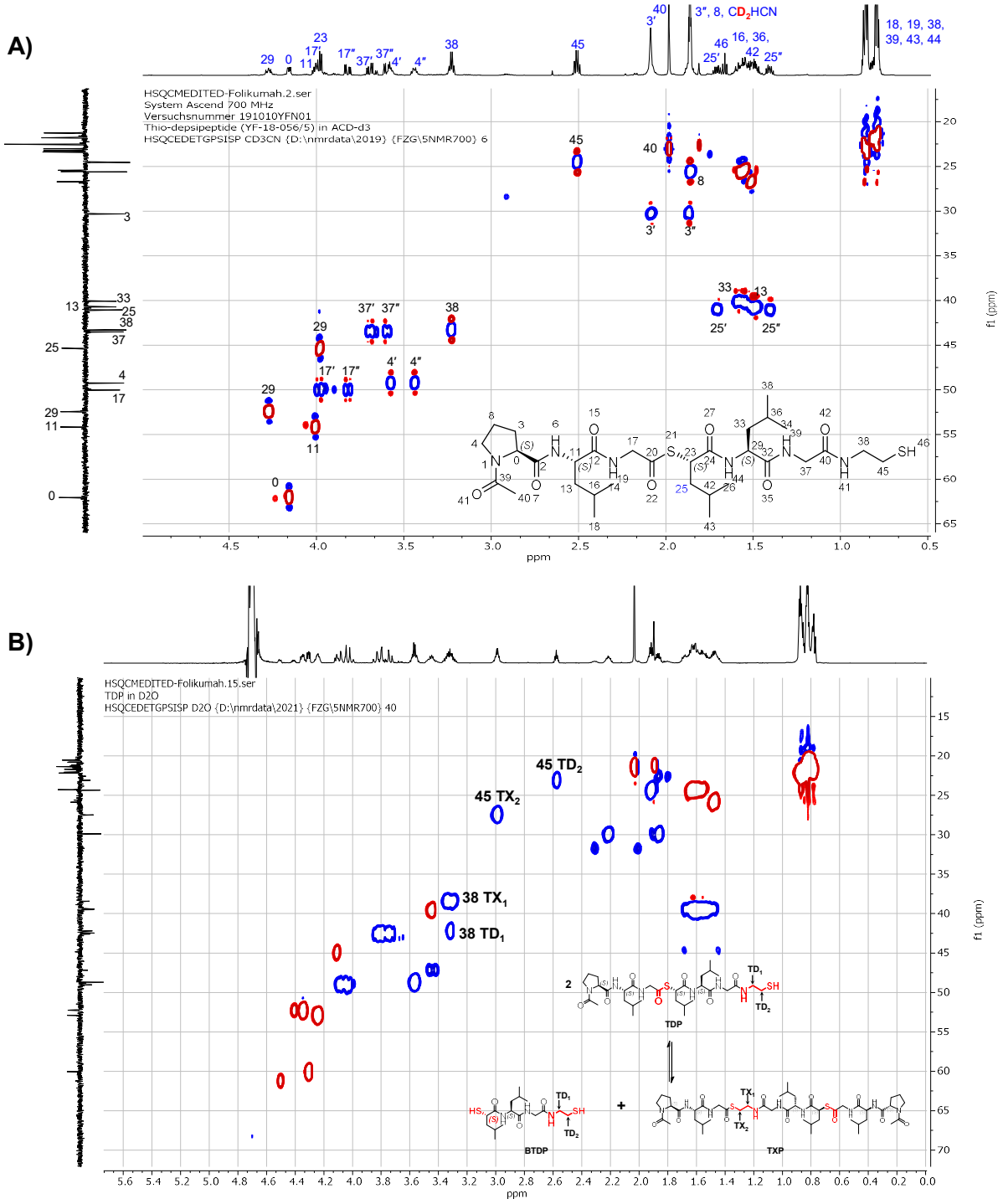

Figure S10. Multiplicity-edited HSQC spectrum of TDP (A) in ACN-d_3 with chemical structure and signal assignments. (B) in D_2O (30 minutes after preparing solution) showing additional resonances **TX_1** and **TX_2** belonging to TXP (insert)

13 Appendix III - Thiol-thioester exchange reaction in precursor enables pH triggered hydrogel formation

Article

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Thiol−Thioester Exchange Reactions in Precursors Enable pH- Triggered Hydrogel Formation

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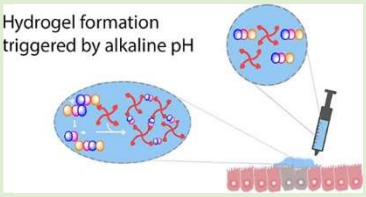
ABSTRACT: Bio-interactive hydrogel formation *in situ* requires sensory capabilities toward physiologically relevant stimuli. Here, we report on pH-controlled *in situ* hydrogel formation relying on latent cross-linkers, which transform from pH sensors to reactive molecules. In particular, thiopeptolide/thio-depsipeptides were capable of pH-sensitive thiol−thioester exchange reactions to yield α,ω-dithiols, which react with maleimide-functionalized multi-arm polyethylene glycol to polymer networks. Their water solubility and diffusibility qualify thiol/thioester-containing peptide mimetics as sensory precursors to drive *in situ* localized hydrogel formation with potential applications in tissue regeneration such as treatment of inflamed tissues of the urinary tract.



INTRODUCTION

Hydrogels can provide functional and structural similarities compared to soft tissues and therefore are potential synthetic extracellular matrix (ECM)-like substitutes. They can be prepared by cross-linking of macromolecules or by polymerizing suitable precursors whereby cross-links are not necessarily covalent bonds but could also be formed by physical interactions such as π−π interactions,[1] hydrophobic interactions, and H- bonding.[2] In addition to similar elastic properties, similarities of hydrogels to ECM include but are not limited to the transport of oxygen and soluble factors[3] and the ability to hold large amounts of water.[4] The capability to transport and to release soluble factors like drugs makes them attractive candidate materials for biomedical applications. Of special interest is a release on demand, which can be achieved by stimuli-sensitive hydrogels responding, e.g., to a change in temperature or pH.[5] However, here, diffusion needs to be considered.[6]

For certain indications, a hydrogel formation on demand is desirable to provide an additional protective layer to favor autoregeneration. For example, urinary tract infections (UTIs) are among the most common bacterial infections acquired in the community and in hospitals.[7] Especially, when caused by infection with urease-producing bacteria like *Proteus* spp., *Klebsiella pneumoniae*, and *Staphylococcus saprophyticus*, an increase in pH > 7 occurs, which can eventually lead to the formation of bladder or kidney stones.[8] The change in the microbiome of the bladder caused by UTIs, especially when recurrent, causes irritation and inflammation of the epithelial layer, which with increasing severity or recurrence is suspected to act as a nucleus for cancer.[9,10] A typical procedure to restore the barrier function is the protection of the extracellular matrix, the glycosaminoglycan (GAG) layer by GAG analogous by hydrogels, either from biopolymers like hyaluronic acid or synthetically derived polymers like *N*-isopropylacrylamide.[11] However, pH sensitivity of most reported hydrogels relates to their response in shape, volume, or swelling characteristics with varying pH conditions. Only few examples report the use of varying pH conditions to trigger hydrogel formation by preferentially making use of noncovalent interactions, which renders them unstable for biological/aqueous environments. Notable examples are pH-triggered hydrogels based on dopamine-functionalized polyallylamine and Fe^{III} ions,[12] injectable pH-triggered hydrogels from aqueous N-palmitoyl chitosan,[13] and pH-triggered, fast responding DNA hydrogels.[14] The very few instances of pH-triggered covalently linked hydrogel systems employ naturally derived hydrogel precursors such as alginate and chitosan.[15,16] Despite the fact that these naturally derived hydrogel precursors are complex in nature and tuning of their material properties[17] is a challenge because of the limited capability of structural variation, they also employ a cross-linking chemistry using quite harmful precursor functionalities like aldehyde groups to gain Schiff-bases or boronic acids. In addition, these reactive groups are present all the time and therefore could react in a non-intended way.

Inspired by drug delivery applications, we questioned ourselves whether a safety feature could be introduced to obtain hydrogel formation only at the site of action. In this way, the concept of prodrugs would be transferred to the synthesis of hydrogels to gain stimuli-induced hydrogel formation on demand.

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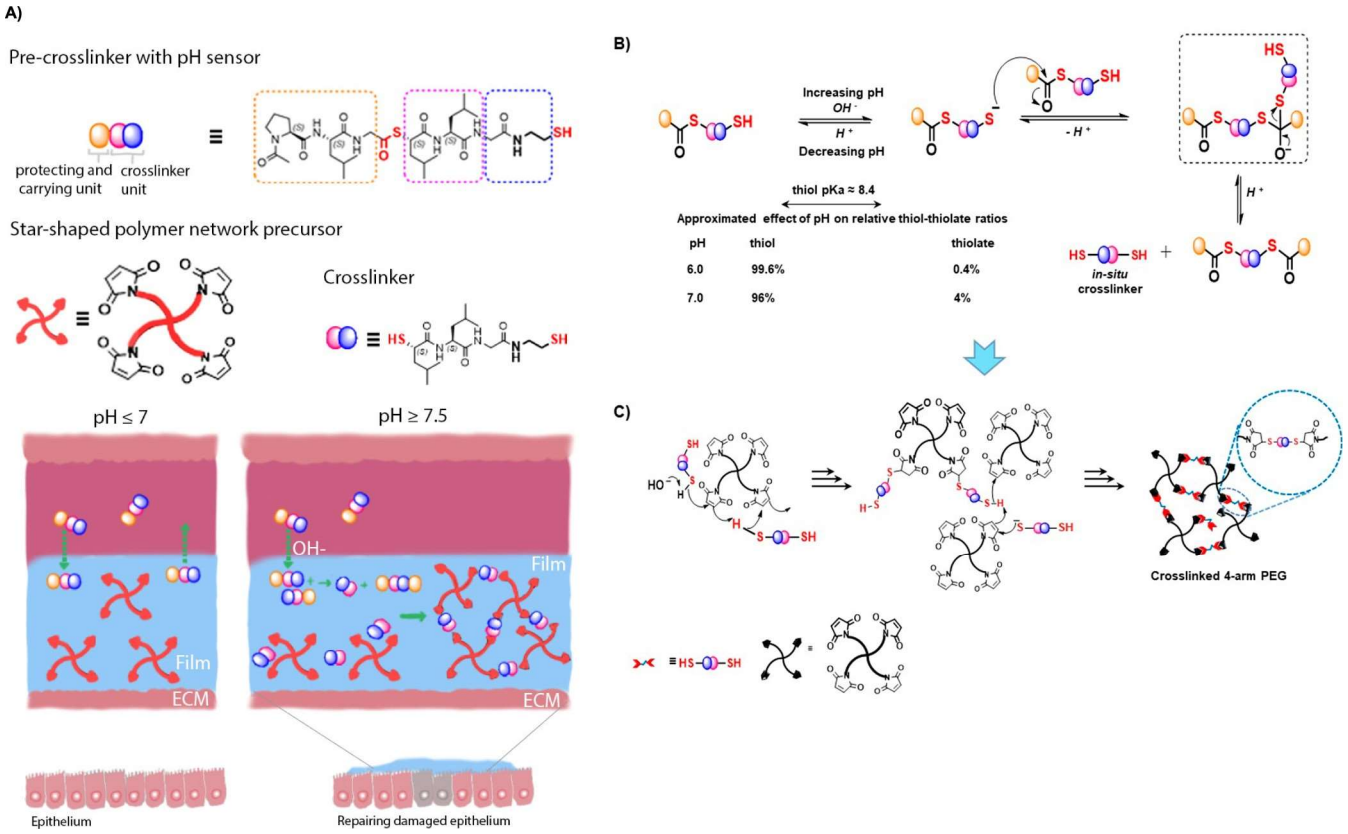
Scheme 1. (A) Concept of a pH-Dependent Hydrogel Formation to Support Healing of Inflamed Tissues like in a Bladder Infection. (B) Mechanism of pH-Dependent "Pseudo" Intramolecular TTE of the Proposed Thio-depsipeptide Initiated by Thiolate Ions. The Driving Force for the Asymmetric Breakdown of the Tetrahedral Intermediate is the Sequence of Amino Acid Residues Attached to the Two Sulfur Atoms of the Tetrahedral Carbon. (C) Formation of PEG Hydrogels with *In Situ* Generated Dithiol Tripeptide from Maleimide-Functionalized Star-Shaped PEG Oligomers. The Maleimide Functional Groups Act as Highly Reactive Michael Acceptors



Prodrugs are bioreversible derivatives of drug molecules with little or no pharmacological activity in their own right but have a built-in structural lability, whether by chance or by design, that permits bioconversion in vivo.[18] This conversion can be initiated by enzymatic or chemical reactions or by combination of the two. By this conversion, the active drug is liberated from a masking promoiety or drug carrier or triggers a structural modification or rearrangement (such as intramolecular reaction or oxidation) such that the resulting molecule is an active metabolite. Two different classes of prodrugs can be identified, carrier prodrugs and bioprecursors.[19] In carrier prodrugs, a transport moiety that is often lipophilic in nature is linked temporarily to the active molecule. A simple hydrolytic reaction cleaves the transport moiety at the correct moment. The transport moiety, also referred to as the carrier group, needs to be nontoxic and should ensure the release of the active molecule with efficient kinetics. Bioprecursors do not provide a linkage between the active moiety and the carrier groups but result from the active moiety itself by a molecular modification. A new compound is generated by this modification and is acting as a substrate for metabolizing enzymes. Once metabolized, it becomes the active moiety.

To transfer the concept of prodrugs to hydrogel formation, the use of highly defined synthetic macromolecular precursors in combination with "latent" small molecule cross-linker moieties is required.

These latent cross-linker precursors could be triggered to become active when pH is varied. At a critical pH, gel formation via covalent linkages can be initiated when appropriate orthogonal reaction partners and an efficient cross- linking strategy are employed. Similar to bioprecursors, these cross-linkers do not present their cross-linking functionalities all the time but would be generated on demand. In an ideal situation, they also would provide a carrier group, which ensures their hydrophilicity.

We speculated that thio-depsipeptide precursors, based on sulfhydryl chemistry, would be able to act as "latent" cross-linkers, which have sensory capability toward pH changes and can form on demand into reactive cross-linkers via thiol−thioester exchange (TTE) reactions in an intramolecular rearrangement controlled by the pH-dependent equilibrium. The TTE reaction is noted for its rapid reaction kinetics in biochemical systems[20] with high efficiency as evidenced by the stoichiometric (1:1 thiol−thioester) interchange of functionality even at low concentrations in the presence of multiple functional groups at room temperature and in aqueous environments.[21−23] A net reaction in TTE exchange reactions occurs only if the incoming and departing thiols have different p*K*a values. By the control of pH, the equilibrium reaction between the incoming and departing thiols can be shifted toward different p*K*a values of incoming and departing species so that a net reaction is gained.

A peptide mimetic-based system was selected as it was speculated that the p*K*a value and this way its suitability as a TTE substrate should be controllable by the sequence of the amino

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acid residues around the thioester, in a particular thio-depsipeptide. In order to realize such a system, two important requirements concerning pH-triggered generation of the cross- linker via TTE and subsequent cross-linking reaction chemistry have to be met.

In the first place, a thio-depsipeptide that is capable of exchange reactions at neutral to basic pH values to yield an α,ω-dithiol-bearing cross-linker must contain both thioester and free thiol moieties (Scheme 1). Secondly, the sequence and nature of amino acid residues around the thioester and thiol moieties should permit TTE reactions, which proceed beyond the formation of the initial tetrahedral intermediate to generate the required α,ω-dithiol cross-linker.

The TTE of the thio-depsipeptide and the required thiol-Michael cross-linking reaction are both pH-dependent and can be tuned by altering the pH of the reaction medium. Since the p*K*a of a particular thiol and the pH of its medium determine the relative amount of thiolate anions to protonated thiols, once the pH exceeds a critical value, the required thiolate ions are generated to initiate both the TTE and the subsequent Michael- thiol addition reactions. Hydrolysis of the thio-depsipeptide should be neglectable as the TTE reaction is three orders of magnitude faster than the hydrolysis reaction.[24]

In this study, we report on the design, synthesis, and ability of thio-depsipeptide (TDP) to undergo TTE reactions controlled by pH. As a proof-of-concept demonstration, the pH-triggered hydrogel formation is envisaged.

The thio-depsipeptide hydrogel formed by the TTE reaction is the first hydrogel formed by application of the prodrug concept using these active molecules as diffusible molecular sensors. As this concept can be applied to other types of cross-linking or rearrangement reactions as well as other stimuli, it is foreseen that it will open numerous hydrogel-based model systems mimicking unique in vivo environments.

MATERIALS AND METHODS

Acetylated thio-leucine was synthesized according to the method described in ref 25 and 26.

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 98%), 1-hydroxybenzotriazole hydrate (HOBt, 97%), 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 97%), boron trifluoride diethyl etherate (98%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99%), 1- octanethiol (98.5%), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 98%), sodium thiomethoxide (95%), diisopropylethylamine (99%), 4-arm polyethylene glycol maleimide (M_n of 20 kDa) (PEG-4MAL, 20K), and deuterated solvents $CDCl_3$ (99.8%) and MeCN-d_3 (99.8%) were all purchased from Aldrich Chemicals (Darmstadt, Germany) and were used as received. Deuterated dimethyl sulfoxide (DMSO-d_6, 99.8%) was purchased from VWR Chemicals (Darmstadt, Germany). Tris- (hydroxymethyl) aminomethane (Tris, 99.8%) and 2-(tritylthio)- ethanamine/*S*-tritylcysteamine (96%) were purchased from Iris- Biotech (Marktredwitz, Germany) and EMD Millipore (Darmstadt, Germany), respectively. Ac-PLG-OH (99.5%) and Fmoc-LG-OH (99.7%) were purchased from Bachem (Bubendorf, Switzerland).

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H NMR (500 and 700 MHz) and ^{13}C NMR (101 MHz) were recorded in $CDCl_3$ (internal standard: 7.26 ppm, ^{1}H; 77.00 ppm, ^{13}C), in MeCN-d_3 (internal standard: 1.94 ppm, ^{1}H; 118.3 ppm, ^{13}C), in DMSO-d_6 (internal standard: 2.50 ppm, ^{1}H; 39.52 ppm, ^{13}C), and in MeOD-d_3 (internal standard: 3.31 ppm, ^{1}H; 49.15 ppm, ^{13}C) on Bruker Avance- 500 MHz and 700 MHz spectrometers. Chemical shifts (δ) were reported as parts per million (ppm), and the following abbreviations were used to indicate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, sept. = septet, m = multiplet, and b = broad and all combinations thereof can be explained by their integral parts.

Fourier Transform Infrared Spectroscopy (FT-IR). The Fourier transform infrared spectra of TDP were recorded on a Nicolet 6700 Fourier spectrometer (Thermo Scientific, Dreieich Germany) with a SenIR Diamond H-ATR. The thio-depsipeptide was investigated in lyophilized form.

Reverse-Phase High-Performance Liquid Chromatography. The purification of the peptide was performed on a Varian HPLC system (Prostar, Model 701, California, USA) by using a polystyrene/divinylbenzene (PS/DVB) reversed-phase semi preparative column (PLRP-S, pore size: 100 Å, 8 μm; 300 × 25 mm). Each purification run was carried out with a linear gradient of water (0.1% v/v TFA, buffer A) and acetonitrile (0.1% v/v TFA, buffer B) from 10% to 90% B for 50 min at a flow rate of 5 mL·min^{-1}. A wavelength of 220 nm was used for the detection of the peptide with a Varian Prostar 325 UV−Vis detector (Victoria, Australia).

Electrospray Ionization Mass Spectrometry (ESI-MS). ESI-MS (direct injection) spectra were obtained on a Bruker Impact II quadrupole/time-of-flight (QqTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an atmospheric pressure ionization source operating in nebulizer assisted electrospray mode. ESI-MS (direct injection) spectra were obtained in positive/negative ion mode by direct injection of samples into the system using a syringe pump (Cole-Parmer, Vernon Hills, IL) operated at a flow rate of 180 μL·h^{-1}. Internal calibration of the system was carried out using a standard sodium formate mixture. All data were processed with the Bruker Compass Data Analysis software 4.3 (Bruker Daltonics, Bremen, Germany) and Mestrenova 12.0 (Mestrelab Research, S.L., Santiago de Compostela, Spain).

Synthesis of Thio-depsipeptide Ac-Pro-Leu-Gly-SLeu-Leu- Gly-NEtSH. ***Synthesis of Fmoc-Leu-Gly-NEtS-Trt.*** A solution of HATU (16.3 g, 42.75 mmol), Fmoc-LG-OH (14.6 g, 35.63 mmol), and *S*-tritylcysteamine hydrochloride in 150 mL of N,N-Dimethylfor- mamide (DMF) was stirred for 15 min, and N,N-Diisopropropylethyl- amine (DIPEA) (19 mL) was added. The reaction mixture was stirred at room temperature for 1 h. After excess of DMF was removed *in vacuo*, the concentrate was precipitated in a 10% v/v mixture of methanol/ H_2O and filtered off. The precipitate was washed with water to remove excess of DMF and was dried to obtain 23.9 g (94.3%) of colorless powder.

Synthesis of NH$_2$-Leu-Gly-NEtS-Trt. To a solution of Fmoc-Leu-Gly-NEtS-Trt (5.7 g, 8.02 mmol) in 75 mL of dry tetrahydrofuran (THF) were added 1-octanethiol (11 mL, 8 equiv) and DBU (470 μL, 40% mol equiv), and the reaction mixture was stirred at room temperature. Reaction was complete after 2 h (TLC, hexane:ethyl acetate:acetic acid = 14:11:1, R_f = 0.30), and excess of THF was removed *in vacuo*. The crude product was dissolved in a small amount of ethyl acetate and purified on a short path silica gel column. Ethylacetate was first used to wash excess of 1-octanethiol and dibenzofulvene-octanethiol adduct, and the eluent was changed to 80% v/v (methanol/ethyl acetate) to obtain the desired product (3.7 g, 95.0%) of a light yellow spongy gum. ***Synthesis of AcSLeu-Leu-Gly-NEtS-Trt.*** To a solution of thioacetyl- Leu (1.0 g, 5.15 mmol) and HOBt (0.8 g, 6.18 mmol) in 30 mL of DMF cooled to 0 °C, a solution of NH_2-Leu-Gly-NEtS-Trt in 20 mL of DMF was added and subsequently stirred for 5 min. The reaction was further cooled to −10 °C, and EDC·HCl (1.2 g, 6.18 mmol) was added in one portion. Afterward, the mixture was stirred whilst it was allowed to warm to room temperature. Excess of DMF was removed *in vacuo*, and the crude product was extracted with ethyl acetate, washed with water and brine, and dried over anhydrous sodium sulfate. A total of 3.2 g of a colorless crude product was obtained after removal of solvent and was used as is.

Synthesis of HSLeu-Leu-Gly-NEtS-Trt. A solution of AcSLeu-Leu- Gly-NEtS-Trt (3.5 g, 5.23 mmol) under N_2 was added to a solution of NaSMe (0.7 g, 5.23 mmol) in 5 mL of MeOH, and the reaction mixture was stirred for 2 h. The reaction mixture was acidified with 0.1 M HCl and was extracted with ethyl acetate. The organic phase was washed with water and brine and dried over anhydrous Na_2SO_4. Subsequent purification over a silica gel column [hexane:ethyl acetate:MeOH:- acetic acid = 15:9:1:0.2] yielded a colorless foam after drying *in vacuo* (2.5 g, 77.5%).

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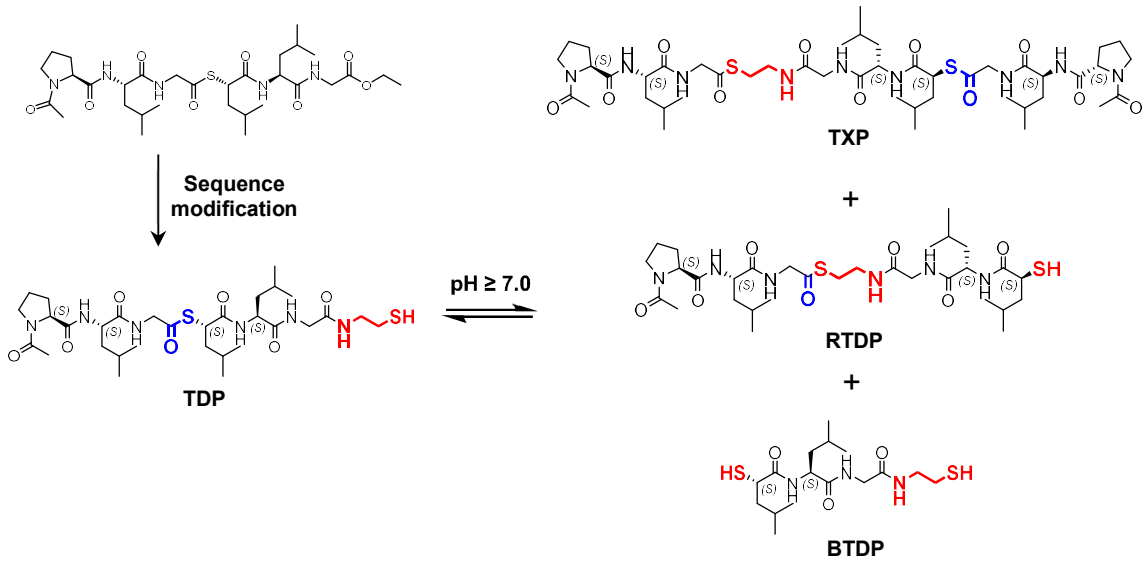
Scheme 2. Modification of the Thio-depsipeptide Ac-Pro-Leu-Gly-S-Lou-Leu-Gly-OEt to TDP[a]



[a]It is speculated that TDP undergoes the TTE reaction to produce the α,ω-dithiol cross-linker BTDP

Synthesis of Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtS-Trt. HATU (2.5 g, 6.68 mmol), Ac-PLG-OH (2.0 g, 6.07 mmol), and HSLeu-Leu-Gly-NEtS-Trt (3.8 g, 6.07 mmol) in 50 mL of DMF were stirred for 15 min, and DIPEA (2 mL) was added. The reaction mixture was stirred at room temperature for 1 h. After excess of DMF was removed *in vacuo*, the crude product After excess of DMF was removed in vacuo, the crude product was extracted with ethyl acetate, washed with water and brine and dried over anhydrous sodium sulfate. The crude product (5.5 g) was an off-white to yellow foamy product, which was obtained after removal of solvent, and was used as is

Synthesis of Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP). A mixture of BF_3·Et_2O (49 μL) and Et_3SiH (3.5 mL) in 60 mL of HFIP was added to Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtS-Trt (5.3 g, 5.70 mmol) and stirred for 30 min. The resulting flaky precipitate was filtered and washed with the filtrate dried over a vacuum evaporator to obtain a light yellow foamy gum (3.9 g). Samples (200 mg) of this crude product were then purified by HPLC using gradient elution with water (0.1% v/ v TFA, buffer A) and acetonitrile (0.1% v/v TFA, buffer B) from 10% to 90% B for 50 min at a flow rate of 5 mL·min^{-1}.

Formation of PEG Hydrogels Using Ac-Pro-Leu-Gly-SLeu- Leu-Gly-NEtSH. pH values to explore pH-dependent hydrogel formation were adjusted by the pH values of the buffer solutions. For hydrogels formed with a maleimide:thiol molar ratio of 1.1, 100 μL solutions of 20 mg PEG-4MAL in bis-Tris (150 mM, pH 6.5 and 7.0) and Tris (150 mM, pH 7.5, 8.0, and 8.5) buffers were mixed with 100 μL of 20 mM TDP in bis-Tris and Tris buffer of corresponding pH, and gelation time was monitored by visual inspection. The experiment was repeated using a similar procedure for hydrogels with a maleimide:thiol mole ratio of 1:2 with 100 μL of 40 mM TDP solutions. Additional hydrogels were prepared in the presence of bromothylmol blue indicator. Demonstrator samples were prepared in a similar procedure using a 1:2 maleimide:thiol mole ratio in 150 mM Tris/bromothymol blue (two drops of bromothymol blue indicator per 3 mL of Tris buffer).

Hydrogel Characterization. Rheology measurements were performed on a Haake Rheowin Mars III (Thermo Scientific, Karlsruhe, Germany) with a parallel plate geometry (Platte PP08) of 8 mm diameter at 25 °C. Amplitude sweep measurements with controlled deformations were carried out to determine the region of linear viscoelastic behavior and obtain estimates of complex viscosity (η^*), storage (G'), and loss (G'') moduli. The degree of swelling based on the hydrogel mass (Q_M) was calculated using eq 1:

$$Q_M = \frac{W_t - W_o}{W_o} \qquad (1)$$

where W_t is the hydrogel mass after swelling and W_o is the hydrogel mass before swelling. Q_M was used to calculate the volume−swelling ratio (Q_V) according to eq 2:

$$Q_V = 1 + \frac{\rho_p}{\rho_s}(Q_M - 1) \qquad (2)$$

where ρ_p is the density of the dry hydrogel (1.12 g·cm^{-3} for PEG) and ρ_s is the density of the solvent (1 g·cm^{-3} for water).

The molecular weight between crosslinks (M_c) and crosslink density (q) were calculated using equations 3 and 4 respectively.

$$\frac{1}{\overline{M_c}} = \frac{2}{\overline{M_n}} - \frac{\frac{\bar{v}}{v_1}(\ln(1 - v_2) + v_2 + \chi_1 v_2^2)}{v_2^{1/3} - \frac{v_2}{2}} \qquad (3)$$

$$q = \frac{\overline{M_r}}{\overline{M_c}} \qquad (4)$$

where M_n is the number-average molecular weight of the polymer, v_1 is the molar volume of the solvent (18 cm^3·mol^{-1} for water), v_2 is the polymer volume fraction in the equilibrium swollen hydrogel (the reciprocal of Q_V), υ is the specific volume of the polymer (ρ_s/ρ_p), and χ_1 is the polymer−solvent interaction parameter.

General Description of Error Analysis. Data were reported as mean value ± standard deviation of three measured quantities.

RESULTS AND DISCUSSION

To meet the requirements of a TDP capable of TTE, we selected the isosteric form of the peptide (Ac-Pro-Leu-Gly-Leu-Leu-Gly-OC_2H_5) derived from the substitution of the Gly-Leu amide nitrogen −NH− with a sulfur atom[27] as a reference compound. In order to enable the "pseudo" intramolecular or "self" thiol−thioester exchange reaction of the peptide mimetic, a free thiol moiety capable of stabilizing the exchangeable acyl group is required. Modification of the original thio-depsipeptide with a cysteamine unit creates a free-thiol-bearing thio-depsipeptide (TDP) with both thiol and thioester functional groups (Scheme 2). A detailed characterization of the thio-depsipeptide is provided in the Supporting Information (Chapter 2).

In line with p*Ka* requirements28 for TTE reactions and from steric considerations, it was anticipated that the Ac-Pro-Leu-Gly- acyl unit should preferentially base on the cysteamine thiol.

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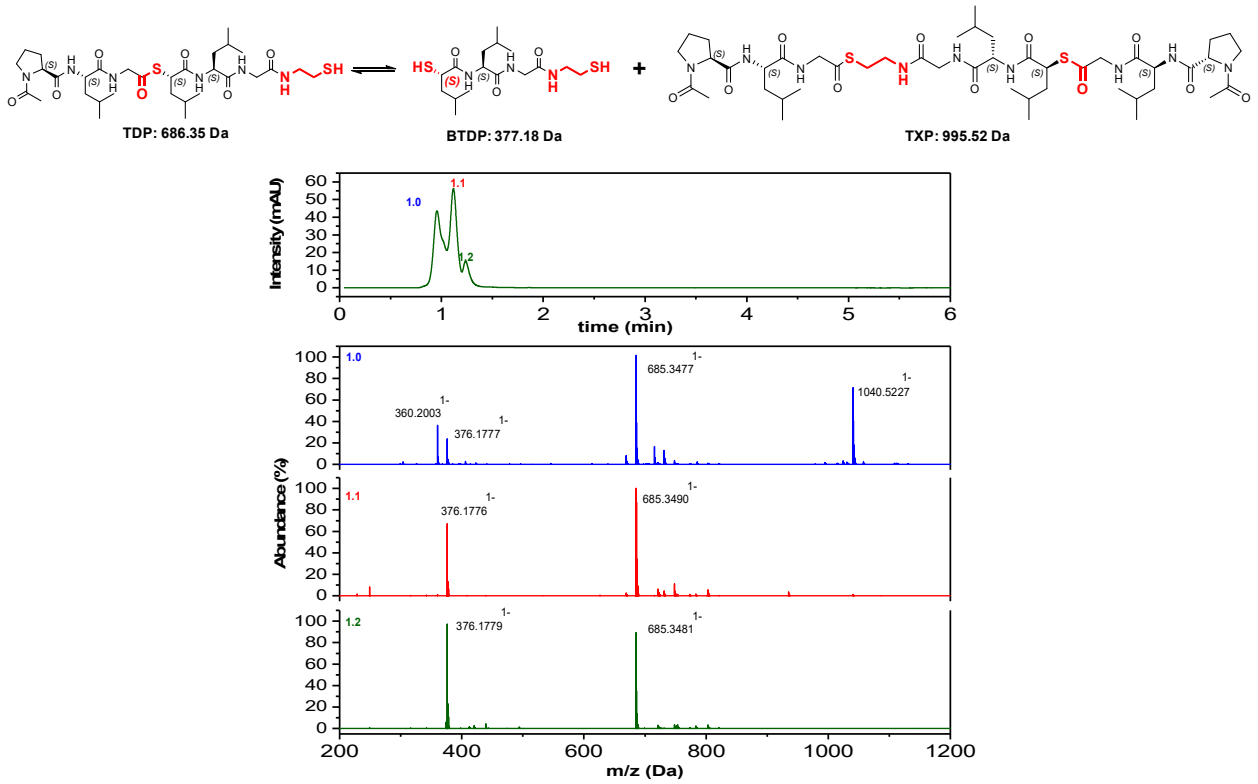

Figure 1. HPLC chromatogram of Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH and the corresponding mass spectra showing the presence of TTE products.

A mixture of products including a rearranged thio-depsipeptide (RTDP) and α,ω-dithiol-bearing -Leu-Leu-Gly- unit capable of cross-linking reactions with Michael acceptor-functionalized multi-arm macromolecules was obtained.

Evidence of Thiol−Thioester Exchange Reactions. In order to obtain the single peptide-mimetic TTE substrate, the ethoxy unit in the original thio-depsipeptide was substituted by a cysteamine unit of comparable length bearing an active thiol group. It is anticipated that after the TTE reaction, the cysteamine thiol has a higher p*K*a value compared to the departing thiol. In addition, the linear structure aids its attack on the thioester unit because of less steric hindrance. A higher p*K*a value of the attacking thiolate ion than that of the displaced or departing has been noted as a requirement for the breakdown of the tetrahedral intermediate to form exchanged products.[24] The detailed NMR and FT-IR characterization of the synthesized TDP can be found in Figures S1−S5 of the Supporting Information.

To test the ability of TDP to undergo TTE reactions, aqueous solutions of the peptide were prepared and analyzed with HPLC-ESI-MS. In the obtained chromatograms, a merged three-peak product signal was observed. These three peaks can be assigned to the peptide structures, TDP and the cross-linker bis-thio-depsipeptide (BTDP). Both were observed as ions with loss of a proton, whereas TXP occurred as a formate ion adduct (Figure 1). This observation was taken as a hint for the ability of the TDP to undergo intramolecular TTE, yielding the intended α,ω-thiol-bearing peptide mimetic.

Further proof of TDP to undergo the TTE reaction was seen in the NMR spectra of the freeze-dried aqueous solution of TDP. In the ^{1}H NMR spectra of the recovered TDP, no signal, which can be attributed to an active thiol of the cysteamine, was observed whilst the amide region of TOCSY spectrum (Figure 2A) contained more than the five expected amino residue spin systems.

A careful examination of the TOCSY spectrum reveals the presence of two pairs each of spin systems belonging to amino acid residues, G_1 and L_1 (Figure 2B). Assuming loss of thiol protons via oxidation and disulfide bond formation, the same number of and/or overlapping spin systems as would have been for the desired structure is expected when no secondary structures or isomers have been formed. In the event of formation of secondary structures or isomers, the difference in chemical shifts for the spin systems in each case (G and L residues) was expected to be small. The observed differences in chemical shifts for the two pairs of G and L spin systems were however large enough to indicate potential changes in the intended structure. From these observations, it can be concluded that the peptide marked in the red box of Figure 2 is one of the products when TDP undergoes the TTE reaction. The sequential correlation of the amino acid residues in the thio-depsipeptide could not be precisely confirmed from the analysis of the NOESY (Figure S6, Supporting Information) spectrum because of the additional glycine and leucine spin systems.

As an additional proof for the occurrence of the TTE, orienting pre-experiments were carried out, in which a set of model thiols was mixed with TDP in equimolar quantities. A key finding from this study by detailed electrospray ionization spectroscopy characterization was the dependence of the fate of the exchange products on the relative p*K*a of the external thiols to that of the attacking TDP thiol. Thiols with p*K*a values similar to that of the attacking TDP thiol caused an increase in the peak intensity of BTDP as observed in mass spectra. Acidic thiols however resulted in a decrease in the peak intensity of BTDP. This could be explained by the lack of breakdown of their

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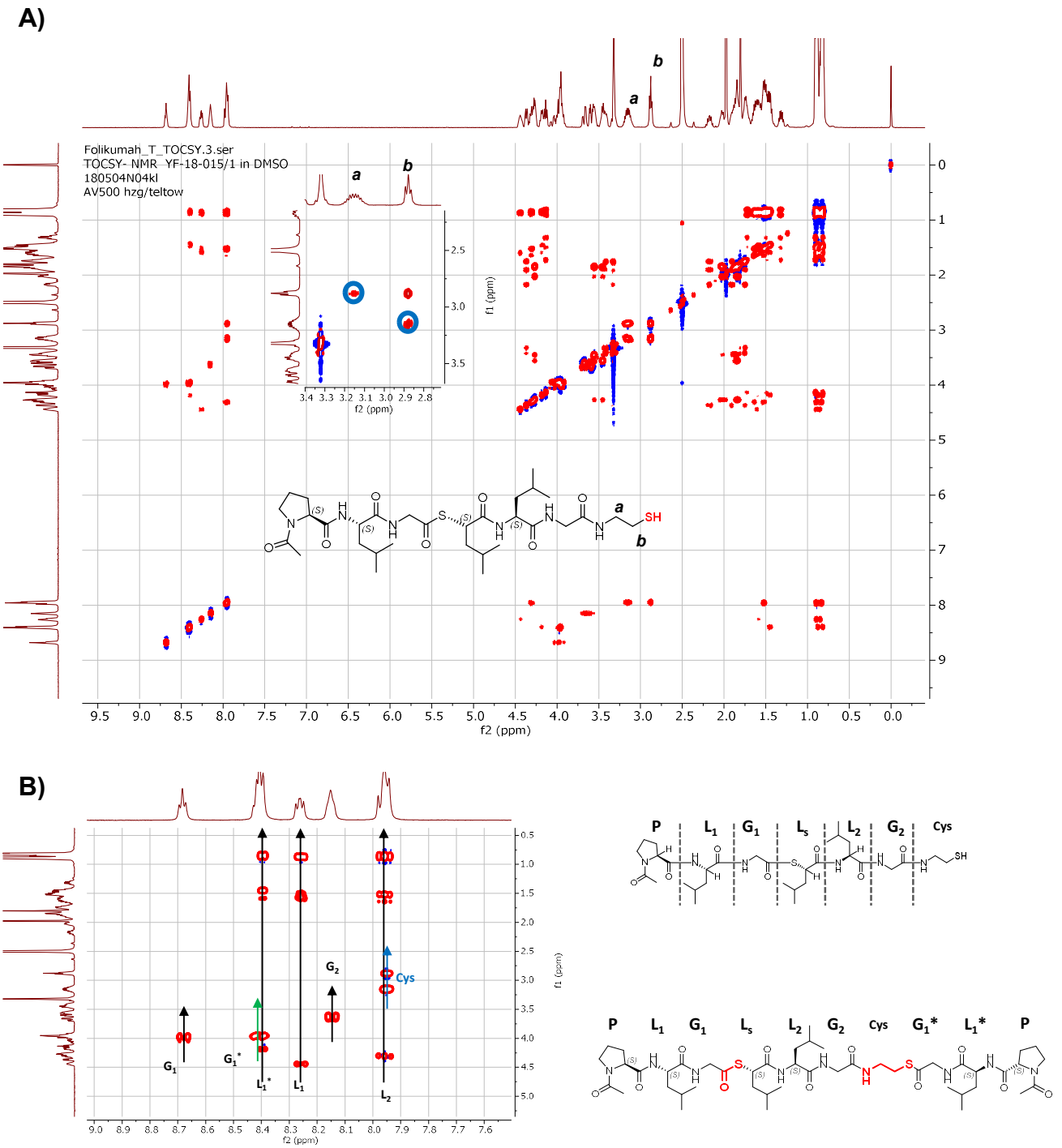

Figure 2. 2D TOCSY spectrum of TDP after dissolving in water and subsequent freeze-drying of the aqueous solution. (A) Spin correlations for free thiol protons could not be observed. (B) Two sets of spin systems assigned to G_1 and L_1 amino acid residues were spotted

respective TTE tetrahedral intermediates, which were clearly visible in the recorded spectra.

pH-Triggered PEG Hydrogel Formation with Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH. Gel formation reactions were conducted with 10 wt % solutions of 20 kDa 4-arm polyethylene glycol functionalized with maleimide (PEG-4MAL) using 1:1 and 2:1 molar ratios of active thiols to maleimide functional groups, assuming 100% PEG functionalization and 100% thio- depsipeptide cleavage to yield the cross-linker BTDP. Since the concentration of the required thiolate ions is directly related to the amount of initial thiol present at a given pH, the increased thiol:maleimide molar ratio of 2:1 should ensure rapid cross-linking compared to the equimolar ratio. As a control experiment, similar trials were conducted with thio-depsipeptide (Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt) and L-glutathione. This thio-depsipeptide and glutathione were the carefully selected peptides to stress the significance of both thioester and thiol functional groups in obtaining a prodrug-type protected precursor. Although this thio-depsipeptide provides a similar sequence structure and a thioester unit, it lacked the needed thiol to initiate the TTE reaction. L-glutathione, on the other hand, contains an active thiol but no thioester unit and would

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Table 1. Summary of Gelation Time and Swelling Ratio of TDP:PEG-4MAL Gelation Trials

	MAL:SH (1:1)			MAL:SH (1:2)		
pH	gelation time (h)	swelling ratio	cross-link density	gelation time (min)	swelling ratio	cross-link density
6.5[a]	no gelation	ND[d]	ND	no gelation	ND	ND
7.0[b]	no gelation	ND	ND	[c]	ND	ND
7.5[b]	24	34 ± 3	2.2 ± 0.1	30	36 ± 3	2.2 ± 0.3
8.0[b]	12	29 ± 2	2.3 ± 0.2	5	16 ± 3	2.8 ± 0.2
8.5[b]	3	18 ± 2	2.7 ± 0.4	3	11 ± 5	3.8 ± 0.4

[a]Bis-Tris-HCl (150 mM). [b]Tris-HCl buffer (150 mM). [c]Only viscous mixture observed after 24 h. [d]ND: not determined.

not generate a dithiol cross-linker on its own in a TTE reaction. A thiol-Michael addition reaction between maleimide and thiol occurs but does not result in cross-linking. All cross-linking reaction trials with the thio-depsipeptide and L-glutathione at all pH values investigated (pH 6.5−8.5) did not yield hydrogels even after extended reaction time periods exceeding 48 h. This confirms the significance of both thiol and thioester functional groups for the generation of α,ω-thiol-bearing cross-linker. Table 1 summarizes the results of TDP hydrogel formation trials with PEG-4MAL. From the gelation times, dependence of TTE, and thiol-Michael reactions, hence the dependence of the cross- linking reaction on the pH of the reaction medium can be observed. This is in line with the fact that both reactions required *a priori* generation of thiolate ions for the initial nucleophilic attack on the thioester carbonyl carbon[24] followed by a subsequent addition reaction to the maleimide units attached to the PEG, assuming a base-catalyzed addition mechanism.[29] When the TDP content was increased, faster gelation times were observed. Equilibrium swelling measurements were conducted to determine the average molecular weight between cross-links (M_c) and cross-link density (q) with the PEG−water interaction parameter (X) taken as 0.426.[30] The effect of increasing thiol content also became apparent for M_c and q. When the molar ratio between the intended *in situ* generated thiols via TTE and the maleimide moieties present of the PEG macromer increased, the degree of swelling and network chain molecular weight decreased whilst the cross-link density and network chain density increased, as seen in Table 1. This is in line with the fact that the increase in q results in less free volume to accommodate water molecules and hence a decreased Q_V because of the lower M_c.

In a demonstration experiment, which enabled the visual monitoring of the extent of cross-linking reaction, an additional set of gels was formed in the presence of the indicator bromothymol blue (Figure 3). The colors of reaction mixtures observed at the start of gelation are characteristic of the indicator color at the operating pH values. When the reaction progressed, gelation occurred. Almost no change in the initial color of the indicator was observed as the buffer capacity of the medium exceeded the change caused by the thiolate ion.

At pH 8.5, completion of gelation was accompanied by a change in the color of the formed gel from blue to pale purple, indicating the occurrence of an additional color. The colors of the gels at pH 8.0 and pH 7.5 were also found to change accordingly, although the rate of color change was slower at lower pH, as found for the rate of gelation. Red colored gels with pH-dependent intensities were obtained after 24 h of monitoring, whilst reaction mixtures at pH 7.0 and 6.5, however, maintained the original indicator colors.

Small-amplitude oscillatory shear experiments were conducted to explore the influence of the pH to the buffered

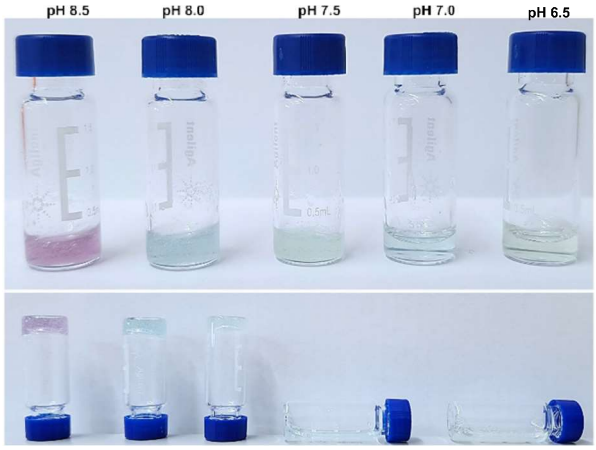

Figure 3. Formation of TDP:PEG-4MAL gels in the presence of bromothymol blue indicator. (A) Reaction mixtures at indicated pH values with characteristic indicator colors. (B) Inverted vials at pH = 8.5, 8.0, and 7.5 showing gel formation

medium and the maleimide:thiol molar ratio on the gel formation. Amplitude sweep experiments were carried out with two of the formed gels to determine the viscosity and complex moduli, G' and G''. Results clearly show the dependence of the storage modulus on the maleimide:thiol molar ratio and initial pH conditions. Since the concentration of thiolate ions at lower pH is low, the rate of TTE and accompanying gelation reactions are therefore slow compared to those formed at higher pH values. Given similar reaction times for gels formed at pH 7.5 and 8.5 (Figure 4), the low storage modulus of gels at pH 7.5 compared to those at pH 8.5 can be attributed to incomplete TTE and gelation reactions. Assuming that more BTDP is generated over time, the expected slow diffusion of these molecules through the already formed network would also impart the final cross-linking and physical properties of the gel.

From the nonlinear behavior of the loss modulus at higher amplitudes, a breakdown in the network structure can be concluded for stiffer gels (gels with storage moduli higher than 5000 Pa).

The sudden drop in all measured quantities, (η^*, G', and G'') even at higher amplitudes, as seen in Figure 4B, can be attributed to a physical rupture of the gels between the measuring plates. This behavior was not observed for softer gels with storage moduli less than 5000 Pa, which maintained almost a linear response throughout the employed amplitude range (Figure 4A).

A direct correlation of thiol content on G' of the gels at each pH could also be drawn by comparing gels with thiol:maleimide ratios of 1:1 to those with thiol:maleimide mole ratios of 2:1.

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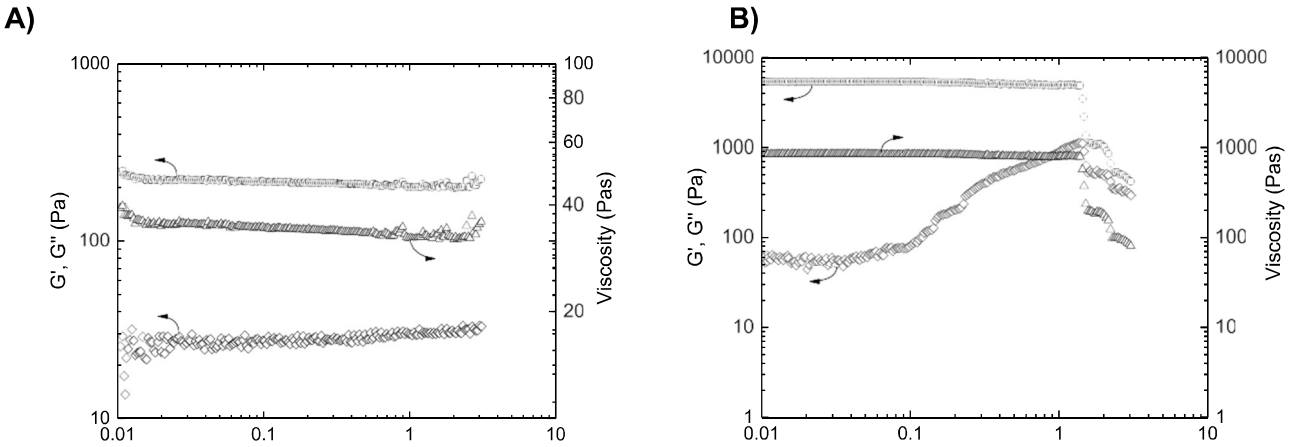

Figure 4. Amplitude sweep tests of TDP:PEG-4MAL hydrogels with a maleimide:thiol molar ratio of 1:2 formed at pH (a) 7.5 and (b) 8.5. The sudden drop in all quantities, G' (open circle), G'' (open diamond), and η^* (open triangle), at higher amplitudes can be attributed to the physical rupture of the gel.

(Figures S7−S10, Supporting Information). The values of initial G' for all gels ranged between 200 and 5000 Pa (Table 1), which falls in the range of elasticities of certain tissue microenvironments, for example, brain tissue with 200−1000 Pa and adipose tissue with 2500−3500 Pa.[31] This might be attributed to the combination of peptide-based cross-links and hydrophilic network constituents. Further evidence of the dependency of G' on pH was found in *in situ* gelation rheology measurements carried out for 20 wt % PEG-4MAL concentrations and 1:1 maleimide:thiol at pH 8.5 and 8.0 (experiment description of In Situ Gelation Rheology and Figures S11−S13 in the Supporting Information). Although both systems exhibit similar G' plateau values, completion of gelation was seen for pH 8.5 in approximately 100 s, whereas at 8.0 pH, gels formed in approximately 200 s. ^{1}H NMR *in situ* gelation monitoring results (NMR In Situ Gelation Measurements and Figure S14 in the Supporting Information) also confirm the observed pH-dependent differences in gelation rates. Reactions at pH 8.5 proceeded so fast that the reaction is almost completed before the measurement starts, while at pH 7.5 in the same time period, almost no decrease in the peak at $\delta = 6.8$ ppm can be observed. At pH 8.0 however, a small decrease in the maleimide proton signal intensity at $\delta = 6.8$ ppm was observed, which was in accordance with the gentle slope and formation of plateau seen toward the completion of gelation in rheology measurements.

The potential of our system for on demand generation of a hydrogel layer, e.g., for the treatment of urinary tract infections, has been demonstrated in two experiments, which are based on *in situ* hydrogel formation at the interface between PEG-4MAL and TDP solutions. To ensure proper exchange of reactants and gelation at the interface, a polymer weight concentration of 10 wt % and maleimide:thiol ratio of 1:1 were used for both experiments. For visualization of the pH, bromothymol blue was added as an indicator. Its color is yellow for pH 6 and blue for pH 8.

In the first experiment, droplets of TDP and PEG-4MAL were introduced to silicon oil to obtain well-separated compartments containing the aqueous solutions of the two reactants, capable of reacting at the interface. When the two droplets were forced to merge, the less dense TDP droplet at pH 8.5 formed a perfect layer over PEG-4MAL. Gelation occurred solely at the interface, noticeable by a thin pale layer between the droplet compartments (Figure 5 and Video S1).

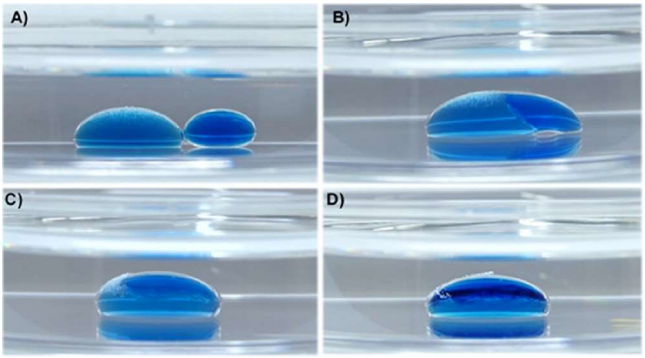

Figure 5. Interfacial formation of TDP:PEG-4MAL gels in the presence of bromothymol blue. (A) TDP (right) and PEG-4MAL (left) droplets at pH 8.5. (B) Two droplets merge by virtue of attractive force between them. (C) TDP droplet forms a layer over the denser PEG-4MAL droplet. (D) Gelation on the interface tracked by the formation of violet color, which is the result of combination of colors from the gel (red) and the indicator at pH 8.5 (blue).

The role of pH in the activation of the TDP solution was shown by performing two different experiments using PEG- 4MAL droplets (pH 8.5) in contact with TDP droplets at pH 8.5 (already activated) on the one hand and TDP droplets at pH 6.0 (non-activated) on the other hand. Gel formation is expected when both droplets provide a pH of 8.5 but not when the pH value of the TDP droplet is at pH 6.0.

In this mixed pH experiment, no gel formation was observed until the TDP layer was activated with two drops of 50 mM aqueous NaOH although the maleimide moiety is regarded as one of the most reactive Michael acceptors and reacts with thiols at pH values as low as 6.5[32] (Figure 6 and Video S2).

Although higher polymer weight concentrations would normally result in fast gelation, it was observed that, at PEG- 4MAL concentrations higher than 10 wt %, no gels were formed. However, the precipitation of a colorless substance in the TDP

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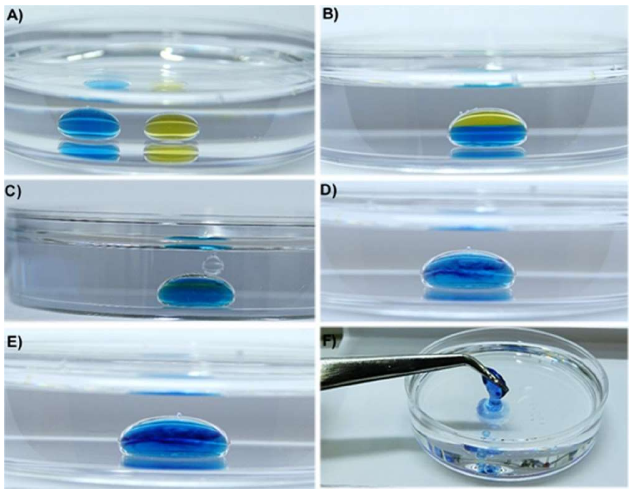

Figure 6. Interfacial formation of TDP:PEG-4MAL gels in the presence of bromothymol blue in silicone oil. (A) TDP (right) and PEG-4MAL (left) droplets at pH 6.0 (yellow color) and 8.5 (blue color), respectively. (B) Two-layered TDP and PEG-4MAL droplet after merging. (C) Activation of TDP layer with two drops of 50 mM aq. NaOH. (D) Initiation of gel formation at the interface. (E) Increased gel formation evidenced by increase in color intensity. (F) Gel layer pulled out of the silicon oil with tweezers from the unreacted PEG- 4MAL droplet.

droplet was detected (Figure S15, Supporting Information). It is assumed that the PEG-4MAL concentration increases the viscosity and surface tension of the droplet, which hinders the diffusion and cross-linking reaction and in this way causes some precipitation of the dithiol. In this case, the water-soluble TDP precursor can be regarded as effectively playing its role as a carrier of the slightly hydrophobic active dithiol cross-linker similar to prodrugs. The generated dithiol could therefore be chemically unavailable when wrongly activated at undesired sites with no orthogonal reaction partner, hence limiting its toxicity.

CONCLUSIONS

A thio-depsipeptide Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) was designed as a precursor with a sensory capability toward pH to produce α,ω-dithiol-bearing peptide mimetic *in situ* at a critical pH. If this reaction activation occurs in the presence of multi-arm macromolecules functionalized with Michael acceptors, the generated dithiol acts as cross-linker to form covalently cross-linked hydrogels. The design of such thio- depsipeptide was to aid a "pseudo" intramolecular thiol− thioester exchange reaction particularly in aqueous medium using pH as the driving stimulus. HPLC-ESI-MS of TDP reveals the presence of all expected TTE reaction products of TDP. Model reactions of TDP with a set of external thiols confirmed the ability of TDP to undergo TTE reactions with results reflecting the role of the p*K*a of thiols in the fate of exchange products. Further evidence of exchange reaction was exploited in the formation of PEG hydrogels at almost neutral to slightly basic pH conditions in buffered media. Results thus far signify the prospects of using thio-depsipeptide as suitable water- soluble TTE substrates, which do not release foul-smelling small-molecular-weight thiols. As the TTE reaction becomes known in material synthesis, the development of substrates or reaction partners useful for application in biological systems gains more attention. We could achieve a sequentially coupled functional system by proper design of the TTE reaction, in which the pH sensitivity of a reactive precursor could be coupled with hydrogel formation by a Michael addition reaction. Similar to the action of prodrugs, the thio-depsipeptide enabled a successful delivery of the slightly hydrophobic active moiety on demand in aqueous media. Depending on the design of the precursors, this concept could be extended to the controlled delivery of active molecules needed for other robust and high- yielding cross-linking reactions for biomedical applications. Application for this sequentially coupled functional system could be seen, e.g., in the treatment of inflamed tissues associated with urinary tract like bladder infections for which pH levels above 7 were reported.[8] By the inclusion of cell adhesion peptide motifs, the hydrogel network formed at this pH could act as a new support layer for the healing of damage epithelium as shown in demonstration experiments. Although the synthesized TDP is a derivative of the commonly used protease activity peptide Ac-Pro-Leu-Gly-SLeu-Leu-Gly-OEt, preparations for toxicity investigations are currently underway to ascertain applicability of TDP in biological studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.0c01690.

Methods (*In Situ* Gelation Rheology and ^{1}H NMR *In Situ* Gelation Measurements); Synthesis and Characterization of TDP (^{1}H NMR Spectrum of TDP; Overlay of ^{13}C and DEPT-135 Spectra of TDP; Multiplicity-Edited HSQC Spectrum of TDP; Amide Region of the TOCSY Spectrum of TDP; and FT-IR Spectrum of TDP); Evidence of TTE Reactions of TDP Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (Amide Region NOESY Spectrum of Freeze-Dried TDP after Dissolving in Water); Rheology and *In Situ* Characterization of Formed Hydrogels (Amplitude Sweep Experiments, *In Situ* Gelation Rheology, and ^{1}H NMR *In Situ* Gelation Measurements) (PDF)

Interfacial gelation at pH 8.5 (MP4)

Mixed pH interfacial gelation (MP4)

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Notes

The authors declare no competing financial interest

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Supporting Information for

Thiol-thioester exchange reaction in precursor enables pH triggered hydrogel formation

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1. Methods

In-situ gelation rheology

Small strain oscillatory shear experiments were performed on the rheometer described in the experimental section using a plate−plate (PP08, ø=8 mm) geometry at 25 °C and under an humidified atmosphere. PEG-4MAL (20 wt. %) and TDP precursor (for 1 : 1 maleimide : thiol stoichiometrically balanced concentration) solutions were freshly prepared in tris-buffer at pHs 8.5 and 8.0. 25 µL of PEG-4MAL solution was poured onto the center of the bottom plate followed 25 µL of the TDP solution. The two precursors were quickly mixed and the upper plate was immediately lowered to a gap size of 0.9 mm to start the dynamic oscillating measurement at constant frequency of 0.5 Hz and strain and 0.05 respectively. The storage G‘ and loss G’’ moduli were monitored and recorded for 25 minutes.

^{1}H-NMR in-situ gelation measurements

Pseudo-2D ^{1}H-NMR reaction monitoring spectra were acquired in arrayed mode using the Bruker zg2d pulse program (pseudo-2D) with 20 spectra recorded in 1.5-minute intervals in the 500 MHz Bruker Avance. 250 µL of each freshly prepared PEG-4MAL and TDP were mixed briefly in stoichiometrically balanced amounts and transferred into a 5 mm NMR tube. The NMR tube was transferred immediately into the instrument. Each experiment/spectrum was acquired with 8 scans and a 3 s relaxation delay. Duration between addition of reagents and completion of the first spectrum was approximately 5 minutes. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane (TMS), referenced to the chemical shifts of residual solvent resonances. Reported errors on conversions were calculated assuming a ±5 % accuracy of signal integrals.

2. Synthesis and characterization of TDP

^{1}H-NMR spectrum of TDP

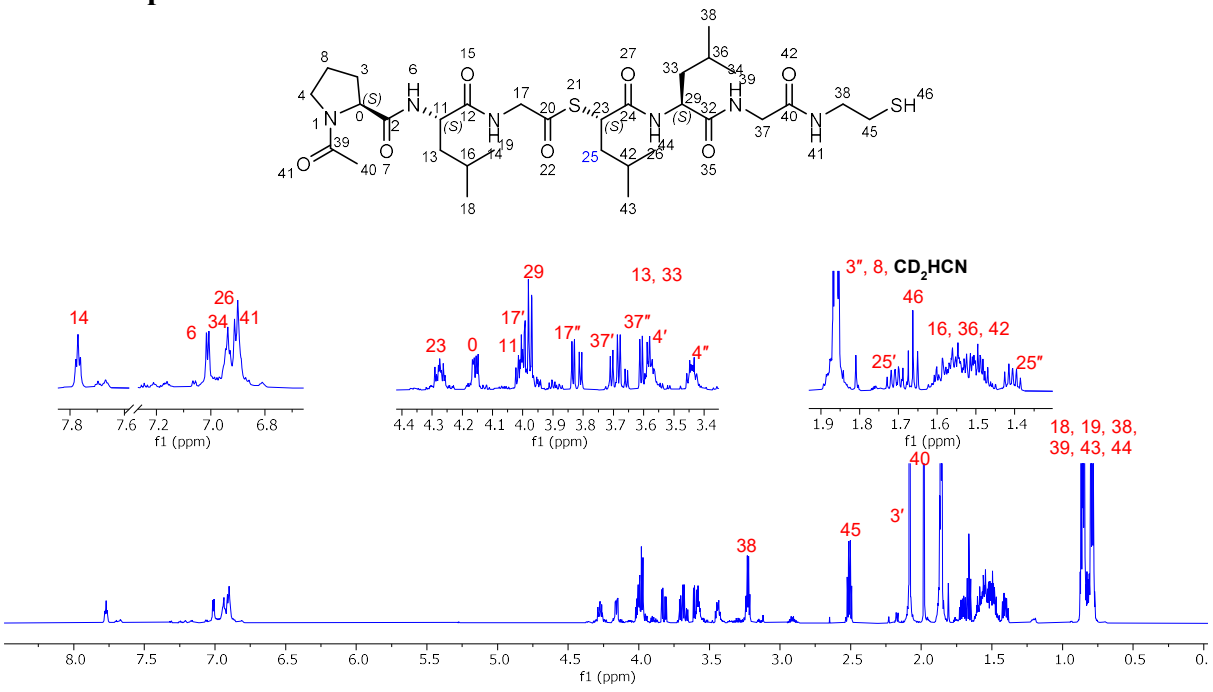


Figure S1. ^{1}H-NMR spectrum of TDP with assignment of signals.

Overlay of ^{13}C and DEPT-135 spectra of TDP

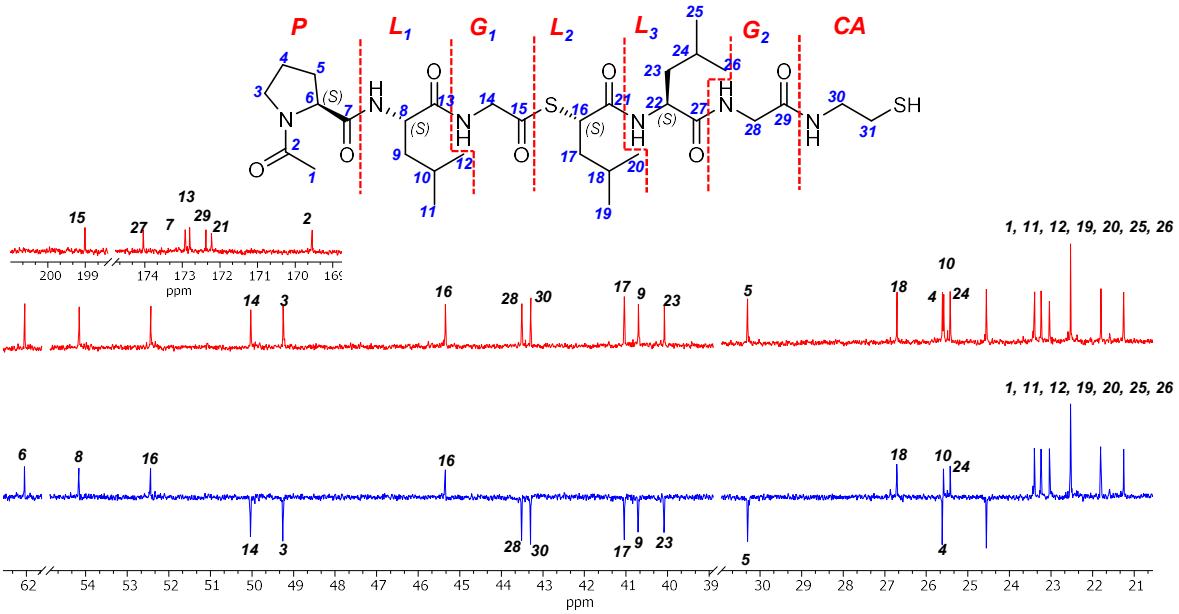


Figure S2. Overlay of ^{13}C and DEPT-135 spectra of TDP with assignment of signals. Region corresponding to carbonyl signals was omitted for clarity since carbonyl carbon signals are absent in DEPT-135. The zoomed-in region (^{13}C) is the rest of the spectrum showing the 8 carbonyl carbon signals.

Multiplicity-edited HSQC spectrum of TDP

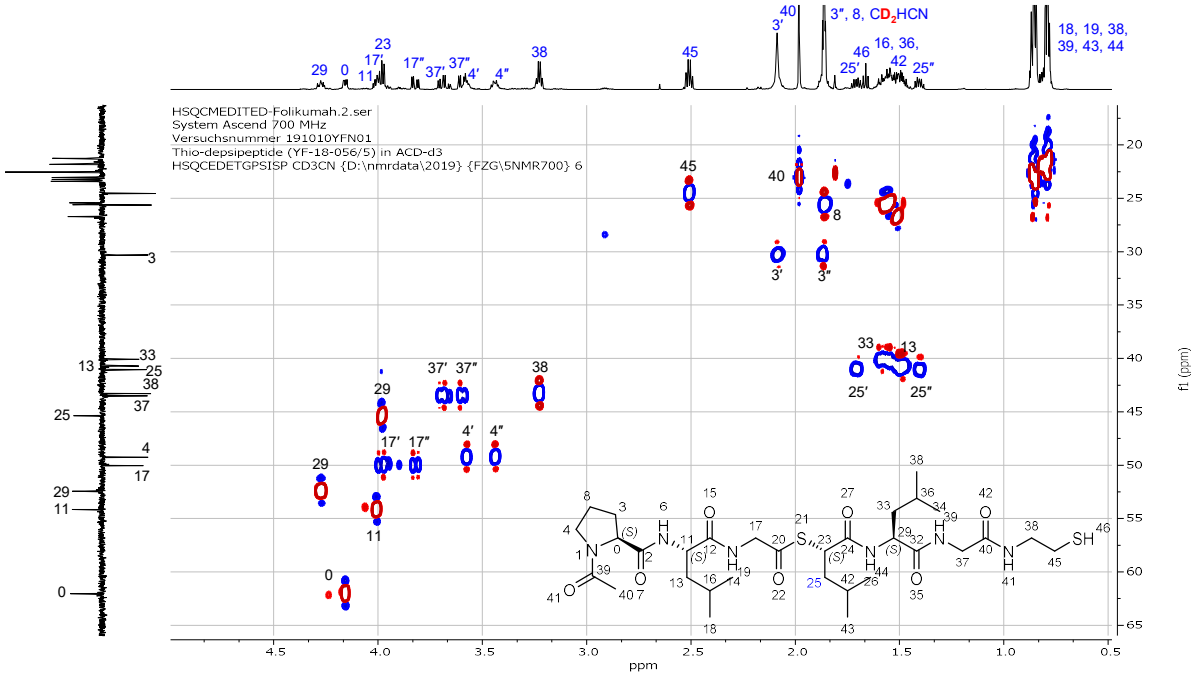


Figure S3. Multiplicity-edited HSQC spectrum of TDP with chemical structure and signal assignments.

Amide region of the TOCSY spectrum of TDP

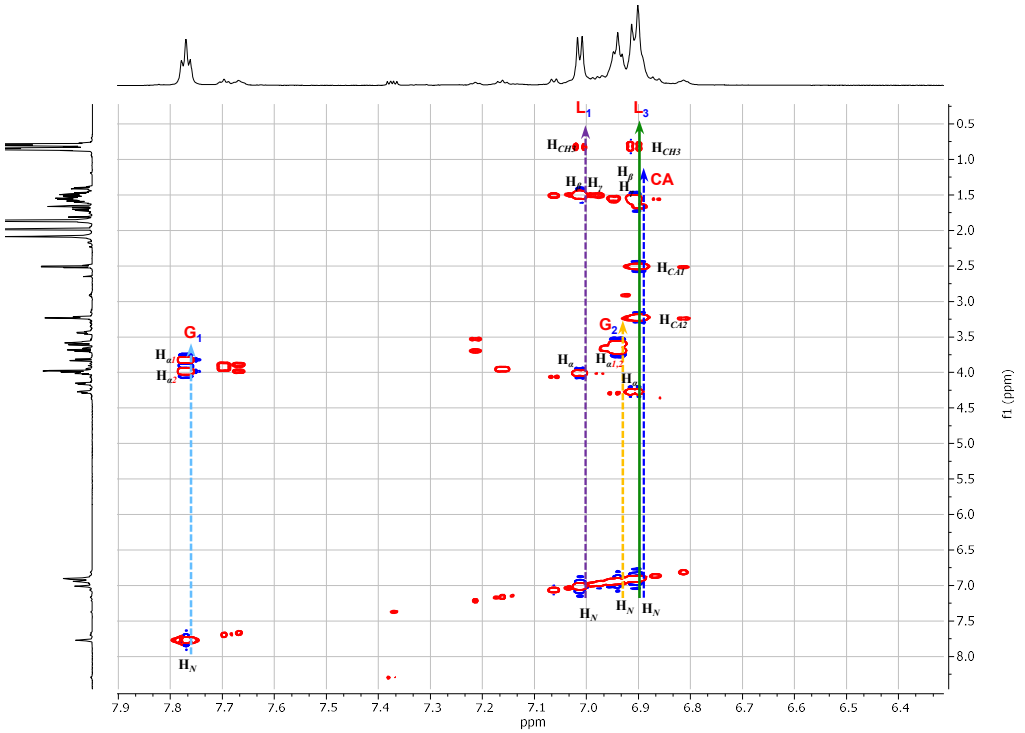


Figure S4. Amide region of TOCSY spectrum of TDP with assignment of amino acid residue spin systems. L and G refer to leucine and glycine amino residues whilst CA refers to the C-terminal cysteamine unit as represented in the chemical structure in figure S2.

FTIR spectrum of TDP

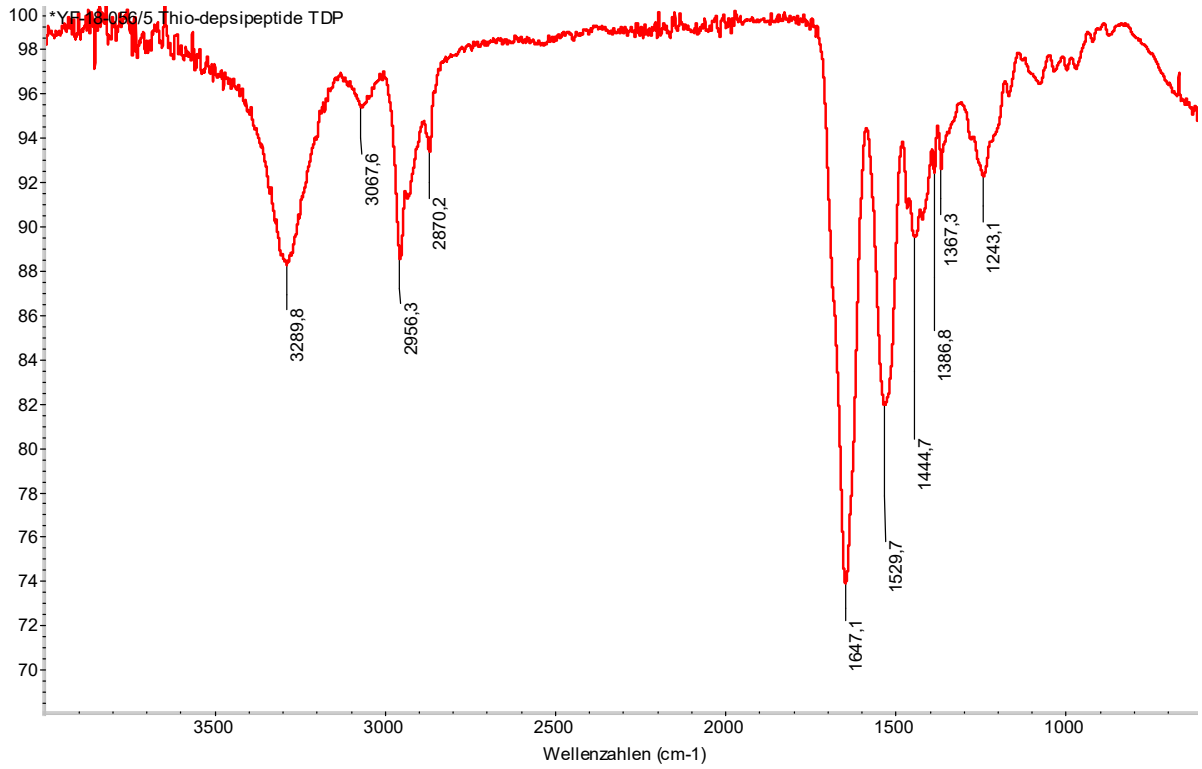


Figure S5. FTIR spectrum of TDP.

3. Evidence of TTE reactions of TDP, Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH

Amide region NOESY spectrum of freeze-dried TDP after dissolving in water

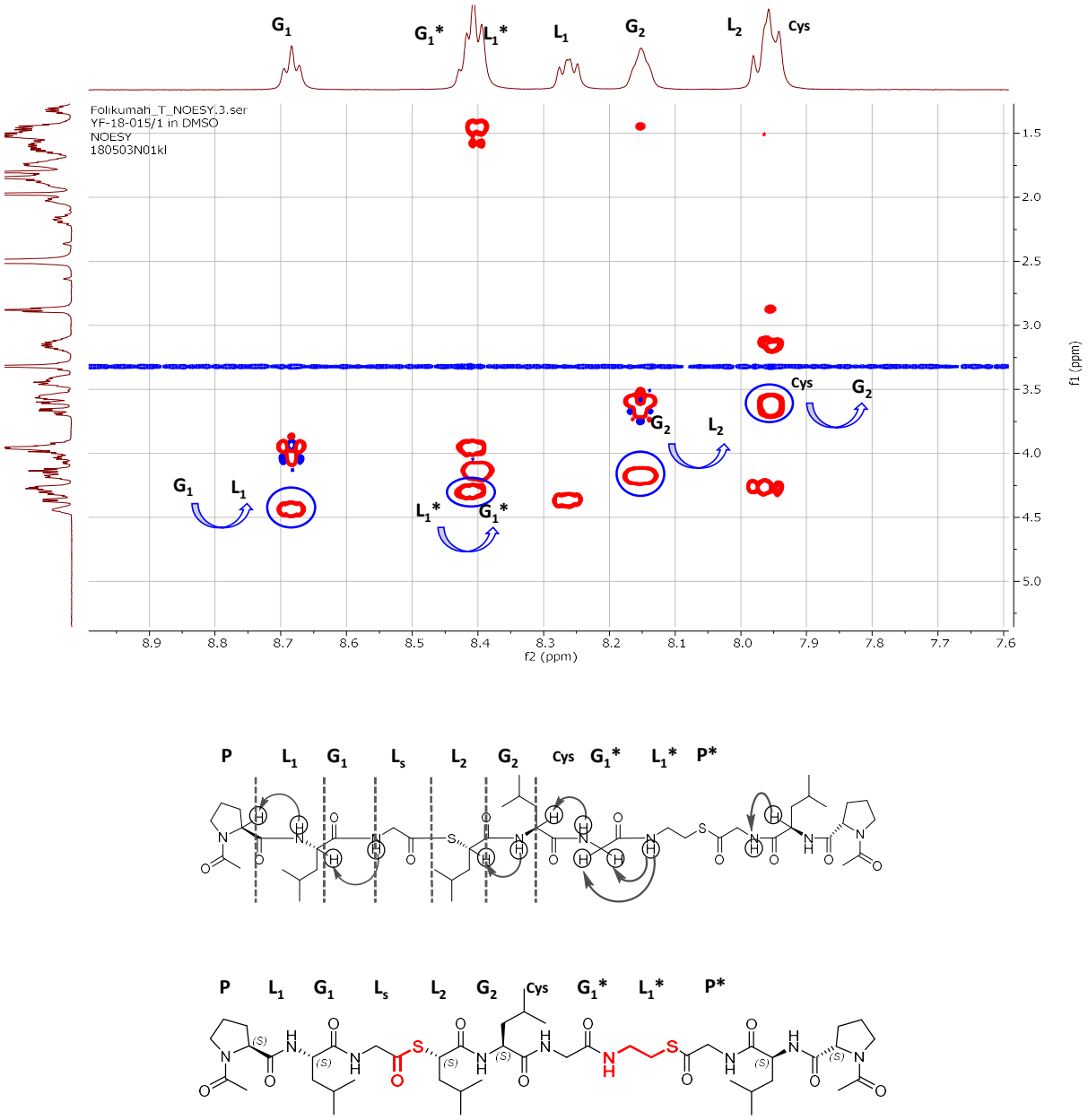


Figure S6. Amide region of NOESY spectrum TDP after dissolving in water and subsequent freeze-drying of the aqueous solution with possible representation of in-space spin correlations.

4. Rheology and in situ characterization of formed hydrogels

Amplitude sweep experiments

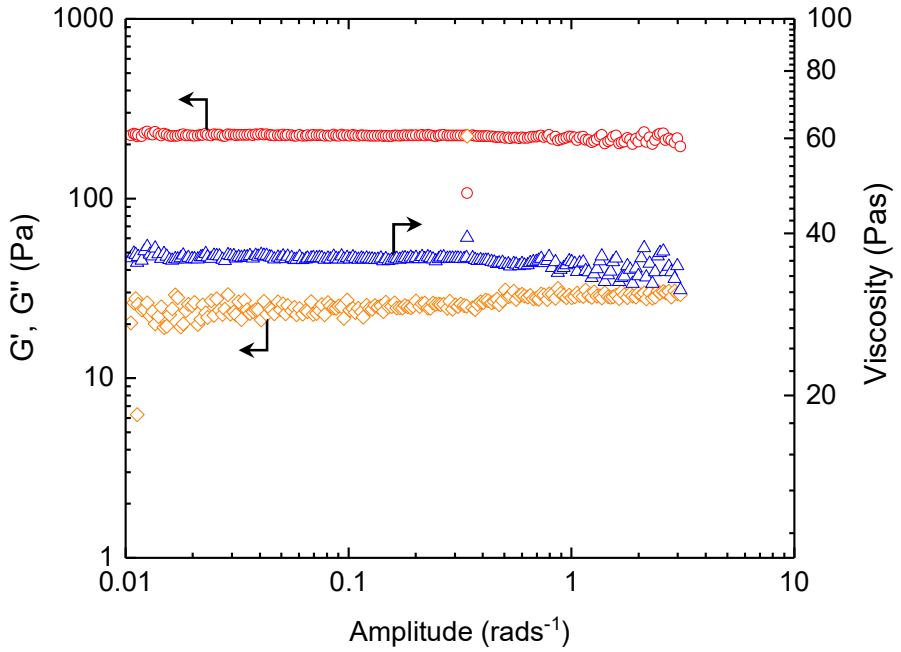


Figure S7. Amplitude sweep rheology experiment of TDP-PEG4MAL hydrogel formed in pH 7.5 tris-HCl buffer (150 mM). Thiol : Maleimide molar ratio is 1 : 1.

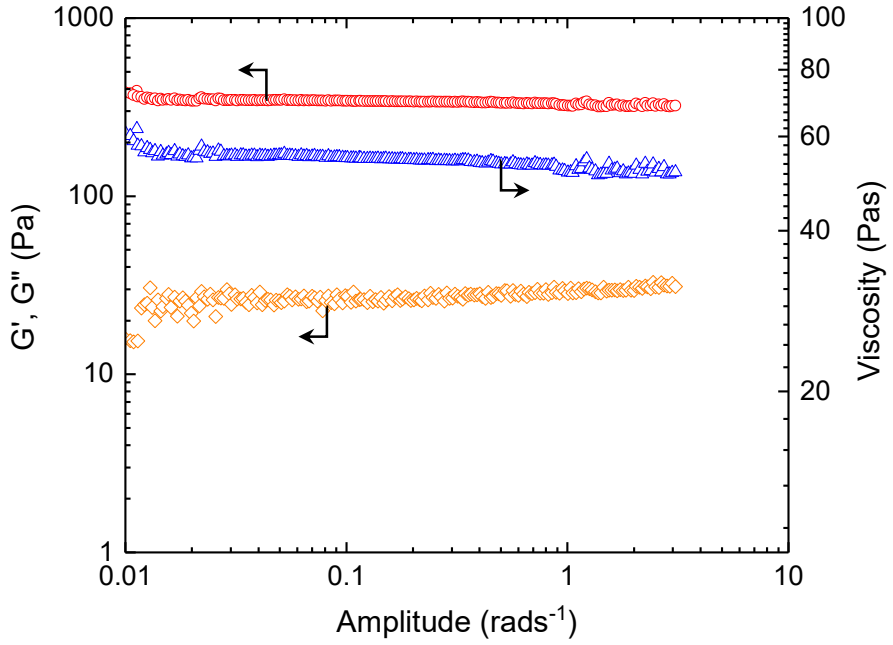


Figure S8. Amplitude sweep rheology experiment of TDP-PEG4MAL hydrogel formed in pH 8.0 tris-HCl buffer (150 mM). Thiol : Maleimide molar ratio is 1 : 1.

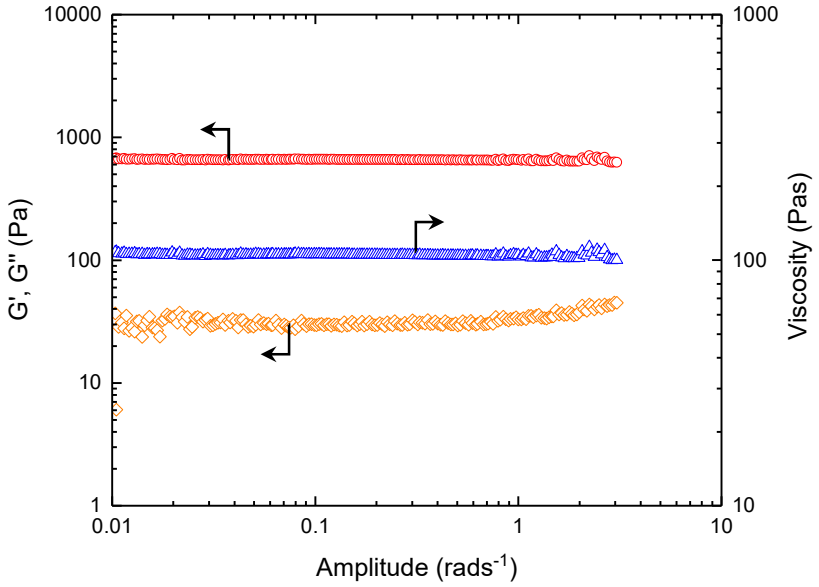


Figure S9. Amplitude sweep rheology experiment of TDP-PEG4MAL hydrogel formed in pH 8.5 tris-HCl buffer (150 mM). Thiol : Maleimide molar ratio is 1 : 1.

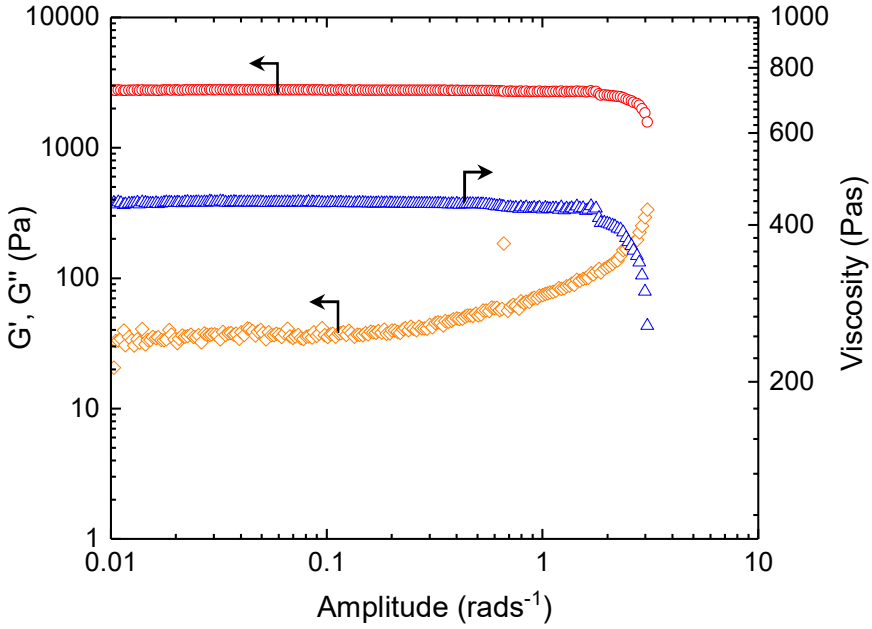


Figure S10. Amplitude sweep rheology experiment of TDP-PEG4MAL hydrogel formed in pH 8.0 tris-HCl buffer (150 mM). Thiol : Maleimide molar ratio is 2 : 1.

In-situ gelation rheology

a) b)

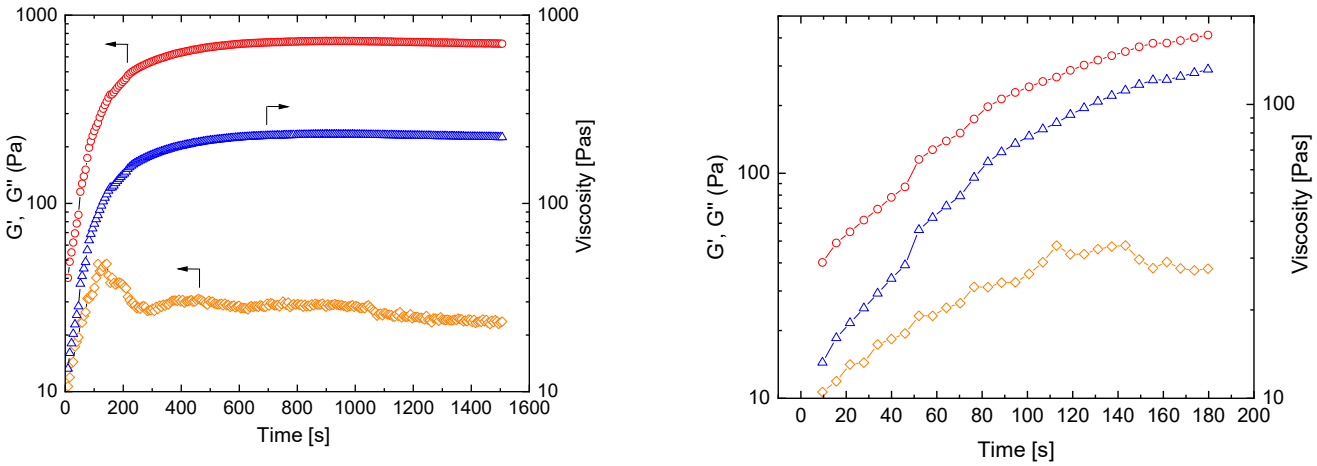


Figure S11. In-situ gelation time sweep experiment of TDP-PEG4MAL hydrogel in pH 8.0 tris-HCl buffer (150 mM) a) and b), b) enlargement of a) for the first 180 s. Thiol : Maleimide molar ratio is 1: 1. Storage modulus G' (red), loss modulus G'' (blue), complex viscosity $|\eta^*|$ (orange).

a) b)

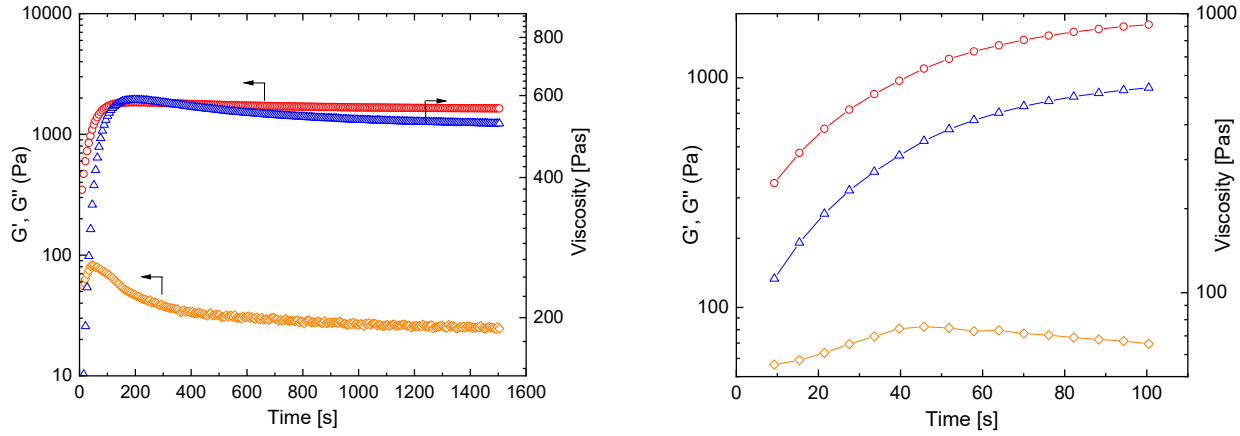


Figure S12. In-situ gelation time sweep experiment of TDP-PEG4MAL hydrogel in pH 8.5 tris-HCl buffer (150 mM) a) and b), b) enlargement of a) for the first 100 s. Thiol : Maleimide molar ratio is 1: 1. Storage modulus G' (red), loss modulus G'' (blue), complex viscosity $|\eta^*|$ (orange).

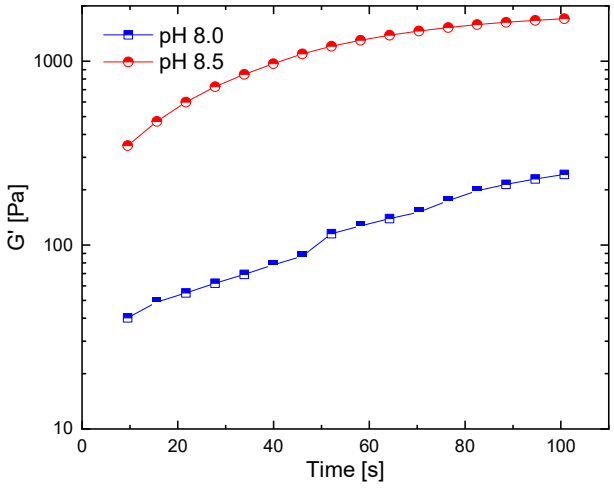


Figure S13. Comparison of storage moduli of In-situ gelation time sweep experiment from Fig. S11 and Fig. S12, pH 8.0 (blue squares) and pH 8.5 (red circles)

^{1}H-NMR in-situ gelation measurements

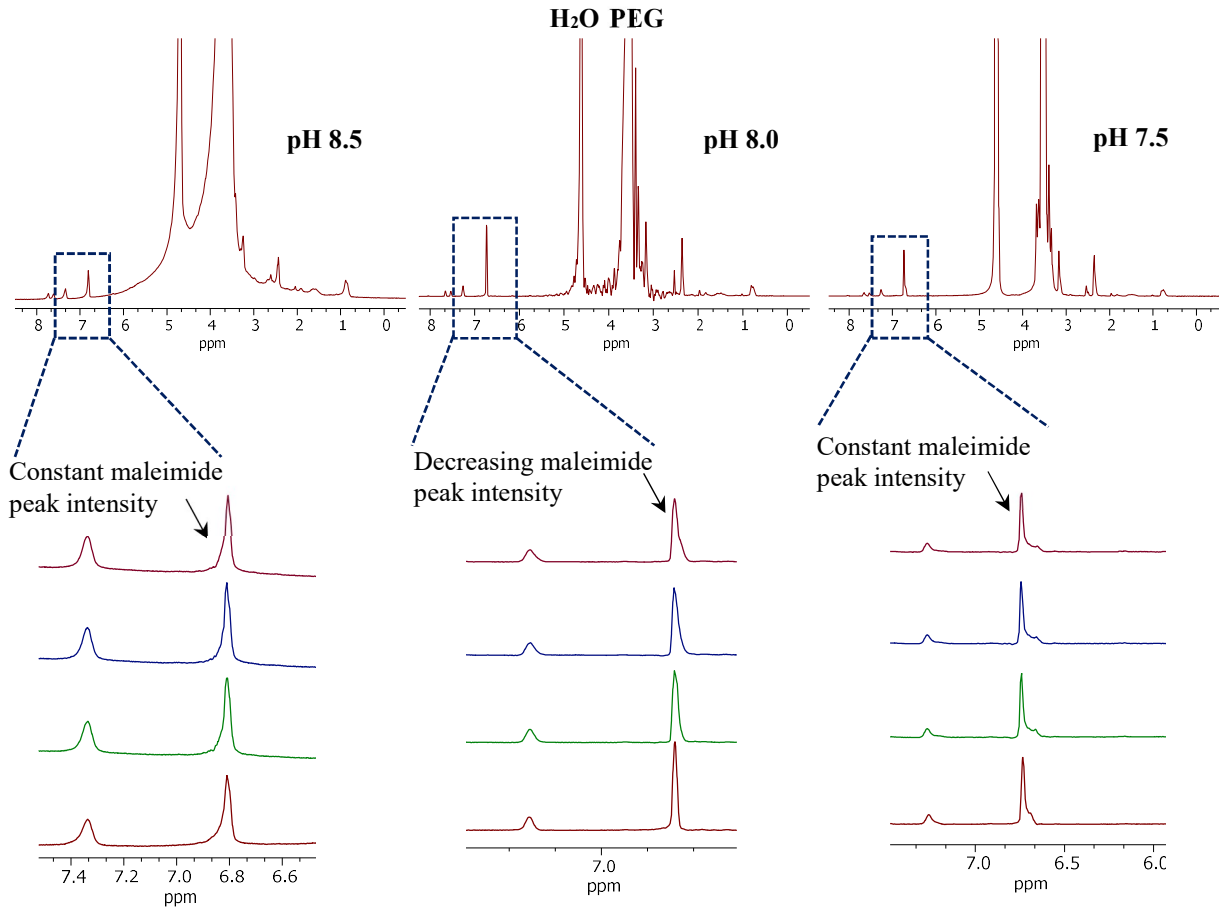


Figure S14. In-situ gelation ^{1}H-NMR spectra of TDP-PEG4MAL hydrogel in pH 8.5, 8.0, and 7.5 tris-HCl buffer (150 mM). Thiol : Maleimide molar ratio is 1: 1.

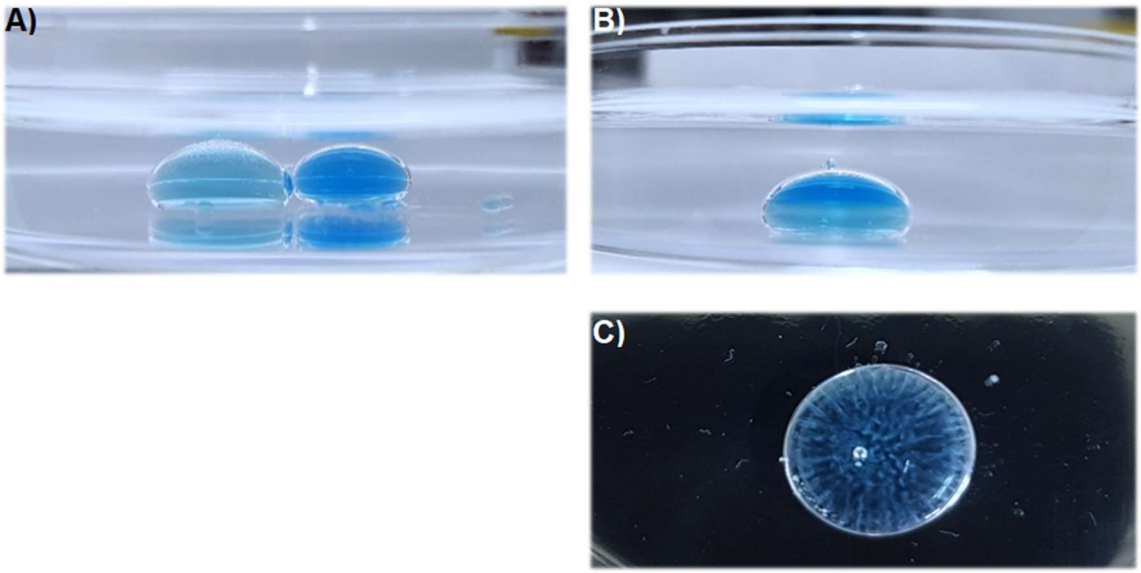
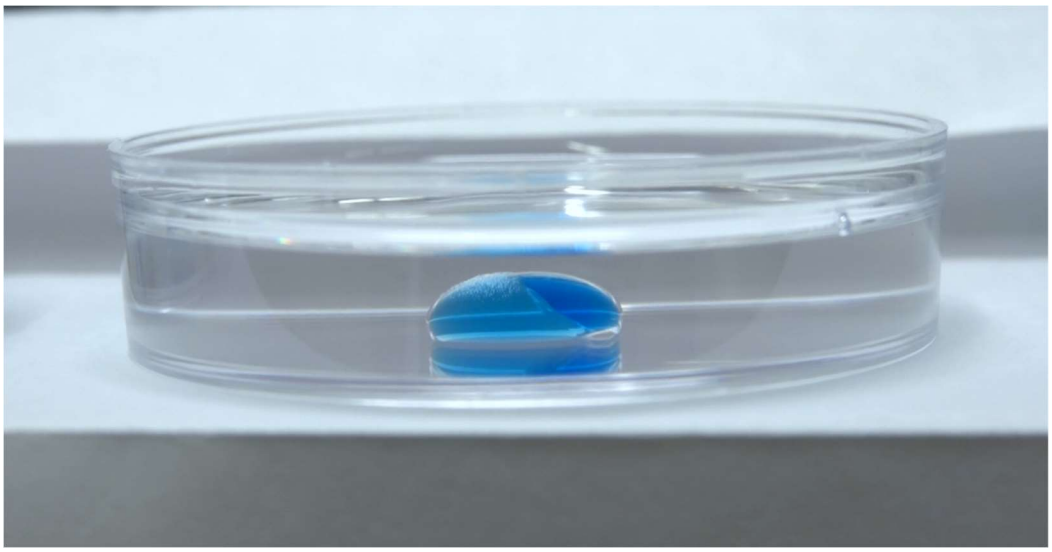
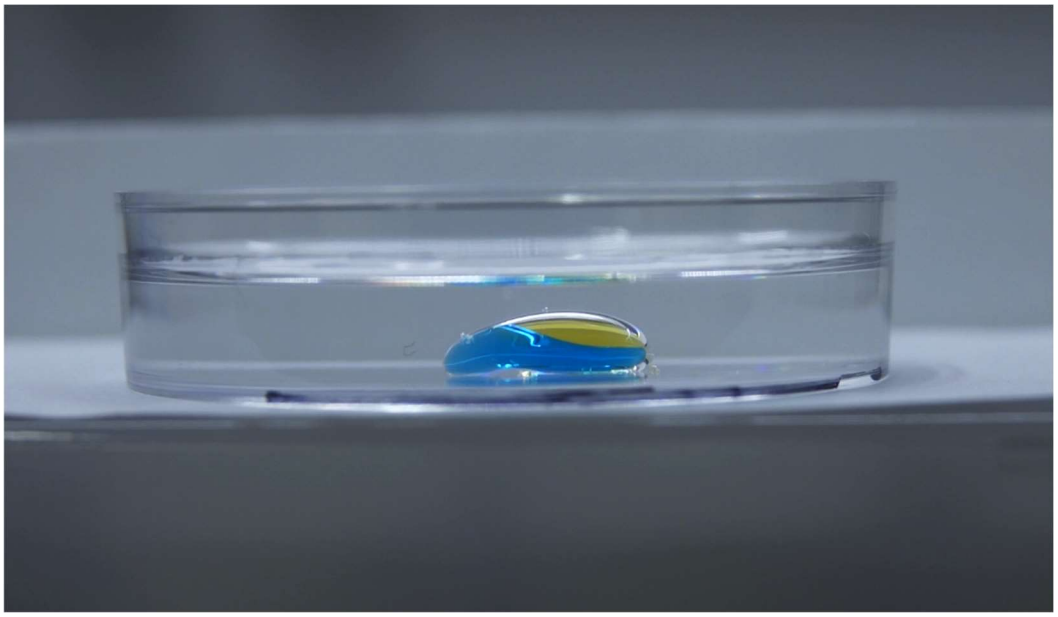


Figure S15. A) PEG-MAL (left, 20 wt. %) and TDP (right) droplets at pH 8.5 forced to make contact B) Two-layered PEG-4MAL/TDP droplet C) Formation of colorless precipitate, viewed from top over black background. This could be to the reason for the inability of the generated dithiol to diffuse and react with the PEG-4MAL in lower part of the droplet.



Video S1. Interfacial gelation of PEG-4MAL (light blue) and TDP (dark blue) droplets at pH 8.5 in a silicon oil matrix at room temperature.



Video S2. Interfacial gelation of PEG-4MAL (pH 8.5, blue) and TDP (pH 6.0, yellow) droplets in the presence of bromothymol blue in silicon oil at room temperature with subsequent increase of pH by addition of aqueous NaOH till a clear color change of the indicator.

14 Appendix IV - A 4-Arm PEG-thiodepsipeptide Precursor Enables Gelatinase-promoted Hydrogel Formation

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Keywords: hydrogel; matrix metalloproteinase; thiodepsipeptide; enzymatic crosslinking

Abstract

In situ hydrogelation of injectable precursors upon biological stimulus is of interest. .Here, we report on a prodrug type peptide-based crosslinker precursor activation and covalent gel formation using gelatinases.

For this, a 4-arm PEG-thiodepsipeptide conjugate, PEG4TDP$_o$ containing the Ac-Pro-Leu-Gly-SLeu-Leu-Gly- thiodepsipeptide sequence was used. The sequence contains an MMP – cleavable thioester which generates thiol- bearing crosslinker for orthogonal reaction with Michael-acceptor functionalized macromolecules to form hydrogels PEG4TDP$_o$ was synthesized with a simple PEG-functionalisation protocol involving convergent and divergent synthetic steps without the need for rigorous purification procedures. PEG4TDP$_o$ in the presence of 10 wt. % maleimide functionalised 4-arm polyethylene glycol formed hydrogels upon addition of MMP-2 or -9 with average gelation times of 28 and 40 minutes respectively. The much faster gelation times compared to the enzyme-free system showed the specific input of the enzymatic reactions.

The MMP-assisted activation and crosslinking using peptide sequences can be paired with the simultaneous release of active payloads for specific application. A promising area for its

application is the development of new biomaterials especially in the area of cancer research as work with MMP inhibitors is plagued with numerous challenges.

Introduction

Successful syntheses of biological compounds, which are otherwise difficult or impossible to synthesize by classical synthetic routes[1] has led to increasing interest in enzymatic reactions. This has also inspired the use of enzymes for crosslinking of hydrogels, mainly due to the mildness of this type of reaction[2]. The mild reaction conditions of enzyme-catalysed reactions imply that they can be used to crosslink natural polymers such as proteins that cannot withstand harsh chemical conditions.[2, 3] Because enzymes are being produced by cells in lieu of normal physiological functions or by virtue of certain biochemical/physical stimuli, enzyme catalysed crosslinking can be used in the design of smart enzyme-responsive systems.[2] In this regard, multi enzyme-sensitive hydrogels can be designed as suitable mimics of the native ECM with dynamic remodelling characteristic.

Enzymatic crosslinking also presents a mild, less toxic and more precise pathway of realizing *in situ* gelation based on bioactive gel precursors that can be administrated safely via a syringe[4] particularly for minimally invasive procedures. Enzymes commonly used in crosslinking reactions and biomaterial syntheses are limited mainly to those involved in crosslinking reactions of the native ECM. These are represented mostly by the oxidoreductases (EC 1.)[1,5-9] and transferases (EC 2.)[10-13] and are usually implemented via functionalized polymer-polymer systems (natural/synthetic) carrying specific peptide motifs as substrates. The use of short peptide sequences (small molecular hydrogelators) for the formation of hydrogels[14-21] via enzymatic bond breaking and supramolecular assembly has received increased attention recently, as these small molecules exhibit properties commonly seen in hydrogels made from natural or synthetic polymers.

The broad range of structure-function relationship afforded by peptide primary/sequence structure, small size and ease of synthesis of these short peptides over conventional polymers makes their incorporation in the formation of hydrogels even more interesting.

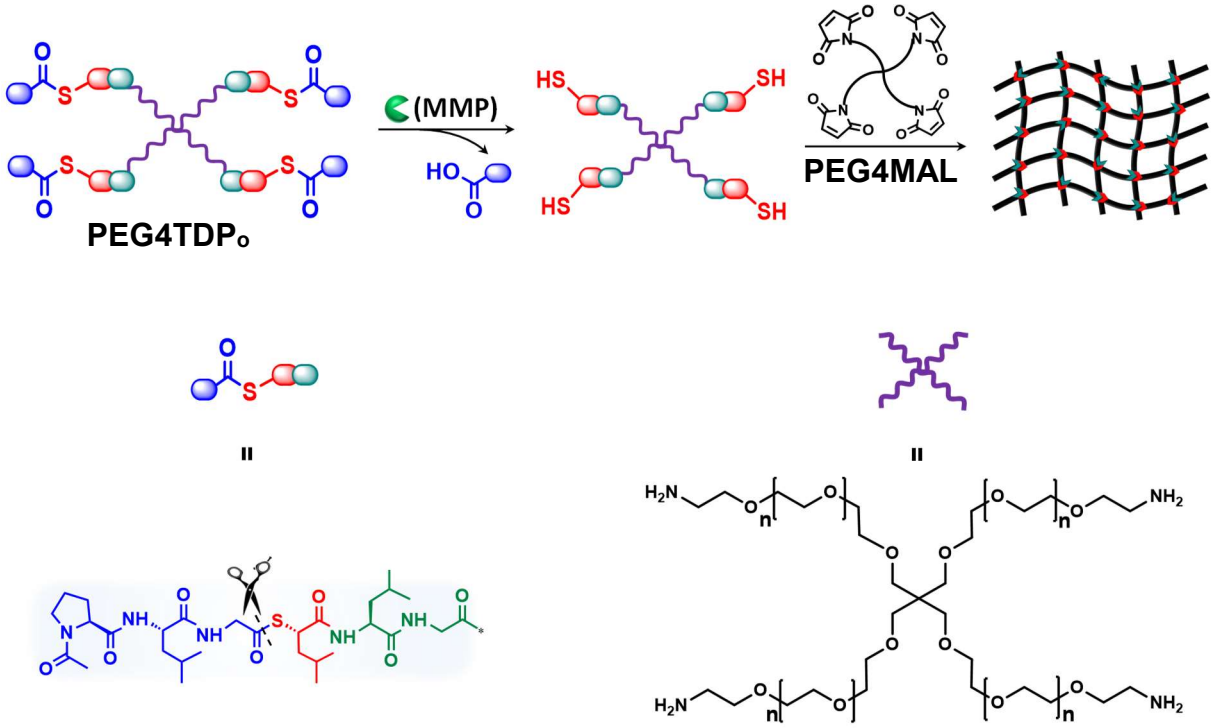
Although research has been far advanced on these enzyme-sensitive supramolecular hydrogelator precursors, it is also of interest to see whether this 'cleavage-leading-gelation' approach can be employed in suitably functionalised telechelics. This strategy compared to supramolecular hydrogelators has the added benefit of producing covalent networks which are more stable to dilution and constant changes in physical conditions experienced in physiological environments.

We have previously reported a prodrug type gelation of maleimide functionalised 4-arm PEG polyethylene glycol (PEG4MAL) using pH-sensitive thiodepsipeptide crosslinker precursor activated by thiol-thioester exchange reaction.[22] In this study, the utility of the used thiodepsipeptide sequence, Ac-Pro-Leu-Gly#SLeu-Leu-Gly (TDP_o) was further extended for gelatinase-promoted *in situ* gelation of PEG4MAL based on the reported sensitivity of TDP_o towards MMP cleavage. The *G-L* bond is one of the most commonly cleaved bonds in both natural and synthetic MMP substrates. [23-25] Consequently, TDP_o which is the isosteric form of the collagenase substrate, Ac-Pro-Leu-Gly#Leu-Leu-Gly was found to be more susceptible to cleavage with k_{cat}/K_m ratio in excess of 10^4 $M^{-1}s^{-1}$.[24] The usefulness of TDP_o for the design of an MMP-sensitive crosslinker precursor arises from its cleavage to yield thiol-bearing peptide capable of reacting in thiol-Michael reaction.

To enable multiple points of reaction after cleavage, we designed a 4-arm PEG-thiodepsipeptide conjugate ($PEG4TDP_o$) using a small molecular mass (2 kDa) PEG-amine core specifically to enhance the solubility of the substrate. When paired with PEG macromers of similar architecture functionalized with highly reactive Michael acceptors such as a

maleimide,[26] an MMP-promoted hydrogel system is envisaged assuming more than a single active thiol units from a PEG4TDP$_o$ molecule can be generated (Scheme 1).

The small PEG core was preferred over larger ones in order to prevent possible formation of secondary structures such as micelles, which have the propensity to shield the peptide sequence from the enzymes. Our choice of thiol-Michael addition has been necessitated by the versatility and robustness it affords in hydrogel synthesis,[27-30] surface and polymer modification.[31] Enzymatic cleavage studies with flourometric thiol assay confirmed the susceptibility of PEG4TDP$_o$ towards MMP-2 and -9.



Scheme 1: General schematic representation of proposed concept. The MMP-sensitive PEG4TDP$_o$ generates a thiol-based active crosslinker assuming at least two peptide units per PEG4TDP$_o$ molecule are successfully cleaved. The resulting crosslinker reacts immediately with available maleimides of PEG4MAL to yield crosslinks.

In this report, the design, synthesis and characterisation of the MMP-sensitive PEG4TDP$_o$ is presented. Susceptibility of PEG4TDP$_o$ towards cleavage by gelatinases (MMPs -2 and -9) as well as the MMP-promoted gelation of PEG4MAL were investigated.

Materials and Methods

Diisopropylethylamine (99%), 4-arm polyethylene glycol maleimide, M_n 20 kDa (PEG4MAL 20 kDa), 11-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 97%), 4-arm polyethylene glycol amine ($PEG4NH_2$ 2 kDa) and were all purchased from Aldrich Chemicals (Darmstadt, Germany) and were used as received. Deuterated dimethyl sulfoxide, DMSO-d_6 (99.8%) was purchased VWR Chemicals (Darmstadt, Germany). Tris(hydroxymethyl) aminomethane (Tris, 99.8%) was purchased from Iris-Biotech (Marktredwitz, Germany). Ac-PLG-OH (99.5%) and Boc-LG-OH (99.7%) were purchased from Bachem (Bubendorf, Switzerland). Acetylated thio-leucine was synthesized according to the method described in ref. [32, 33]. MMPs -2 and -9 (activated) were purchased from Aldrich Chemicals (Darmstadt, Germany). Invitrogen Measure-iT^{TM} thiol assay kit was purchased from Thermo Fisher Scientific (Darmstadt, Germany).

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR (700 MHz) and ^{13}C-NMR (101 MHz) were recorded in DMSO-d_6 (internal standard: 2.50 ppm, ^{1}H; 39.52 ppm, ^{13}C) on Bruker Ascend - 700 MHz spectrometer. Chemical shifts (δ) were reported as parts per million (ppm) and the following abbreviations were used to indicate the multiplicities: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *sept*. = septet, *m* = multiplet, *b* = broad and all combinations thereof can be explained by their integral parts.

Electrospray Ionization Mass Spectrometry (ESI-MS)

ESI-MS (direct injection) spectra were obtained on a Bruker Impact II quadrupole/time-of-flight (QqTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an atmospheric pressure ionization source operating in the nebulizer assisted electrospray mode. ESI-MS (direct injection) spectra were obtained in positive/negative ion mode by direct

injection of samples into the system using a syringe pump (Cole-Parmer, Vernon Hills, IL) operated at a flow rate of 180 $\mu L \cdot h^{-1}$. Internal calibration of the system was carried out using standard sodium formate mixture. All data were processed with the Bruker Compass Data Analysis software 4.3. (Bruker Daltonics, Bremen, Germany) and Mestrenova 14.0 (Mestrelab Research, S.L., Santiago de Compostela, Spain).

Reverse-Phase High Performance Liquid Chromatography

Peptide purification was performed on an Agilent HPLC system (1290 Infinity II Series, Germany) equipped with a preparative binary pump, an automatic sample injection and fraction collection system (1290 Prep ALS/FC), a variable wavelength detector (1290 VWD) and InfinityLab mass detector (LC/MSD XT). Purification was carried out using a polystyrene/divinylbenzene (PS/DVB) reversed-phase semi-preparative column (PLRP-S, pore size: 100 Å, 8 µm; 50*30 mm). Each purification run was carried out with a linear gradient of water (0.1% v/v FA, buffer A) and acetonitrile (0.1% v/v FA, buffer B) from 10 % to 90 % B in 40 min at a flow rate of 5 $mL \cdot min^{-1}$. A wavelength of 214 nm was used for the detection of the peptide.

Enzymatic Cleavage Kinetics – Fluorescence Spectroscopy

Enzymatic cleavage studies were performed with Invitrogen Measure-iT^{TM} thiol assay kit on a Tecan Infinite M200 Pro microplate reader. In a typical experiment, the substrate ($PEG4TDP_o$, 0.25 – 8 µM), activated enzyme (0.2 µg/mL) and thiol fluorescent probe were added to each well and the final volume made up to 150 µL using the working buffer (50 mM tris-buffer, pH 7.5, 10 mM $CaCl_2$, 0.05% Brij-35). The plate was incubated at 37 °C for 5 min and fluorescence measurements (excitation/emission – 490/520 nm) taken in 2-minute intervals for 48 minutes.

Final fluorescence data are averages of two replicates minus substrate control. Concentration of cleavage products was calculated with glutathione standard curves.

Synthesis of *4-arm-PEG- (AcPro-Leu-Gly-thio-4-methylpentanoyl-Leu-Gly-methylamino) ethane*

Synthesis *of 4-arm- PEG-(Gly-Leu-Boc)*

A solution of Boc-Leu-Gly-OH (1.08 g, 3.75 mmol), HATU (1.2 eq., 1.71 g, 4.50 mmol), and PEG4NH_2 (0.2 eq., 1.50 g, 0.75 mmol) in 20 mL DMF was stirred for 15 min and DIPEA (3 eq., 1.45 g ~2 mL) was added. The reaction mixture was stirred at room temperature for 2 h. After excess of DMF was removed *in vacuo*, the concentrate was dissolved in DCM and washed thoroughly with 0.5 M HCl, aqueous saturated $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation to obtain 2.15 g (90.9 %) yellowish gummy solid. The product with appreciable purity was used as in subsequent reaction synthesis step.

Synthesis of 4-arm- PEG-(Gly-Leu-NH_2)

To a solution of 4 –arm PEG-(Gly-Leu-Boc) (2.05 g, 0.66 mmol) in 20 mL MeOH, oxalyl chloride (1.2 eq., 0.72 g, 8.67 mmol) was gently added and the reaction was stirred for 6 hours. After the removal of excess solvent on the rotary evaporator, the crude product was purified with ultrafiltration (cellulose ultrafiltration membrane, cutoff 1.5 kDa) using 10% v/v ACN. Acetonitrile was removed on a rotary evaporator and the product was freeze dried to obtain 1.62 g (94.4 %) yellowish gum.

Synthesis of Ac-PLG-thio-4-methylpentanoic acid

A solution of AcPro-Leu-Gly-OH (1.32 g, 4.02 mmol), HATU (1.2 eq., 1.68 g, 4.42 mmol), and thio-Leu (1.2 eq., 0.75g, 4.83 mmol) in 15 mL DMF was stirred for 15 min and DIPEA (3 eq., 1.45 g ~2 mL) was added. The reaction mixture was stirred at room temperature overnight. After excess of DMF was removed *in vacuo*, the concentrate was dissolved in ethylacetate and washed with distilled water and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated on rotary evaporator to obtain 1.73 g brownish crude product. 200 mg samples of this crude product were then purified by HPLC using gradient elution with water (0.1% v/v FA, buffer A) and acetonitrile (0.1% v/v FA, buffer B) from 10% to 90% B in 50 min at a flow rate of 5 $mL \cdot min^{-1}$. White fluffy product (0.98 g, 53.2 %) was obtained after freeze-drying.

Synthesis of 4-arm PEG-(AcPro-Leu-Gly-thio-4-methylpentanoyl-Leu-Gly-methylamino) ethane

Ac-PLG-thio-4-methylpentanoic acid (0.07 g, 0.16 mmol) and 4-arm PEG-(Gly-Leu-NH_2) ethane (0.08 g, 0.03 mmol) in 10 ml DMF was stirred for 15 min and TEA (3 eq. of the acid, 0.05 g, 0.48 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. After excess of DMF was removed *in vacuo*, the crude product was purified with ultrafiltration (cellulose ultrafiltration membrane, cutoff 3.5 kDa) using 10 % v/v ACN. Acetonitrile was removed with a rotary evaporator and the product was dried over freeze dryer to obtain 120 mg (85.7 %) light yellow gum.

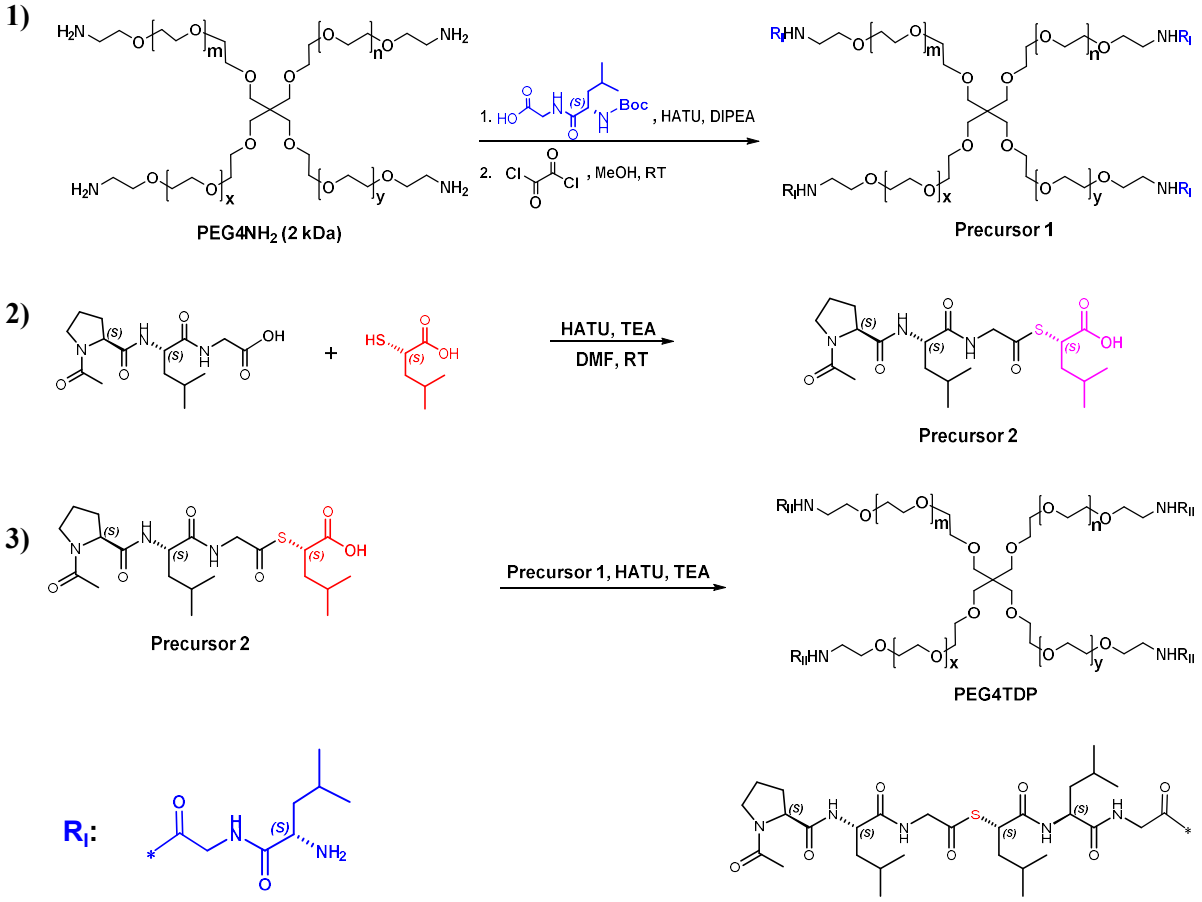
MMP-promoted hydrogel synthesis

MMP-mediated hydrogels were formed with a thiol: maleimide molar ratio of 1:1. 100 µL solutions of 3 mg $PEG4TDP_o$ in 10 % DMSO (tris buffer 50 mM, pH 7.56, 10 mM $CaCl_2$, 0.05% Brij-35) containing 3 and 6 µg/ml of the enzymes were added to 10 mg PEG4MAL (20

kDa) and incubated on a shaker at 37 °C. The reaction vials were constantly monitored for onset of gelation and final hardening (point where the vials could be overturned without flowing of reaction materials).

Results and Discussion

Synthesis and characterisation of TDP



Scheme 2: Synthetic route for TDP, step 1 combines the peptide coupling and Boc deprotection in two separate syntheses.

While the synthesis of the thioester bearing peptide, Ac-Pro-Leu-Gly#SLeu-Leu-Gly-OEt has been previously reported,[24, 34] it is very sensitive to hydrolysis and exchange reactions, so that the sequence incorporation into functional molecules requires careful consideration.[22] The functionalization of $PEG4NH_2$ to $PEG4TDP_o$ for enzyme promoted hydrogel formation as planed here demanded a specialized methodology. This was accomplished via a multi-step solution phase peptide coupling as

depicted in Scheme 2 using the uronium based coupling agent, HATU. To maximise the efficiency of the synthesis, a mixed/combined convergent and divergent synthetic steps were employed. PEG4NH_2 was first functionalized with the dipeptide Boc-Leu-Gly-OH followed by mild Boc deprotection using oxalic chloride to the corresponding free amine. To avoid complications of carrying out the more difficult thiol coupling on the functionalized 4-arm PEG-Gly-Leu-NH_2 intermediate, Ac-Pro-Leu-Gly-SLeu was synthesized and purified in a separate step. The final step of the synthesis then comprised the standard acid-amine coupling of functionalized 4-arm PEG-Gly-Leu-NH_2 and Ac-Pro-Leu-Gly-SLeu-OH to yield PEG4TDP_o.

Structure of PEG4TDP_o was determined with ESI-MS and detailed NMR studies. Both 1D- and 2D-NMR spectra of PEG4TDP_o revealed apparently a double signal set due to the prolyl *cis* and *trans* conformers, a common phenomenon often seen in proline containing peptides[35] but not previously reported for the Ac-Pro-Leu-Gly#SLeu-Leu-Gly-OEt sequence. Analysis of the NMR spectra suggests >95 % functionalisation of the amino groups of the starting PEG4NH_2 core while retaining the full TDP_o sequence structure without any evidence of. PEG4TDP_o hydrolysis products

The total correlation spectroscopy (TOCSY), and nuclear overhauser effect spectroscopy (NOESY) spectra were for peptide unit of PEG4TDP_o, figure 1 were observed to be similar to that the single thiodepsipeptide sequence, Ac-Pro-Leu-Gly#SLeu-Leu-Gly-NEtSH (TDP) used in our pH-promoted gelation studies.[22] An equilibrium mixture of *cis* and *trans* conformers with 40 % *cis* to 60 % *trans* distribution was observed in PEG4TDP_o. The tripeptide, Ac-Pro-Leu-Gly-OH and its (*S*)-2-mercapto-4-methylpentanoic acid extended analogue, Ac-Pro-Leu-Gly-SLeu-OH similarly exhibits 40% *cis* to 60% *trans* isomer proportions as observed for PEG4TDP_o.

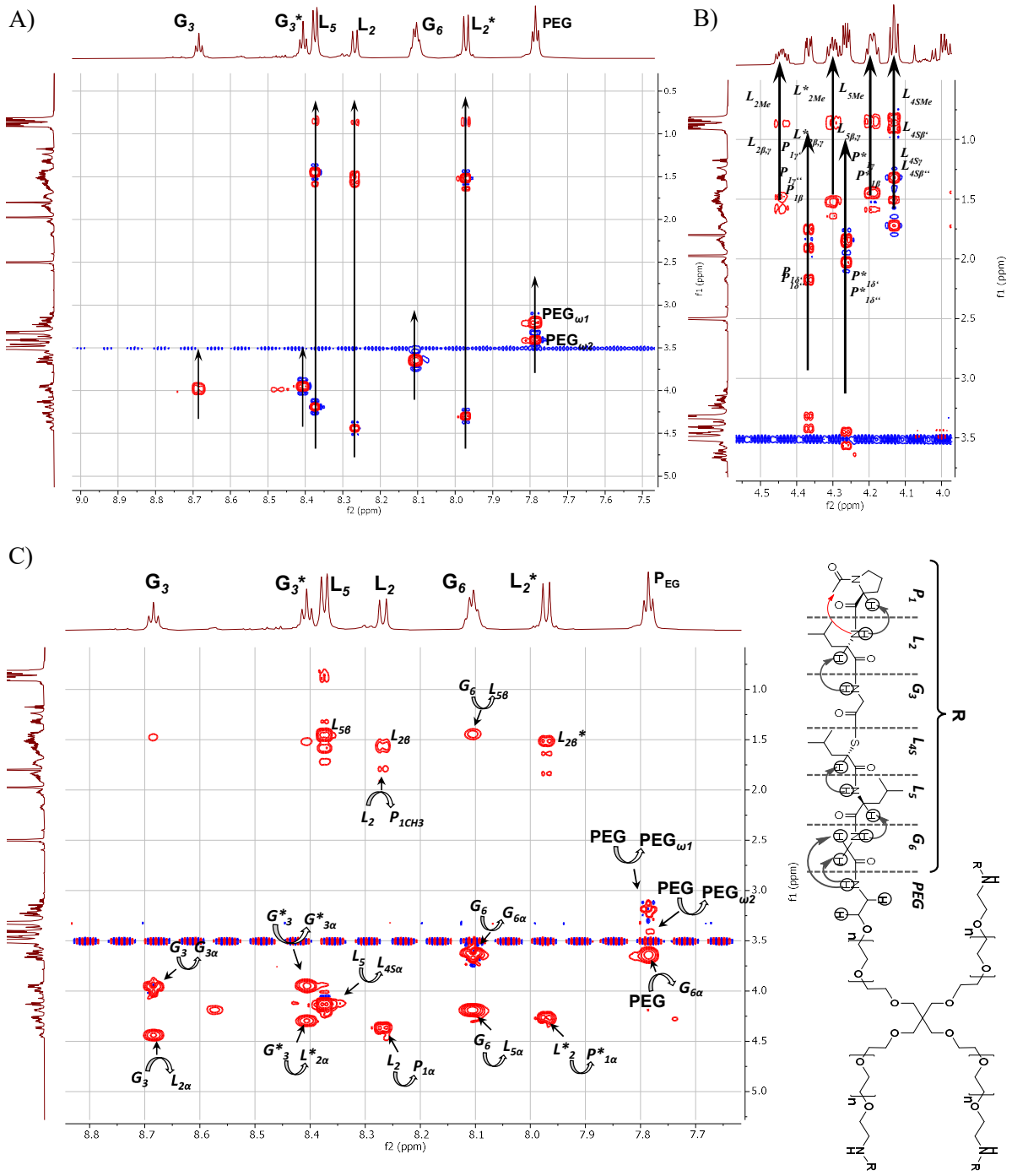


Figure 1: A) Amide region of TOCSY spectrum of PEG4TDP$_o$ showing two sets of resonances for G_3 and L_2, signals for *trans* conformers are designated with * B) α- H region TOCSY spectrum of PEG4TDP$_o$ with relevant spin assignments C) NOESY spectrum of amide region of PEG4TDP$_o$ with NOE assignments schematic representation of possible spin interactions of the chemical structure

Further evidence of PEG4TDP$_o$ complete structure was observed in the mass spectra data, figure 2. Although PEG4NH$_2$ yielded +4 ions as highest charged species, +5 charged species were observed for both *L-G*-functionalised PEG4NH$_2$ (precursor 2) and PEG4TDP$_o$.

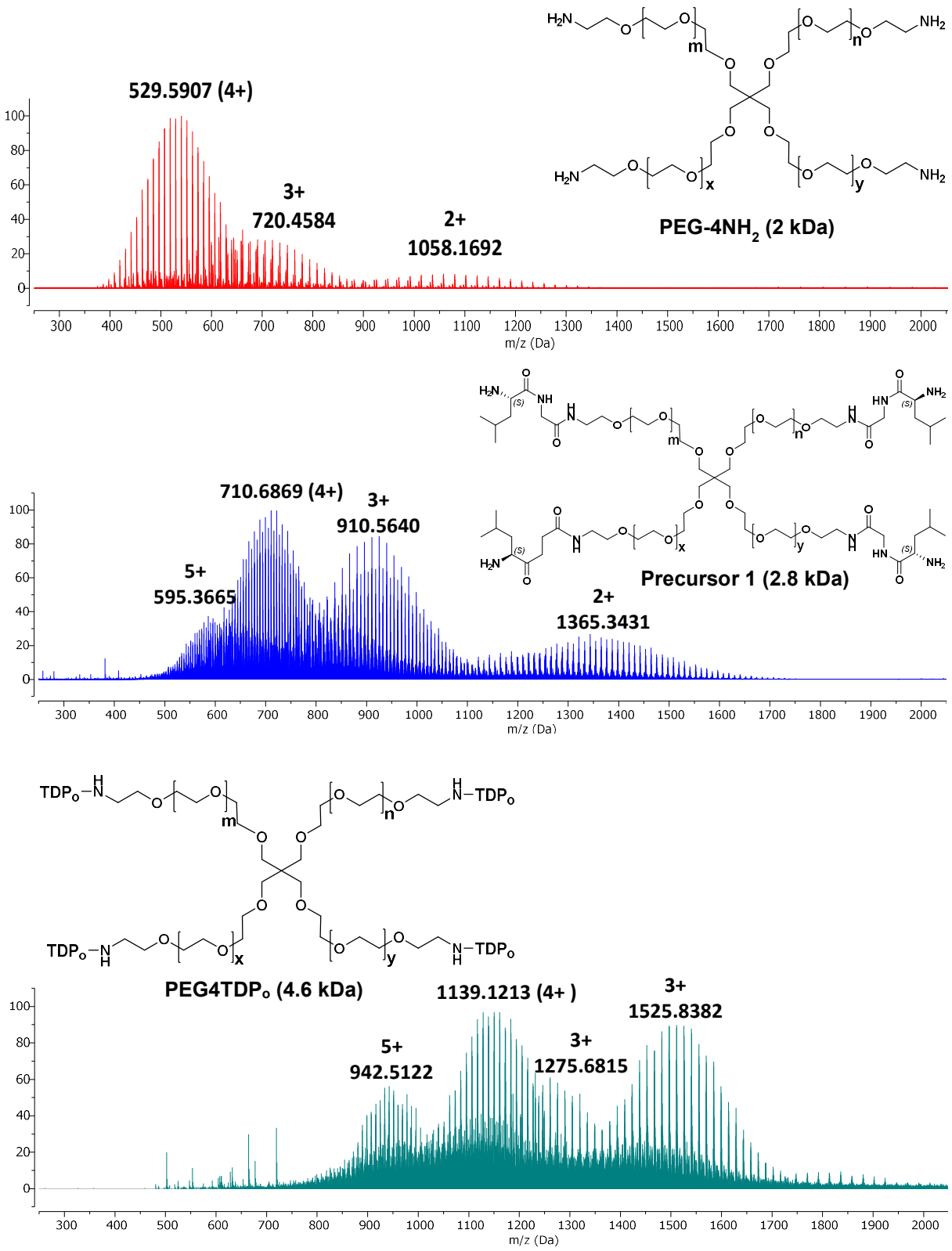


Figure 2: ESI-MS spectra of from top PEG4NH$_2$, *L*-*G*-functionalised PEG4NH$_2$ and TDP$_o$-functionalised PEG4NH$_2$.

PEG4TDP$_o$ MMP Cleavage and Gel Formation

In a first step, the susceptibility of the PEG4TDP$_o$ towards enzymatic cleavage was investigated. Although the advantage of simultaneously monitoring the appearance of cleavage fragments and disappearance of initial substrate[36] makes quantitative reverse phase HPLC attractive, limitations on analyte detection at reduced concentrations and rigorous efforts in development of suitable instrumentation and protocol pose challenges. The ability of vertebrate collagenases to cleave both ester and thioesters was exploited in the past for the development of spectrophotometric assay to continuously monitor the cleavage of thiodepsipeptides in the presence of Ellman's reagent,[37] since thiodepsipeptides hydrolyse to produce active mercaptans/thiols. In this work, a flourometric thiol assay with increased sensitivity towards thiol detection than 4,4'-dithiodipyridine or Ellman's reagent was employed for the continuous monitoring of PEG4TDP$_o$ cleavage products.

In line with earlier studies on synthetic substrates for vertebrate collagenases with respect to their preference for peptide length and type of amino acid at each subsite, we have decided to maintain the sequence of the reference compound, Ac-Pro-Leu-Gly#SLeu-Leu-Gly. Earlier studies[38] suggested the preference of lipophilic sequences by these class of enzymes. Therefore, in order to not significantly alter the lipophilicity of our MMP-sensitive thiodepsipeptide crosslinker precursor, we made use of the small molecular weight 1,1,1-tris(aminomethyl)ethane (TAME) as the core for the synthesis of a three-arm thiodepsipeptide. Although the substrate was sensitive to enzymatic cleavage (MMP-2 and MMP-9) at reduced concentrations (0.25 to 8 μM), its poor solubility at the required minimum concentration for the formation of hydrogels led to its inability to be used for enzyme-promoted hydrogel formation. Modification of the precursor design was carried out with a 4 –arm amine functionalized polyethylene glycol core of 2000 Da. The choice of medium sized hydrophilic core was to improve the solubility of the thiodepsipeptide without considerably altering its susceptibility towards enzymatic cleavage. It is also reasoned here that, the rigidity afforded by the short PEG arms should prevent possible formation of micelle-like structures, which could arise in the case of high molecular weight multi-arm PEG cores due to increased flexibility of the polymer chains. Formation of micelle-like structures could be problematic as the hydrophobic part, which is the peptide segment of interest, becomes embedded in the micelle core, thereby restricting access by the enzymes.

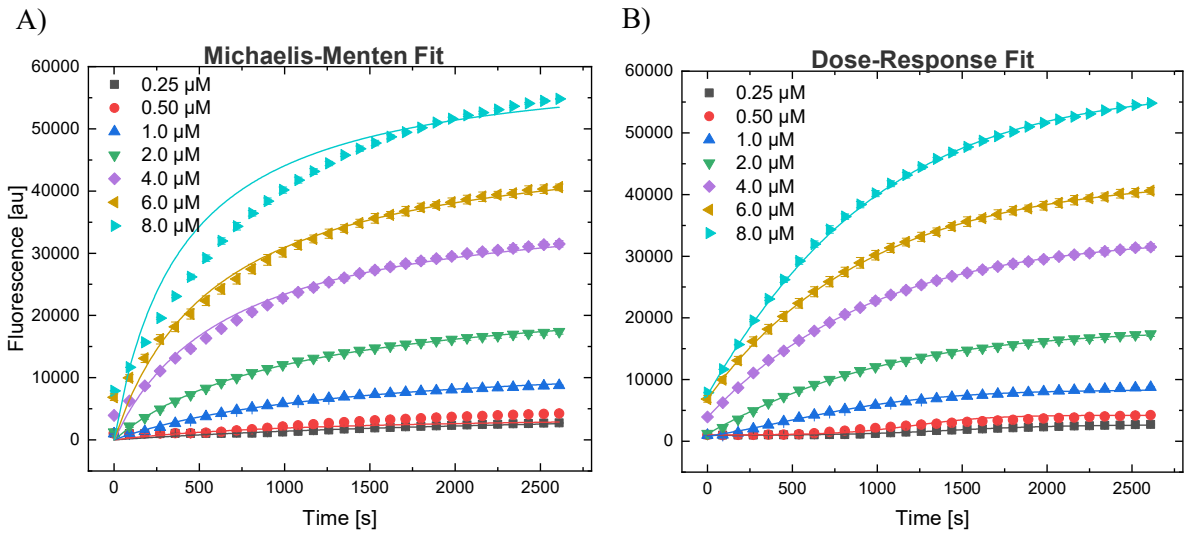


Figure 3: Enzymatic cleavage of PEG4TDP at various concentrations using MMP-9 (0.2 µg/mL). Error margins are within size of each data point. Experimental data are represented with closed symbols. Solid lines are (A) Michaelis-Menten fits and (B) Dose-response fits

Figure 3 shows the results of enzymatic cleavage of PEG4TDP$_o$ at concentrations between 0.25 µM and 8 µM using 0.2 µg/mL MMP-9. Although results clearly depict that PEG4TDP$_o$ was susceptible to enzymatic cleavage, the hydrolysis kinetics did not follow the usual Michaelis-Menten kinetics. Large deviations between experimental and Michaelis-Menten fits were observed at all concentrations especially as substrate concentration increased. Consequently, the extraction of kinetic parameters via the Lineweaver-Burk plot was not possible. Interestingly, the data followed a dose-response fit commonly used for competitive binding assays although different dimensions are used. Both PEG4TDP$_o$, tri-arm TDP$_o$ on TAME core and blanks also exhibited similar sigmoid type cleavage kinetics, thereby leading us to assume that the observed lag could be due to an unknown process in the flourometric detection used. Additional studies would be required to fully characterise the cleavage kinetics of PEG4TDP$_o$.

Although studies with the flourometric assay revealed successful cleavage of PEG4TDP$_o$, additional evidence of hydrogel formation would form a solid foundation for the design TDP-based crosslinker precursors for MMP-related biomedical applications. In a typical enzyme-promoted gel formation, a gelling mixture comprising PEG4MAL, PEG4TDP$_o$ and enzymes in 100 µL tris buffer was monitored

for gel formation during incubation at 37 °C. Table 1 shows results from gel formation in the presence of varying concentrations of MMPs -2 and -9. Both t_{onset} and t_{gel}, which are times for onset of gelation and final hardening of gelling mixtures respectively, were lower for reactions involving MMP-2 than MMP-9. Maleimide functionalised PEG macromers in reaction with thiol-based crosslinkers have been reported to afford fast forming gels within seconds.[39, 40] Although these gelation times are ideal for *in situ* applications[41, 42], lack of control over the structural homogeneity of these gels and difficulty in handling make them less unattractive. We have made a case for a regain of this control by the combination of gel forming precursors with fast reacting orthogonal moieties but with an intermediate orthogonal activation step for one of those moieties.[22] The maleimide-thiol coupling system, which included an intermediate thiol activation via thiol-thioester exchange reaction, made it possible to obtain gelation times between 3 and 30 minutes depending on pH and thiol: maleimide molar ratio variations. The notable shift in concentration of substrates from dilute regime (MMP-cleavage studies with flourometric assay) to increased concentration required for gel formation introduces some level of complexity that could hamper gel formation. In the first place, the required 10 wt.% polymer concentrations for gel formation results in significant increase in viscosity of the reaction medium that could impair enzyme diffusion and subsequent substrate recognition. Secondly, since thioesters are prone to non-enzymatic hydrolysis, it is important that enzymatic hydrolysis rates at substrate concentrations required for gel formation exceeds that of the background hydrolysis if the system is to be considered as enzyme-mediated.

Although gelation rates are not fast, the rates observed are about two times higher than those observed for MMP-assisted supramolecular hydrogelation of amphiphilic peptide precursors.[43] Moreover, this two-step/cascade reaction system provides a more controlled gel formation. Variations of polymer weight concentrations, thiol: maleimide molar ratio and use of different molecular weight maleimide functionalized polymer could be essential to control not only the gelation times, but also the final physical properties of the gels formed.

	6 µg/mL		3 µg/mL	
	t_{onset}/min	t_{gel}/min	t_{onset}/min	t_{gel}/min
MMP-2	27	30	33	36
MMP-9	38	40	44	47
Control	58	62		

Table 1: Gelation times of $PEG4TDP_o$ and PEG4MAL (10 % w/v) mixtures using MMPs -2 and -9. Control experiment was without any of the enzymes.

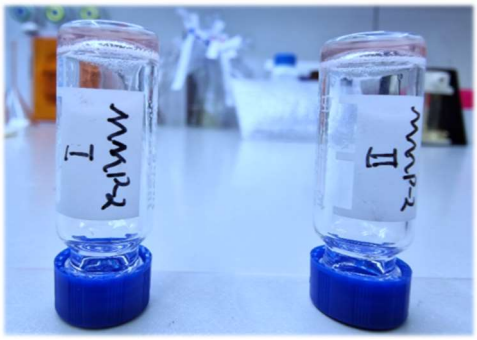


Figure 4: $PEG4TDP_o$ and PEG4MAL (10 % w/v, 100 µL) gels using MMPs -2 and -9 using 3 µg/mL (I) and 6 µg/mL (II) enzyme concentrations.

Interestingly, control experiments (with no enzyme), also gelled within 1 hour indicating possible non-enzymatic hydrolysis of $PEG4TDP_o$. Weingarten et. al.[24] reported that non-enzymatic hydrolysis contributed not more than 25 % to the total observed hydrolysis (first 5 minutes) of Ac-Pro-Leu-Gly#SLeu-Leu-Gly-OEt using vertebrate collagenase between pH 6.5 and 7. Contributions by non-enzymatic hydrolysis in our cleavage studies using flourometric assay were limited to 30 % even at 8 µM substrate concentration and after 45 minutes of reaction time. This contribution becomes significant for extended reaction times as the substrate concentration increases about 100 fold (for 1:1 thiol: maleimide molar ratio) and could explain the gelation seen in the control experiments after 60 minutes. Hydrogelation systems catalysed my MMPs using amphiphilic peptide derivatives rely basically on physical interactions as the driving force.[43] Although results are promising as potential alternative avenue for the synthesis of soft biomaterials in addressing cancer metastasis, the physical nature of the gels and relatively slow crosslinking rates remain a challenge. The use of thiodepsipeptide based crosslinker precursor not only addresses these issues but could provide a basis for the development of

superior approach especially if all aspects of substrate recognition are fully understood.

Conclusion

The utility of thiodepsipeptide sequence Ac-Pro-Leu-Gly-SLeu-Leu-Gly as MMP-sensitive peptide was exploited for the design of a multi-arm PEG-thiodepsipeptide crosslinker precursor with a sensory capability towards MMP activation. The proposed MMP-promoted activation reaction in the presence of multi-arm macromolecules functionalized with Michael acceptors, should aid the formation of hydrogels in thiol-Michael addition reaction.

The simplicity of such a system encompasses all aspects from the design and synthesis of multi-arm thiodepsipeptide crosslinker precursor to its application for MMP-promoted hydrogel formation. The multi-step synthesis of PEG4TDP$_o$ was accomplished using standard solution phase peptide coupling strategy with the uronium based HATU coupling agent giving rise to intermediates and final product in good to excellent yields without rigorous purification procedures. Detailed NMR studies of PEG4TDP$_o$ revealed a *cis-trans* isomerization of the thiodepsipeptide moiety resulting in the presence of 40 % *cis* to 60 % *trans* isomers.

Enzymatic cleavage studies conducted with flourometric assay indicated the susceptibility of PEG4TDP$_o$ towards cleavage by MMPs -2 and -9 albeit with non-Michaelis-Menten type reaction kinetics. The observed sigmoid type reaction behaviour would require additional work outside the scope of the study for the accurate determination of enzyme cycling parameters k_{cat} and K_m. Additional work would be required to fully characterize the mode of binding of multi-arm TDP substrates with MMPs. We could demonstrate successful MMP-promoted gelation of 4-arm maleimide functionalized PEG (PEG4MAL, 20 kDa) with PEG4TDP$_o$ using MMPs -2 and -9. Average gelation time using 10 % w/v polymer solutions was about 30 minutes with possible room to tune by the variation of polymer molecular mass, polymer weight concentration and polymer: PEG4TDP$_o$ molar ratios. Detailed rheological studies to investigate the gelation kinetics and physical properties of the formed are planned for future work.

The presented approach which provides a simple but unexplored way for *in situ* synthesis of covalently crosslinked soft materials, could lead to the development of an alternative pathway in addressing cancer metastasis making use of MMP overexpression as a trigger. This goal has so far not being reach with MMP inhibitors despite the extensive work this regard. In addition, if coupled with highly sensitive peptide sequences, this system could also double as MMP-related disease diagnostic tool.

Acknowledgements

This work was financially supported by the Helmholtz Association through programme-oriented funding and through Helmholtz Graduate School of Macromolecular Bioscience (MacroBio, VH-GS-503). We would also like to express our profound gratitude to Prof. Andreas Lendlein and Dr. Marc Behl for their helpful inputs and discussions.

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Supporting Information

A 4-Arm PEG-thiodepsipeptide Precursor Enables MMP-promoted Hydrogel Formation

Makafui Y. Folikumah[1,2], Axel Neffe[1]*

Synthesis and characterization of PEG4TDP$_0$

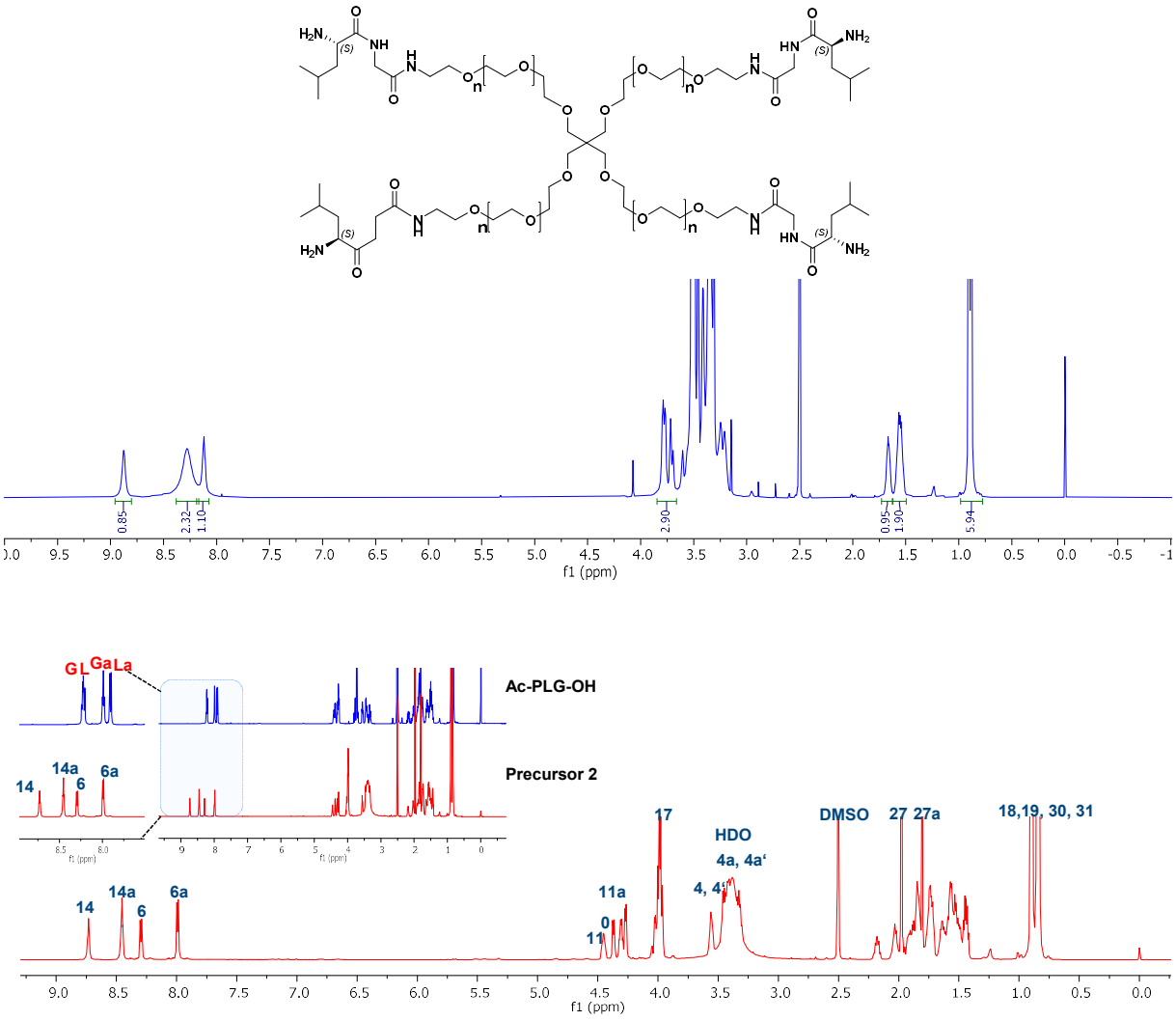

Figure S1: A) ^{1}H-NMR spectrum G-L functionalised PEG4NH$_2$, precursor 1 B) ^{1}H-NMR of Ac-Pro-Leu-Gly-SLeu-OH (precursor 2) showing presence of 2 pairs of resonances mostly for glycine and leucine residues as a result of cis-trans isomerism of acyl-prolyl bond. Similar observation is also seen for Ac-Pro-Leu-Gly-OH

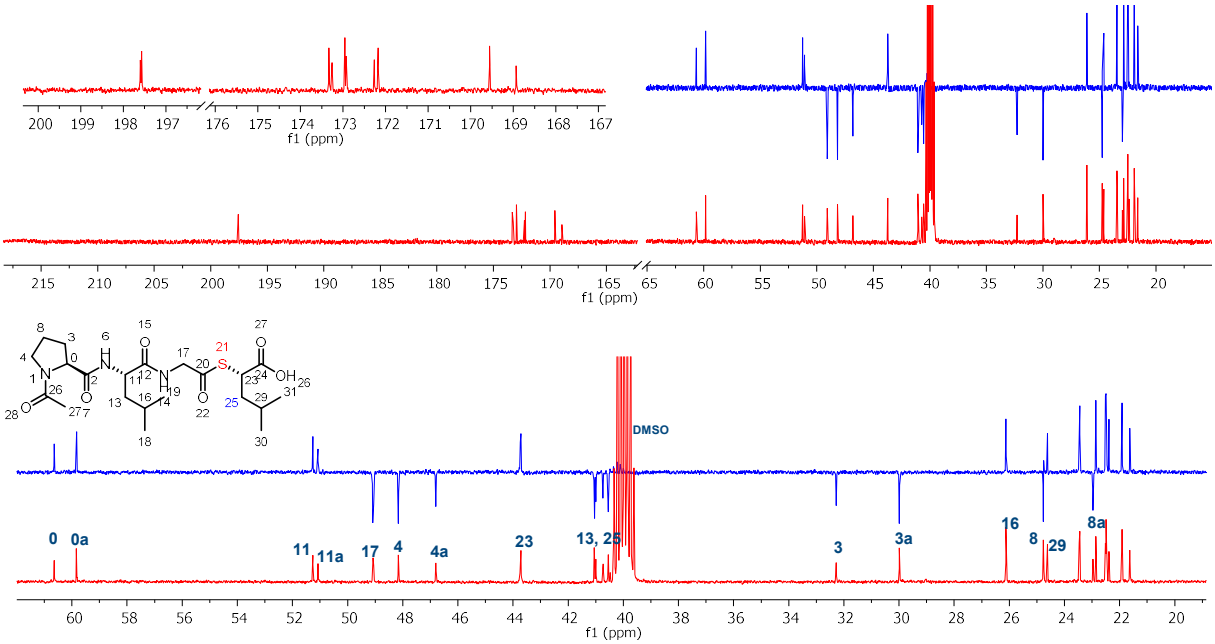

Figure S2: ^{13}C- and DEPT-135 spectra of Ac-Pro-Leu-Gly-SLeu-OH, precursor 2