Folikumah, M.; Neffe, A.; Behl, M.; Lendlein, A.:
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In: MRS Advances. Vol. 4 (2019) 46 - 47, 2515 - 2525.
First published online by Cambridge University Press: July 15, 2019

DOI: 10.1557/adv.2019.308
https://doi.org/10.1557/adv.2019.308

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

Makafui Y. Folikumah[1,2], Axel T. Neffe[1,#], Marc Behl[1], Andreas Lendlein[1, 2*]

[1]*Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Teltow, Germany*

[2]*Institute of Chemistry, University of Potsdam, Potsdam-Golm, Germany*

[#]*Present address: Institute for Technical and Macromolecular Chemistry, University of Hamburg, Hamburg, Germany*

*Correspondence to: Prof. Andreas Lendlein
Institute of Biomaterial Science, Helmholtz-Zentrum Geesthacht, Kantstr. 55, 14513, Teltow, Germany
Email: andreas.lendlein@hzg.de
Phone: +49 (0)3328 352-235

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.

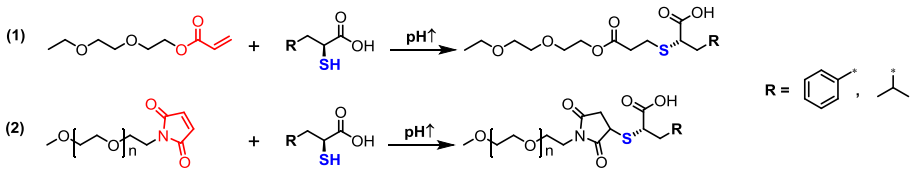
EXPERIMENTAL DETAILS

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 (δ) 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

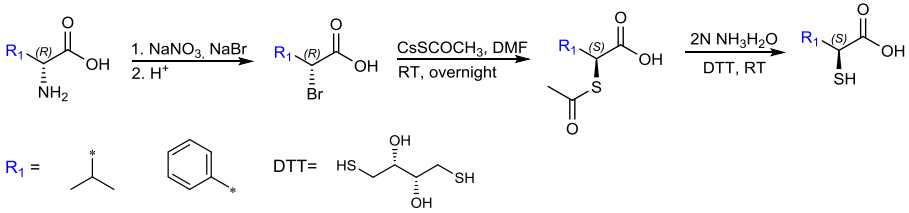
300 µL each of 0.53 M stock solutions of both reagents PEG-AC and SH-Leu in DMSO/D_2O was pipetted into the NMR tube and different mole equivalents (0.1, 0.5 and 1.0) of TEA was added before the experiments were started. Timings for blanks without the TEA catalyst were carried out right after the mixing 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 $\boldsymbol{g}_1$ and $\boldsymbol{h}_1$, which were compared with the corresponding product protons $\boldsymbol{g}_2$ and $\boldsymbol{h}_2$ as seen in Figure 1. Final conversions were however calculated with signals from methylene (-CH_2) protons $\boldsymbol{f}_1$ from the reactant at δ = 4.22 ppm compared with the product methylene protons $\boldsymbol{f}_2$ at δ = 4.13 ppm. Similar shifts in signals can be seen for methylene and methanetriyl protons $\boldsymbol{e}_1$and $\boldsymbol{k}_1$ respectively after product formation to corresponding $\boldsymbol{e}_2$ and $\boldsymbol{k}_2$.

Table 1: Results of Michael-thiol reactions using PEG-AC and SH-Leu in DMSO and D_2O *

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

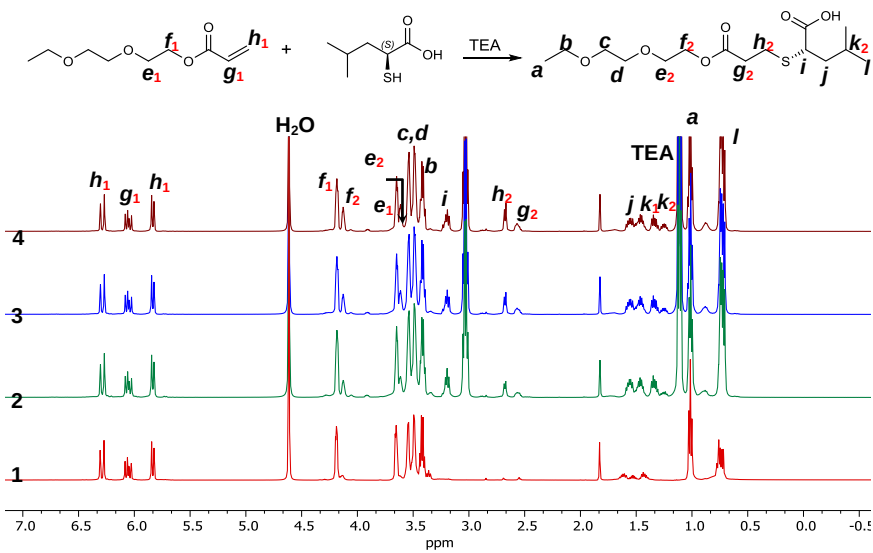


Figure 1: Overlay of ^{1}H-NMR spectra of PEG-AC and SH-Leu reactions in D_2O. (1) (blank without TEA) after 84 mins, 0% acrylate conversion, (entries 2-4 including 0.5 eq TEA, after (2) 8 min 21% ± 1 % acrylate conversion, (3) 44 min 27% ± 1% acrylate conversion, and (4) 84 mins 32% ± 2% acrylate conversion.

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 D_2O 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.

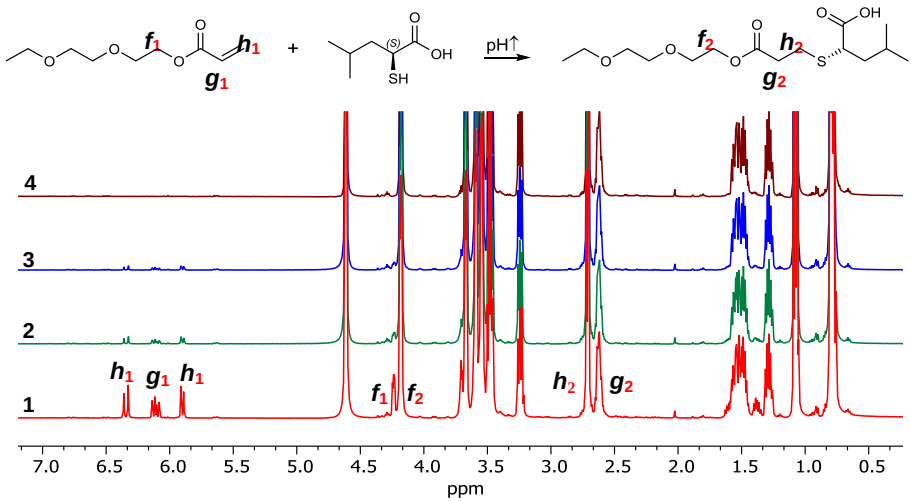


Figure 2: Overlay of ^{1}H-NMR spectra of PEG-AC and pH adjusted SH-Leu reactions in D_2O. (1-3)- Entry PEG-AC-1 (1) after 8 mins, 8.3% ± 0.4% conversion (2) after 44 mins, 25% ± 1% conversion and (3) after 84 mins, and 38% ± 2% conversion compared with entry PEG-AC-2 after 8 mins, quantitative conversion (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 $[M\text{-}H]^-$ for SH-Leu, $[M\text{-}H^+]^-$ and $[2M\text{-}2H^+\text{+}Na^+]^-$ ions whilst $[M\text{+}Na^+]^+$, $[M\text{-}HCO_2^-]^+$ and $[2M\text{-}H^+\text{+}2Na^+]^+$ 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, $[M\text{-}2H^+\text{-}H_2O]^{2-}$. 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.

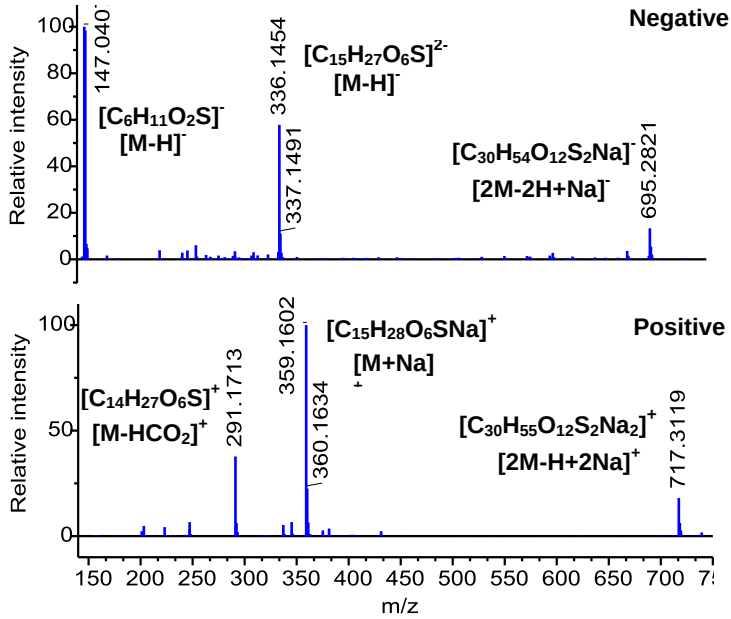


Figure 3: ESI-TOF MS spectrum of PEG-AC/SH-Leu thiol-Michael product in negative and positive ion modes. Peak at m/z 147.0407 corresponds to SH-Leu with loss of a proton

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 $[M\text{+}OH^-]^-$

and $[M\text{-}HCO_2^+]^-$ 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.

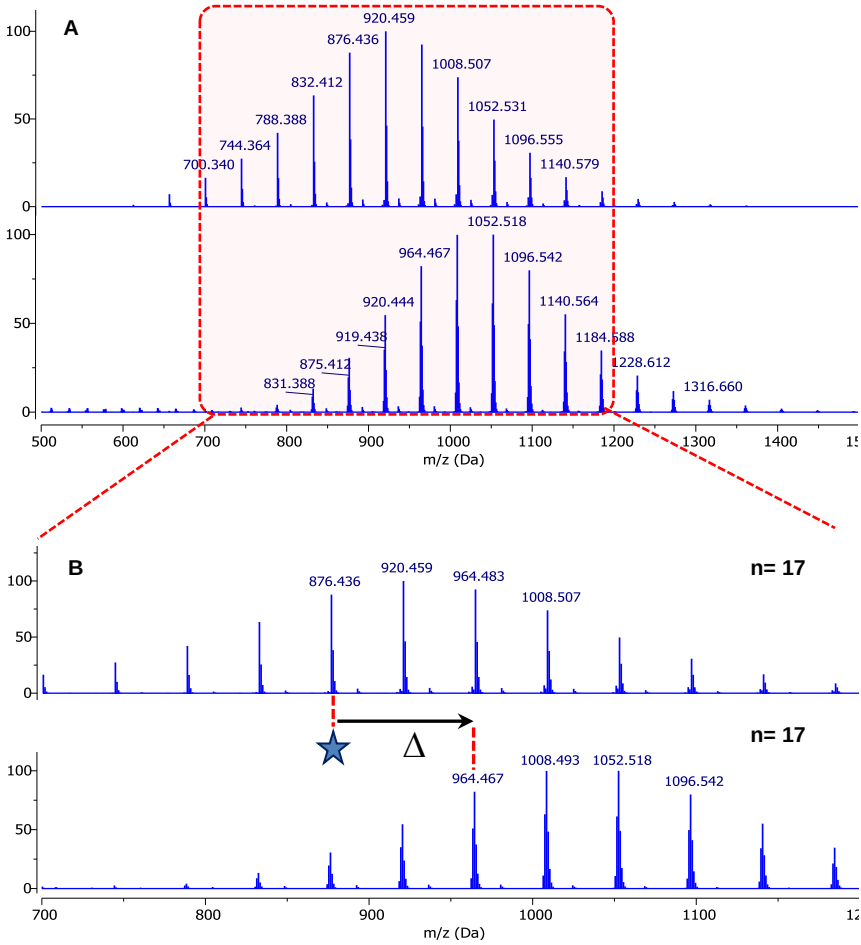


Figure 4: (A) Overlay of ESI-TOF MS spectra of PEG-MAL 750 and thiol-Michael addition product (B) Zoomed 700 – 1200 Da region showing change in mass for n = 17 PEO units

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

Additional RP-HPLC analysis of the reaction mixture PEG-Ac and SH-Lew 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 5 B).

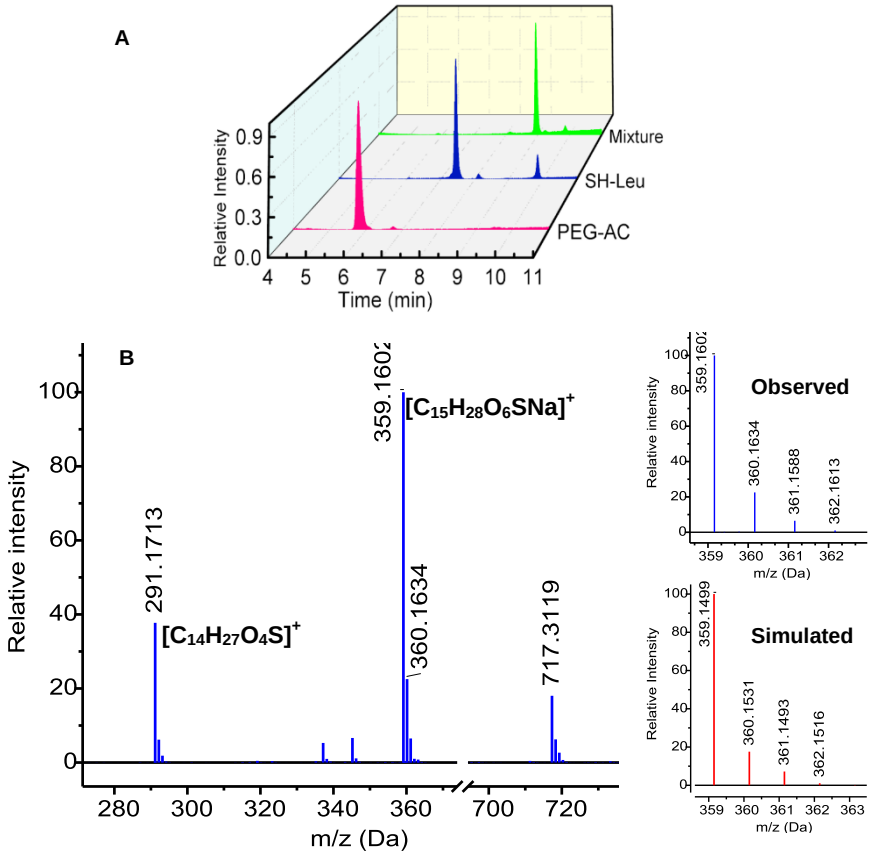


Figure 5: (A) 3D overlay of RP-HPLC chromatograms of diethylene glycol ethyl ether acrylate, thio-leucine (pH 7.5) and reaction mixture (B) ESI-MS of HPLC product fraction/peak with simulated and observed isotopic patterns compared

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.

ACKNOWLEDGMENTS

This work was financially supported by the Helmholtz Association through Helmholtz Graduate School for Macromolecular Bioscience (VH-GS-503) and program-oriented funding.

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