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Thiol-thioester exchange reaction in precursor enables 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 crosslinkers, 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.
1. 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 crosslinking of macromolecules or by polymerizing suitable precursors whereby crosslinks are not necessarily covalent bonds but could also be formed by physical interactions such as π-π interactions, hydrophobic interactions, and H-bonding. In addition to similar elastic properties, similarities of hydrogels to ECM include but are not limited to the transport of oxygen and soluble factors and the ability to hold large amounts of water. 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 of temperature or pH. However, here, the diffusion needs to be considered.

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. Especially, when caused by infection with urease producing bacteria like Proteus spp., Klebsiella pneumoniae, and Staphylococcus saprophyticus, an increase of pH > 7 occurs, which can eventually lead to the formation of bladder or kidney stones. 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. 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.

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 non-covalent 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 crosslinking 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.

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 \textit{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 crosslinker moieties is required. These latent crosslinker 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 crosslinking strategy are employed. Similar to bioprecursors, these crosslinkers do not present their crosslinking 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 sulphhydryl chemistry, would be able to act as “latent” crosslinkers, which have sensory capability toward pH changes and can form on demand into reactive crosslinkers 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\(^\text{20}\) 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\(^\text{21-23}\). A net reaction in TTE exchange reaction occurs only if the incoming and departing thiols have different \(pK_a\) values. By the control of pH, the equilibrium reaction between the incoming and departing thiols can be shifted toward different \(pK_a\) 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 \(pK_a\) value and this way its suitability as a TTE substrate should be controllable by the sequence of the amino 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 crosslinker via TTE and subsequent crosslinking reaction chemistry have to be met.
A) Pre-crosslinker with pH sensor

Star-shaped polymer network precursor

pH ≤ 7

pH ≥ 7.5

Epithelium

Repairing damaged epithelium
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 sulphur 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.
In the first place, a thio-depsipeptide that is capable of exchange reactions at neutral to basic pHs values to yield an \( \alpha, \omega \)-dithiol-bearing crosslinker 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 \( \alpha, \omega \)-dithiol crosslinker.

The TTE of the thio-depsipeptide and the required thiol-Michael crosslinking reaction are both pH-dependent and can be tuned by altering the pH of the reaction medium. Since the pKa 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.\(^2^4\)

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 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 crosslinking 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.

2. Materials and Methods

Acetylated thio-leucine was synthesized according to the method described in ref.\(^2^5, 2^6\).

1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl, 98%), 1-hydroxybenzotriazole hydrate (HOBt, 97%), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphat (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, Mₙ of 20 kDa (PEG-4MAL, 20K), and deuterated solvents CDCl₃ (99.8%) and MeCN-d₃ (99.8%) were all purchased from Aldrich Chemicals (Darmstadt, Germany) and were used as received. Deuterated dimethyl sulfoxide (DMSO-d₆, 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**

¹H-NMR (500 and 700 MHz) and ¹³C-NMR (101 MHz) were recorded in CDCl₃ (internal standard: 7.26 ppm, ¹H; 77.00 ppm, ¹³C), in MeCN-d₃ (internal standard: 1.94 ppm, ¹H; 118.3 ppm, ¹³C), in DMSO-d₆ (internal standard: 2.50 ppm, ¹H; 39.52 ppm, ¹³C), and in MeOD-d₃ (internal standard: 3.31 ppm, ¹H; 49.15 ppm, ¹³C) 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 semipreparative column (PLRP-S, pore size: 100 Å, 8 μm; 300 x 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 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-Dimethylformamide (DMF) was stirred for 15 min, and N,N-Diisopropropylethylamine (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/H₂O 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 NH₂-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, Rᵢ = 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 NH₂-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. Afterwards, 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 N₂ 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 Na₂SO₄. 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, 7.5%).

**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 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 BF₃·Et₂O (49 µL) and Et₃SiH (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 minutes. The resulting flaky precipitate
was filtered and washed with the filtrate dried over 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 in 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 bromothymol blue indicator. Demonstrator samples were prepared in a similar procedure using a 1:2 maleimide:thiol mole ratio in 150 mM Tris/bromothymol blue (2 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 (Qₘ) was calculated using Equation 1:

\[
Q_M = \frac{W_f - W_0}{W_0} 
\]  

\[(1)\]
where \( W_t \) is the hydrogel mass after swelling and \( W_0 \) is the hydrogel mass before swelling. \( Q_M \) was used to calculate the volume-swelling ratio \( (Q_V) \) according to Equation 2:

\[
Q_V = 1 + \frac{\rho_p}{\rho_s} (Q_M - 1) \tag{2}
\]

where \( \rho_p \) is the density of the dry hydrogel (1.12 g·cm\(^{-3} \) for PEG) and \( \rho_s \) 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.

\[
\frac{1}{M_c} = 2 \frac{\bar{v}_2}{\bar{v}_3} \ln(1 - \nu_2) + \nu_2 + \chi_1 \nu_2^2 \tag{3}
\]

\[
q = \frac{M_r}{M_c} \tag{4}
\]

where \( M_n \) is the number-average molecular weight of the polymer, \( \nu_j \) is the molar volume of the solvent (18 cm\(^3\)·mol\(^{-1} \) for water), \( \nu_2 \) is the polymer volume fraction in the equilibrium swollen hydrogel (the reciprocal of \( Q_V \)), \( \bar{v} \) is the specific volume of the polymer \( (\rho_s / \rho_p) \), and \( \chi_1 \) is the polymer-solvent interaction parameter.

**General description of errors analysis**

Data were reported as mean value ± standard deviation of three measured quantities.

**3. 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\(_2\)H\(_5\)) derived from the substitution of the Gly-Leu amide nitrogen -
NH- with a sulfur atom\textsuperscript{27} as a reference compound. In order to enable ‘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).

\begin{align*}
\text{Sequence modification} \quad \xrightarrow{\text{pH} \geq 7.0} \quad \text{TDP} \\
\text{RTDP} + \text{BTDP}
\end{align*}

\textbf{Scheme 2.} Modification of the thio-depsipeptide Ac-Pro-Leu-Gly-S-Lou-Leu-Gly-OEt to TDP. It is speculated that TDP undergoes TTE reaction to produce the $\alpha,\omega$-dithiol cross linker BTDP.

In line with $pK_\alpha$ requirements\textsuperscript{28} 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. A mixture of products including a rearranged thio-depsipeptide (RTDP) and $\alpha,\omega$-dithiol-bearing -Leu-Leu-Gly- unit capable of crosslinking reactions with Michael acceptor-functionalized multi-arm macromolecules was obtained.
3.1 Evidence of thiol-thioester exchange reactions

![Chemical structures](image)

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

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 TTE reaction, the cysteamine thiol has a higher pKα 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 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\textsuperscript{24}. The detailed NMR as well as FT-IR characterization of the synthesized TDP can be found in Fig. 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 3-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 (Fig. 1). This observation was taken as a hint for the ability of the TDP to undergo intramolecular TTE, yielding the intended $\alpha,\omega$-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 $^1$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 (Fig. 2 A) 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$_1$ (Fig. 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 Fig. 2 is one of the products when TDP undergoes the TTE reaction.
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 G₁ and L₁ amino acid residues were spotted.

The sequential correlation of the amino acid residues in the thio-depsipeptide could not be precisely confirmed from the analysis of the NOESY (Fig. 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 pKₐ of the external thiols to that of attacking TDP thiol. Thiols with pKₐ 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 respective TTE tetrahedral intermediates, which were clearly visible in the recorded spectra.

3.2 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 crosslinker 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 crosslinking 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 not generate a dithiol crosslinker on its own in a TTE reaction. A thiol-Michael addition reaction between maleimide and thiol occurs but does not result in crosslinking. All crosslinking 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 crosslinker. 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 crosslinking reaction on the pH of the reaction
medium can be observed. This is in line with the fact that both reactions required \textit{a priori} generation of thiolate ions for the initial nucleophilic attack on the thioester carbonyl carbon\textsuperscript{24} followed by a subsequent addition reaction to the maleimide units attached to the PEG, assuming a base-catalyzed addition mechanism\textsuperscript{29}.

Table 1. Summary of gelation time and swelling ratio of TDP: PEG-4MAL gelation trials

<table>
<thead>
<tr>
<th>pH</th>
<th>MAL:SH (1:1)</th>
<th>MAL:SH (1:2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gelation time (h)</td>
<td>Swelling ratio</td>
</tr>
<tr>
<td>6.5\textsuperscript{a}</td>
<td>No gelation</td>
<td>ND</td>
</tr>
<tr>
<td>7.0\textsuperscript{b}</td>
<td>No gelation</td>
<td>ND</td>
</tr>
<tr>
<td>7.5\textsuperscript{b}</td>
<td>24</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>8.0\textsuperscript{b}</td>
<td>12</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>8.5\textsuperscript{b}</td>
<td>3</td>
<td>18 ± 2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: bis-Tris -HCl (150 mM)
\textsuperscript{b}: Tris-HCl buffer (150 mM)
\*: Only viscous mixture observed after 24 h
ND: Not determined

When the TDP content was increased, faster gelation times were observed. Equilibrium swelling measurements were conducted to determine the average molecular weight between crosslinks ($M_c$) and crosslink density ($q$) with the PEG-water interaction parameter ($\chi$) taken as 0.426\textsuperscript{30}. The effect of increasing thiol content also became apparent for $M_c$ and $q$. When the molar ratio between the intended \textit{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 crosslink density and network chain density increase, 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 $M_c$. 

18
In a demonstration experiment, which enabled the visual monitoring of the extent of crosslinking reaction, an additional set of gels was formed in the presence of the indicator bromothymol blue (Fig. 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 color 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.
**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.

Small-amplitude oscillatory shear experiments were conducted to explore the influence of the pH to the buffered 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 (Fig. 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 crosslinking and physical properties of the gel.

**Figure 4.** Amplitude sweep tests of TDP:PEG4MAL 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 $\eta^*$ (open triangle), at higher amplitudes can be attributed to physical rupture of the gel.
From the non-linear 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, \( \eta^*, G', G'' \) even at higher amplitudes as seen in Fig. 4 (B) 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, Fig. 4 (A).

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 (Fig. S7 – S10, Supporting Information). The values of initial \( G' \) for all gels ranged between 200 – 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.\(^3\) This might be attributed to the combination of peptide-based crosslinks and hydrophilic network constituents. Further evidence of the dependency of \( G' \) on pH was found in \textit{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 \textit{in situ} gelation rheology and Fig. 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 \textit{in situ} gelation monitoring results (\(^1\)H-NMR \textit{in situ} gelation measurements and Fig. 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-MAL 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 in 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 the PEG-4MAL. Gelation occurred solely at the interface, noticeable by a thin pale layer between the droplet compartments (Fig. 5, Video S1).

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 one hand and TDP droplet 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 6.532 (Fig. 6 and Video S2).
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).

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 droplet was detected (Fig. 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 crosslinking 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 crosslinker 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.
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 silicone oil with tweezers from the unreacted PEG-4MAL droplet.

4. Conclusion

A thio-depsipeptide Ac-Pro-Leu-Gly-SLeu-Leu-Gly-NEtSH (TDP) was designed as precursor with a sensory capability toward pH to produce $\alpha,\omega$-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 crosslinker to form covalently crosslinked 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 pK\textsubscript{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 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.\textsuperscript{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.

Supporting Information.

Methods (In situ gelation rheology, \textsuperscript{1}H-NMR in-situ gelation measurements)
Synthesis and characterization of TDP (1H-NMR spectrum of TDP; Overlay of 13C and DEPT-135 spectra of TDP; Multiplicity-edited HSQC spectrum of TDP; Amide region of the TOCSY spectrum of TDP; FTIR 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, 1H-NMR in situ gelation measurements)

Interfacial gelation at pH 8.5 (MP4)

Mixed pH interfacial gelation (MP4)

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References


