Supporting Information

Spacer length shapes drug release and therapeutic efficacy of traceless disulfide-linked ADCs targeting the tumor neovasculature

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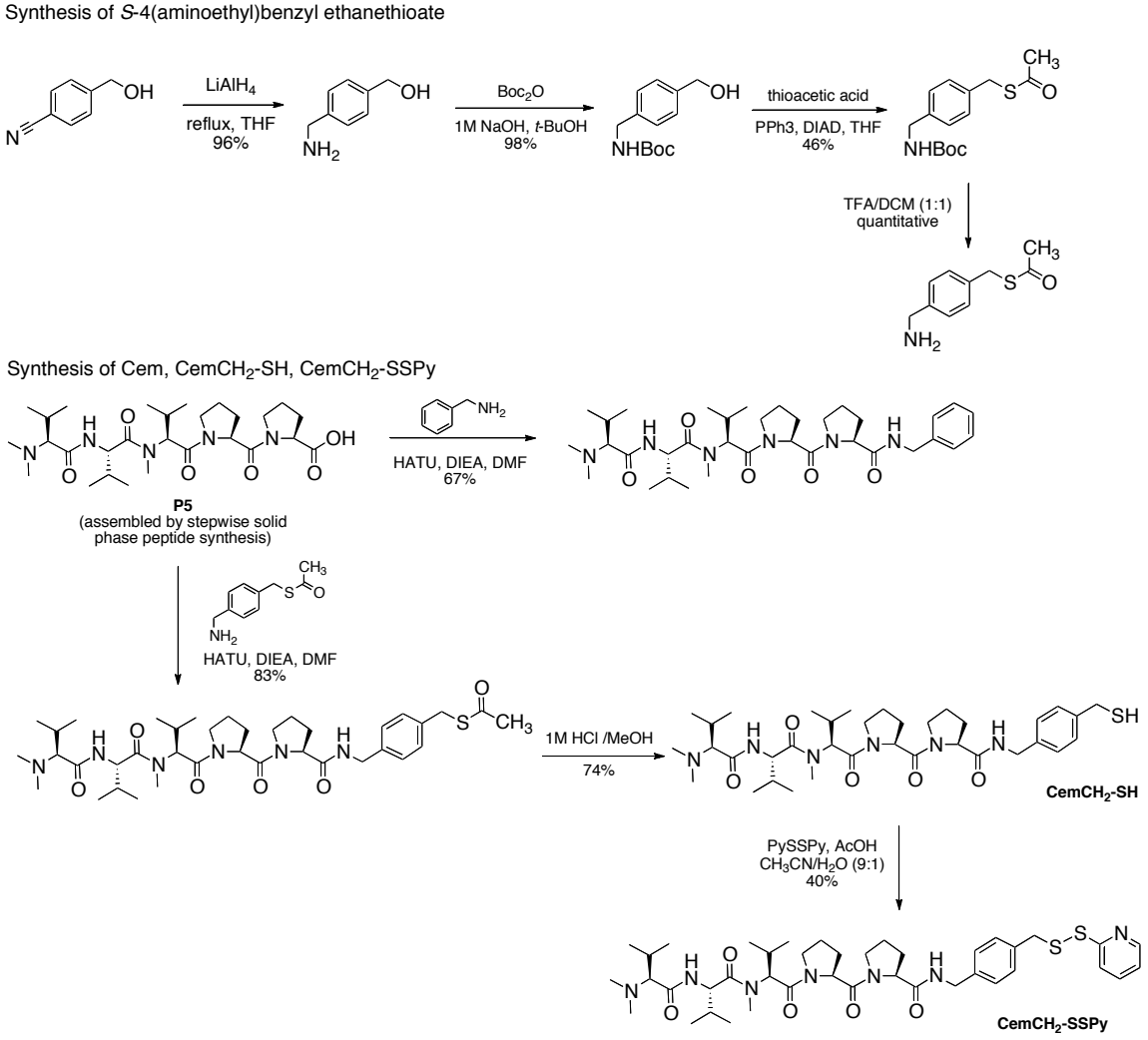
1 Synthesis of Cem, CemCH$_2$-SH and CemCH$_2$-SSPy

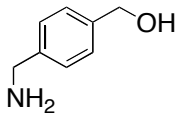
The synthesis of cemadotin and its derivatives is based on previously reported work from our group.[1, 2] In addition, the data for CemCH$_2$-SH has been previously reported.[1]

Material and Methods: Analytical and preparative HPLC were performed on an Alliance HT RP-HPLC with PDA UV detector, using either a Synergi 4 µm, Polar-RP 150*10 mm at a flow rate of 4 mL/min with linear gradients of solvents A and B (A=millipore water with 0.1% trifluoroacetic acid (TFA), B=acetonitrile), or an X-Terra Prep RP-18, 5 µm 150*10 mm at a flow rate of 4 mL/min with linear gradients of A' and B (A'= 0.1 M Triethylammonium acetate pH 7).



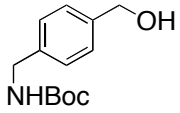
Scheme 1. Synthetic scheme for the synthesis of Cem, CemCH$_2$-SH and CemCH$_2$-SSPy.

(4-(aminomethyl)phenyl)methanol



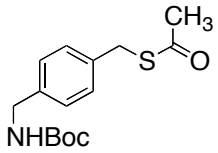
2-Cyanobenzaldehyde was converted into the corresponding amino-alcohol derivative as previously described.[3, 4] A solution of 2-cyanobenzaldehyde (1 g, 7.6 mmol) in anhydrous THF (10 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (1.74 g, 45.8 mmol) in anhydrous THF (30 mL) at 0 ºC. The reaction mixture was heated to reflux and stirred for 5 hours before cooling down again to 0 ºC and quenching with H_2O (2 mL), 15% NaOH (4 mL) and H_2O (6 mL). After stirring for 10 min at room temperature, the mixture was filtered on a pad of celite (washed with EtOAc). Concentration *in vacuo* gave (4-(aminomethyl)phenyl)methanol as a white solid (1.0 g, 96% yield), which was used without further purification. Spectroscopic data was identical to that previously reported.[4] ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.80 (s, 2H), 4.60 (s, 2H), 7.25 (m, 4H); ESI-MS: calcd. for $C_8H_{12}NO$ ($[M+H]^+$) *m/z* 138.08, found 137.79.

***tert*-butyl 4-(hydroxymethyl)benzylcarbamate**



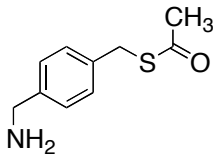
(4-(Aminomethyl)phenyl)methanol was converted to the Boc derivative as previously described.[4] Briefly di-*tert*-butyl dicarbonate (1.99 g, 9.12 mmol) was added to a stirred solution of -(aminomethyl)phenyl)methanol (1.0 g, 7.3 mmol) in *t*BuOH / 1M NaOH (2/1, 50 mL) at 0 °C. The reaction was allowed to warm up to room temperature, and stirred for 2 additional hours. The reaction was then washed with a $NaHCO_3$ saturated solution and the aqueous phase extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, and concentrated *in vacuo* to give a white solid (1.70 g, 98% yield), which was used in the next step without further purification. Spectroscopic data was identical to that previously reported.[4] ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.36 (s, 9H), 4.23 (s, 2H), 4.61 (s, 2H), 7.23 (m, 4H); ESI-MS: calcd. for $C_{13}H_{20}NO_3$ ($[M+H]^+$) *m/z* 238.29, found 237.92.

_S_-4-((tert-Butoxycarbonyl)amino)methyl)benzyl ethanethioate



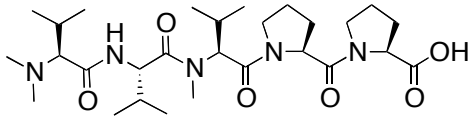
tert-Butyl 4-(hydroxymethyl)benzylcarbamate was converted to the corresponding thioester derivative using Mitsunobu conditions. A two-neck round bottomed flask was charged with *tert*-butyl 4-(hydroxymethyl)benzylcarbamate (1.90 g, 8.0 mmol), triphenylphospine (32 mmol, 4 equiv.) and thioacetic acid (32 mmol, 4 equiv.) in anhydrous THF (100 mL). The flask was immersed in an ice bath and the mixture was stirred under an atmosphere of argon. Diisopropyl azodicarboxylate (DIAD) (32 mmol, 4 equiv.) was then added dropwise. Upon completion of addition, the mixture was allowed to warm to room temperature and was stirred overnight. The reaction mixture was diluted with diethyl ether (100 mL) and washed twice with a $NaHCO_3$ saturated solution. The aqueous layers were combined and re-extracted with diethyl ether (100 mL). The organics were combined, dried with Na_2SO_4, filtered, and concentrated *in vacuo*. The resulting residue was diluted in diethyl ether (40 mL) and hexanes were added (20 mL). The mixture was left standing overnight. The resulting white solid was removed under vacuum and the filter cake washed with a 50% (v/v) solution of hexanes/diethyl ether (100 mL). The solvent was removed under vacuum, and the resulting yellow oil was purified by flash column chromatography (hexanes/EtOAc, 8/1, R_f 0.15). Solvent evaporation resulted in an oil that upon addition of hexanes resulted in a white solid. The product was filtered and washed with cold hexanes to give the desired product as a white solid (1.15 g, 46% yield). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 1.43 (s, 9H), 2.32 (s, 3H), 4.08 (s, 2H), 4.26 (m, 2H), 7.21 (m, 4H); ESI-MS: calcd. for $C_{15}H_{22}NO_3S$ ($[M+H]^+$) *m/z* 296.40, found 295.90.

_S_-4-(Aminomethyl)benzyl ethanethioate



In a 10 mL flask *S*-4-((tert-butoxycarbonyl)amino)methyl)benzyl ethanethioate (0.4 g, 1.35 mmol) was dissolved in DCM (6 mL). TFA (2 mL) was added and the mixture stirred at room temperature for 15 minutes. After the mixture was evaporated and lyophilized from dioxane, the desired product was isolated as a white powder (260 mg, quantitative). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 2.33 (s, 3H), 3.71 (s, 2H), 4.09 (m, 2H), 7.25 (m, 4H); ESI-MS: calcd. for $C_{10}H_{14}NOS$ ($[M+H]^+$) *m/z* 196.28, found 195.83.

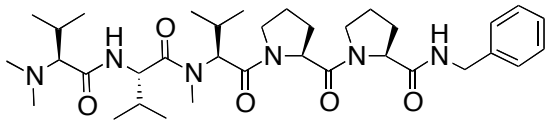
_N,N_-dimethylvalyl-valyl-_N_-methylvalyl-prolyl-proline (P5)



The pentapeptide P5 was assembled by step-wise solid phase peptide synthesis. Commercially available pre-loaded Fmoc-Pro on Tentagel resin (3.88 g, 0.66 mmol, RAPP Polymere) was swollen first in DCM, then in DMF (2 x 3 min x 15 mL, in both cases). The Fmoc group was removed with 20 % piperidine in DMF (2 x 5 min x 10 mL). The resin was washed with DMF.

Prior to coupling to the resin, amino acids were activated: 3 equiv. of each amino acid were pre-activated for 10 minutes with equimolar amounts of a coupling agent in the presence of 6 equiv. *N,N*-diisopropylethylamine (DIEA) at a concentration of 0.15 M in DMF. In the case of Fmoc-Pro-OH and Fmoc-N(Me)-Val-OH single couplings were performed using HBTU/HOBt; in the case of Fmoc-Val-OH and $(Me)_2$-Val-OH prolonged triple and double couplings with HATU, respectively, were required for complete reaction. The pentapeptide was cleaved from the resin after 2 hours incubation with 60 mL of DCM/TFE/AcOH (3/1/1). The resin was filtered, the solvent evaporated and the desired product lyophilized from dioxane (310 mg, 85% yield). ESI-MS: calcd. for $C_{28}H_{50}N_5O_6$ ($[M+H]^+$) *m/z* 552.7, found 552.1.

Cem



In a 25 mL round bottom flask containing P5 (40 mg, 0.073 mmol) dissolved in DMF (2 mL), HATU (28 mg, 0.073 mmol, 1 equiv.) and DIEA (51 µL, 0.290 mmol, 4 equiv.) were added. After 10 minutes pre-activation, benzylamine (10 µL, 0.087 mmol, 1.2 equiv.) was added dropwise. The reaction was judged completed after 2 hours by HPLC chromatography. DMF was removed under vacuum, the crude product re-suspended in 50% CH_3CN / H_2O, and purified over HPLC. The collected fractions were lyophilized, and the desired peptide was isolated as a white powder (32 mg, 67 % yield, HPLC retention time: 10.36 minutes, Synergi column, 20-80% B in 15 minutes, 4 mL/min). ESI-MS: calcd. for $C_{35}H_{57}N_6O_5$ ($[M+H]^+$) *m/z* 641.44, found 641.14.

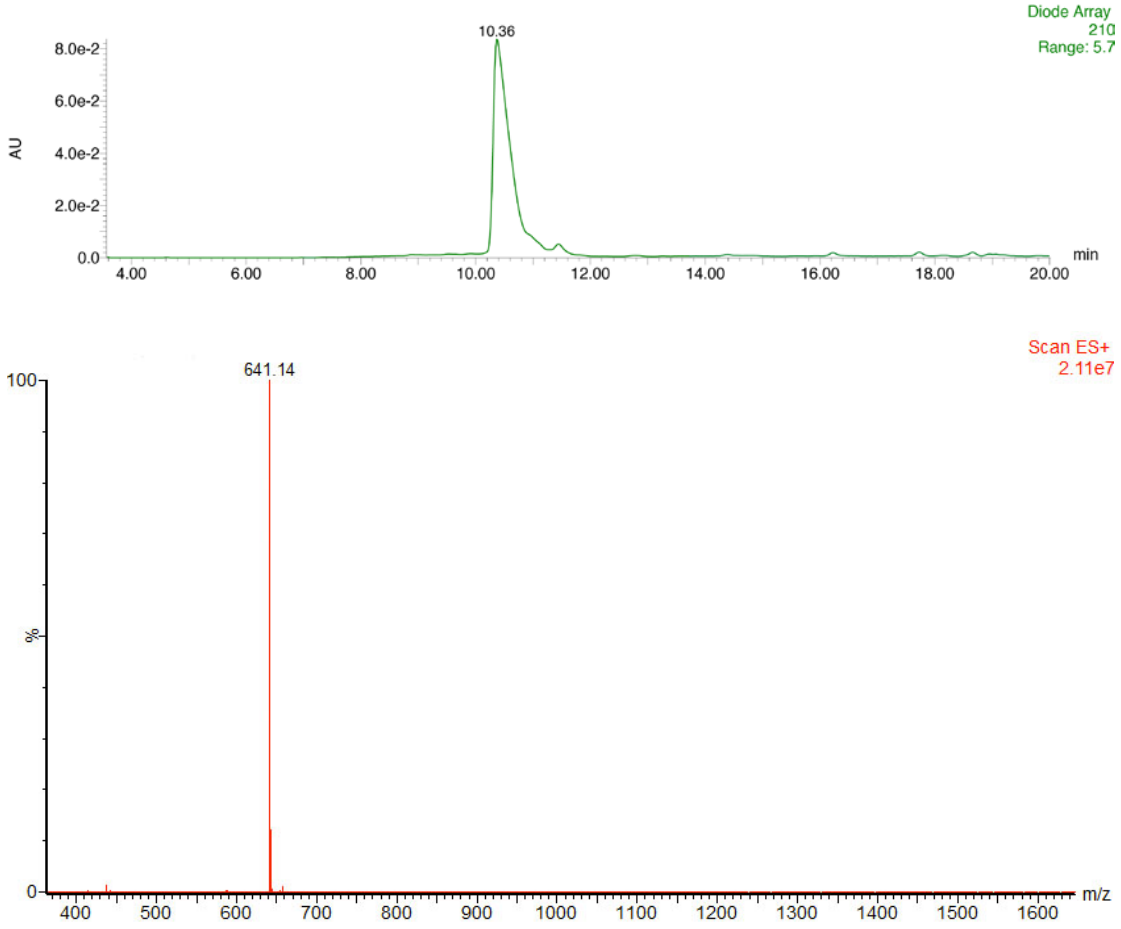
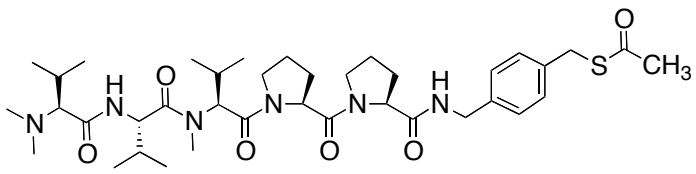


Figure 1. HPLC trace (wavelength of 210 nm) and ESI-MS spectrum of Cem.

CemCH_2-SAc



In a 25 mL round bottom flask containing P5 (100 mg, 0.181 mmol) dissolved in DMF (4 mL), HATU (68.9 mg, 0.181 mmol, 1 equiv.) and DIEA (127 µL, 0.725 mmol, 4 equiv.) were added. After 10 minutes pre-activation, *S*-4-(aminomethyl)benzyl ethanethioate (42.5 mg, 0.218 mmol, 1.2 equiv.) was added as a solid. The reaction was judged completed after 1 hour by HPLC chromatography. DMF was removed under vacuum, the crude product re-suspended in 50% CH_3CN / H_2O, and purified over HPLC. The collected fractions were lyophilized, and the desired peptide was isolated as a white powder (110 mg, 83 % yield, HPLC retention time: 11.52 minutes, Synergi column, 20-80% B in 15 minutes, 4 mL/min). ESI-MS: calcd. for $C_{38}H_{61}N_6O_6S$ ($[M+H]^+$) *m/z* 730.0, found 728.9.

CemCH$_2$-SH

Cem-CH$_2$-SAc (32 mg, 0.044 mmol) was dissolved in MeOH (1.5 mL, degassed). Subsequently NaOH (1.5 mL, 200 mm in water, degassed) was added and the reaction incubated for 30 minutes at room temperature. The reaction was judged to be over by ESI-MS analysis. The reaction was quenched with Tris buffer (3 mL, 1 M, pH=7) and the thiol reduced for 30 minutes with DTT (100 mm final concentration). The crude mixture was purified by HPLC. The collected fractions were lyophilized, and the desired peptide was isolated as a white powder (22 mg, 74% yield, HPLC retention time: 11.22 minutes, Synergi, 20-80% B in 15 minutes). ^{1}H-NMR (500 MHz, DMSO) δ 0.69 (d, 3H, *j*= 6.8 Hz), 0.78-0.9 (m, 12H), 0.9-1 (m, 3H), 1.65-2.3 (m, 11H), 2.75 (s, 6H), 3.07 (s, 3H), 3.5-3.6 (m, 2H), 3.6-3.8 (m, 4H), 4.15 (dd, 1H, J= 14.3, 5.6 Hz), 4.25-4.35 (m, 3H), 4.48-4.6 (m, 2H), 4.97 (d, 1H, J= 11.9 Hz), 7.1-7.3 (m, 4H), 8.23-8.29 (m, 1H), 8.9-9.0 (m, 1H), 9.8 (m, 1H); ESI-MS: calcd. for $C_{36}H_{59}N_6O_5S$: ($[M+H]^+$) *m/z* 687.8, found 687.0.

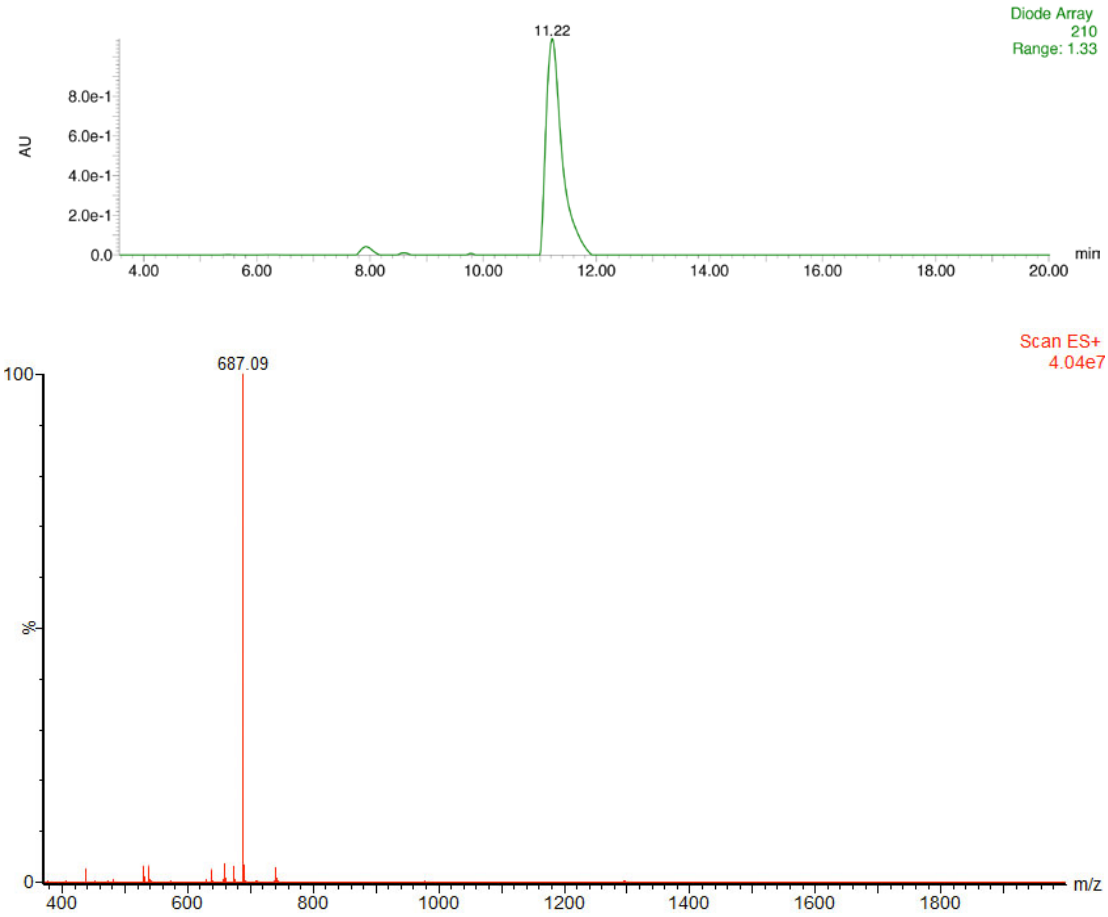


Figure 2. HPLC trace (wavelength of 210 nm) and ESI-MS spectrum of CemCH$_2$-SH.

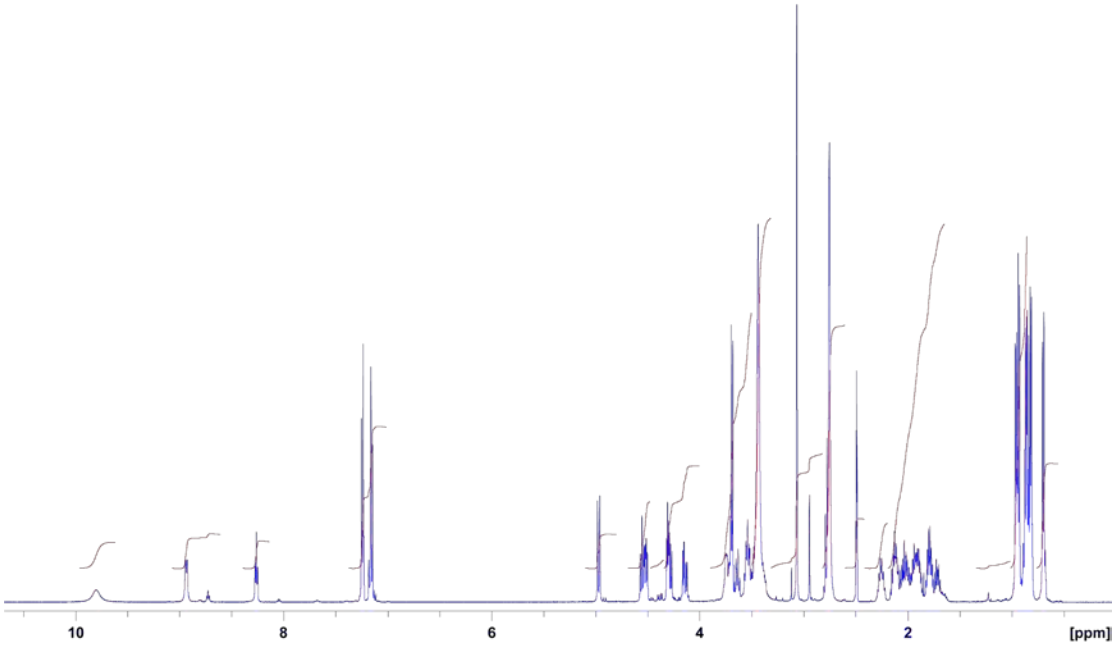
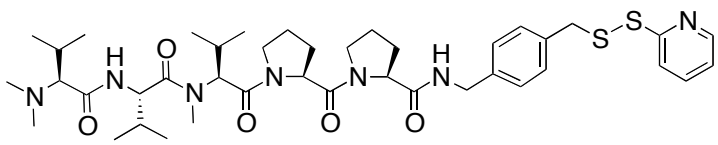


Figure 3. ^{1}H-NMR spectrum of CemCH$_2$-SH.

CemCH$_2$-SSPy



CemCH$_2$-SH (40 mg, 0.058 mmol) dissolved in 9/1 CH_3CN/H_2O was added dropwise to an AcOH (0.35 mL) solution of pyridyl-disulfide (103 mg, 0.466 mmol, 8 equiv.). After 1 h the reaction was evaporated, the crude resuspended in 1/9 CH_3CN/H_2O: the solid (pyridyl-disulfide) was removed by centrifugation, the cleared solution was purified over HPLC and the desired product isolated as a white powder (18.5 mg, 40% yield, HPLC retention time: 12.85 minutes X-Terra, 20-80% B in 15 minutes). ^{1}H-NMR (500 MHz, DMSO) δ 0.63-0.73 (m, 6H), 0.76-1.07 (m, 12H), 1.65-2.17 (m, 10H), 2.17-2.23 (s, 7H), 2.62-2.64 (m, 1H), 3.06 (s, 3H), 3.46-3.62 (m, 2H), 3.62-3.82 (m, 2H), 4.08 (s, 2H), 4.11-4.2 (m, 1H), 4.25-4.36 (m, 2H), 4.44-4.56 (m, 2H), 4.97 (d, 1H, J= 8.5 Hz), 7.1-7.3 (m, 4H), 7.59-7.63 (m, 1H), 7.71-7.78 (m, 1H), 8.06-8.08 (m, 1H), 8.6-8.3 (m, 1H), 8.4-8.5 (m, 1H); ESI/MS: calcd for $C_{41}H_{62}N_7O_5S_2$ ($[M+H]^+$) *m/z* 796.43 Da, found 796.08 Da.

Supporting Information

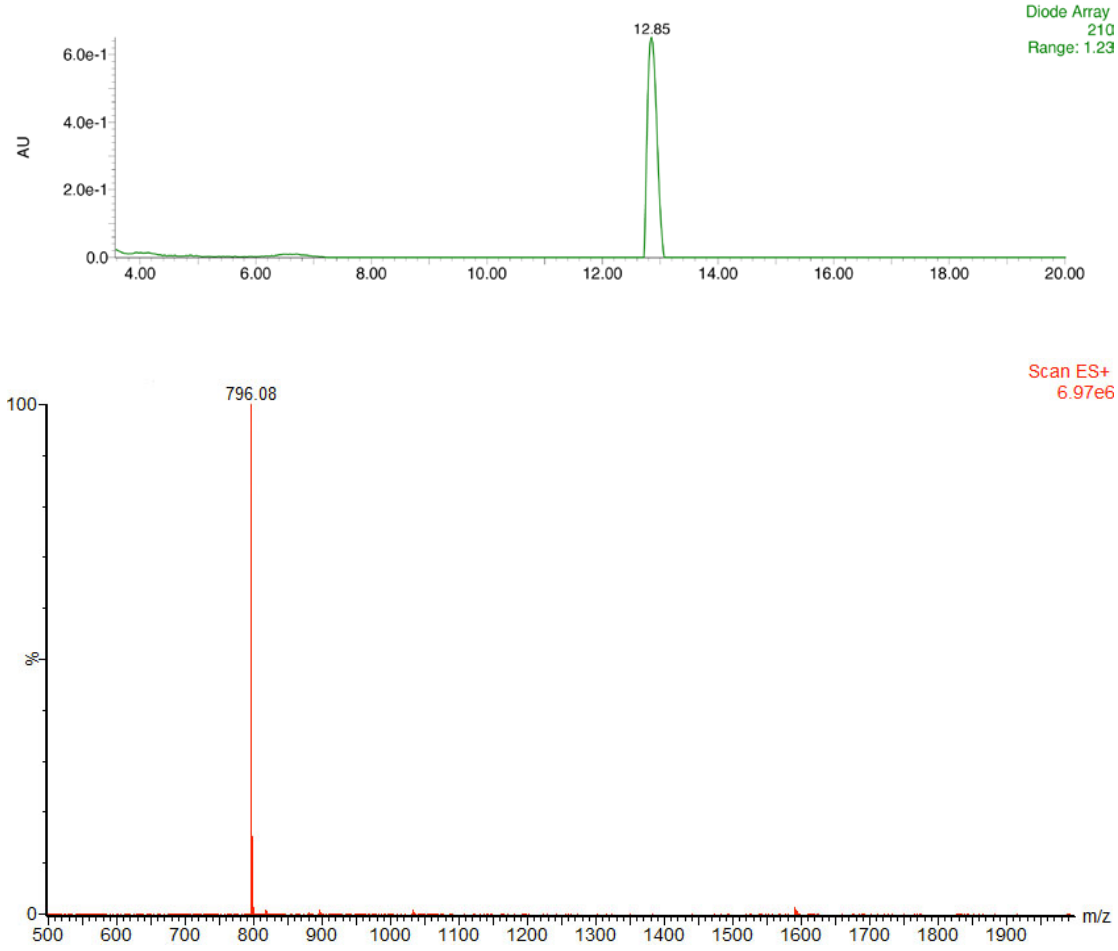


Figure 4. HPLC trace (wavelength of 210 nm) and ESI-MS spectrum of CemCH_2-SSPy.

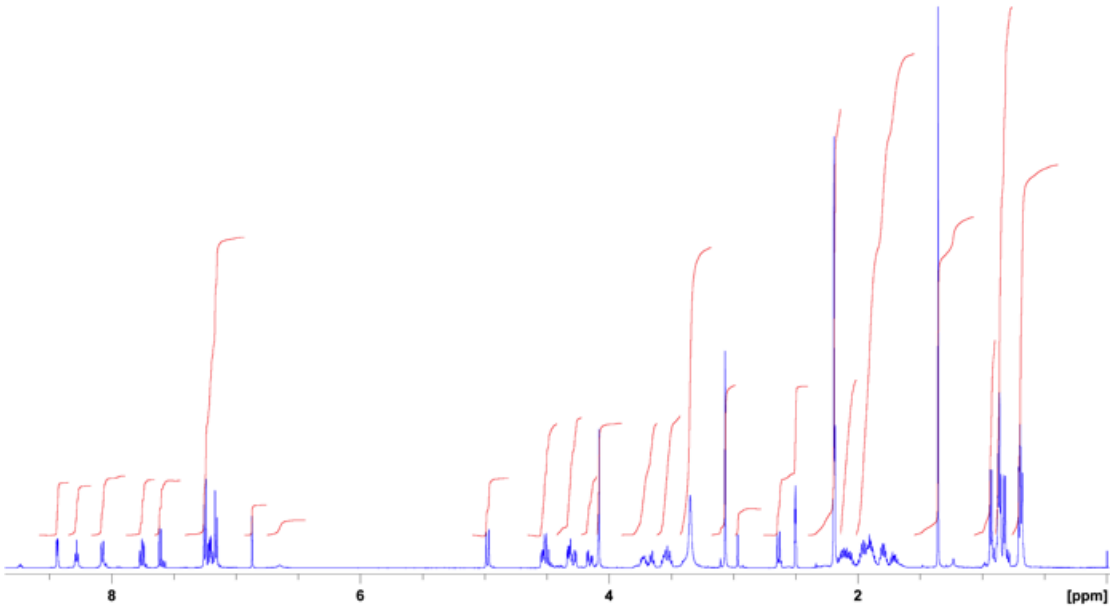


Figure 5. ^{1}H-NMR spectrum of CemCH_2-SSPy.

2 Cell culture

2.1 Incubation and manipulation conditions

Cells were handled under sterile conditions in a laminar air flow hood using disposable sterile plastic pipettes (TPP®), sterile pipette tips and flasks (TPP®). Different sizes of tissue flasks (25 cm^2, 75 cm^2, 150 cm^2 and 300 cm^2) and autoclaved glass flasks were used for cultivating cells. Incubation of cells occurred either in a humidified incubator (series II Water Jacketed CO_2 Incubator, ThermoForma) with a 5% CO_2/95% air atmosphere or in a shaking incubator (Innova™ 4000) at 140 to 180 rpm (only suspension cells). The temperature for protein production was 31 °C and for cell growth 37 °C.

2.2 CHO-S cells

Transfected CHO-S cells were grown in suspension. These required PowerCHO-2CD medium (Lonza) supplemented with 20 mL of HT Supplement (Invitrogen), 11 mL of antibiotic-antimycotic solution (Gibco Invitrogen) and 40 mL ultraglutamine 200 mm (Lonza) according to the manufacturers protocol. As soon as a cell density of 4 – 6×10^6 cells/mL was reached, cells were split and diluted to approximately 0.3×10^6 cells/mL. Cells were incubated in a shaking incubator, 140 rpm, at 37 °C for growth and 31 °C for protein production.

3 Antibody cloning, expression, purification and characterization

The cloning, expression and characterization of Db(F8)GGC, and SIP(F8)GGC and SIP(KSF)GGC antibodies has been previously described,[5, 6] while SIP(F8)GGAGGC and SIP(F8)GGAGAGGC were newly cloned. Briefly, adherent CHO-S cells (Invitrogen) were stably transfected by electroporation with the pcDNA3.1 vector carrying the antibody gene. Selection was carried out in the presence of G418 (0.65 g/L). Clones of G418-resistant cells were screened for antibody expression by ELISA using recombinant EDA of human fibronectin as antigen, and protein A - HRP for detection (GE Healthcare). The best expressing clone was adapted to grow in suspension in supplemented PowerCHO-2CD protein free medium for large scale production of antibody. The antibodies were purified from the cell culture medium by protein A affinity chromatography.

3.1 Protein purification

3.1.1 Supernatant conservation

The cells from transfection as well as the stable cell lines were centrifuged for 15 minutes at 4 °C at 7000 rpm in a SLA 3000 rotor (Sorvall RC 5C Plus, Kendro) and the supernatant was collected in plastic bottles. In case the purification was not immediately started, 0.1 % sodium azide was added to the supernatant and the bottles stored at 4 °C.

3.1.2 Protein A sepharose resin purification

Protein purification was carried out at 4 °C. Protein A sepharose resin and plain sepharose resin were added into separate plastic columns (Sigma). The supernatant was first let over the plain sepharose column (PD10 column, GE Healthcare) (in order to get rid of unspecific sepharose binders and to eliminate cellular debris) followed by the protein A sepharose resin column with a maximal speed of 3 mL/min. Both columns were first washed and equilibrated with PBS pH 7.4. Columns were washed after supernatant was let through. The first washing step was done with PBS pH 7.4 until the OD_{280} of the flow through reached a value below 0.1. The second washing step was carried out with 0.1 M NaCl in PBS pH 7.4, containing 0.1% Tween 20 until the OD_{280} was lower than 0.05. Finally, the column was washed with 200 mL of PBS pH 7.4.

3.1.3 Elution

Depending on the pI of the protein, a different elution buffer was used. If the calculated pI was above 7, 0.1 M glycine (pH = 3) was used and if the pI was below 7, 0.1 M triethylamine (TEA) was used for the elution of the protein. Fractions of 0.5 mL up to 15 mL were collected. The optical density (OD) of the different fractions was measured at 280 nm (Cary300 UV-visible spectrophotometer, Varian) and all fractions with an OD_{280} higher than 0.1 were pooled and subsequently the eluted solution was diluted with PBS pH 7.4 to an OD lower than 1.

3.1.4 Dialysis

The protein was put into dialysis against 5 L PBS pH 7.4 to a final dilution of $1:10^4$ with a SpectraPor® Dialysis Membrane (MWCO 12-14'000) overnight at 4 °C. The OD_{280} of the dialysis product was measured.

3.1.5 Filtration

After dialysis, proteins were filtered with a 0.22 μm^2 filter (Whatman® Fp30/02 CA-S) and the OD_{280} was measured. Aliquots of the filtered protein were snap-frozen and stored at -80 °C.

3.2 Protein characterization

3.2.1 SDS-PAGE

Unmodified and modified antibodies were all analysed on SDS-PAGE under non-reducing and reducing conditions. 24 µL of protein (in case of a concentrated protein solution the sample was diluted with PBS pH 7.4) were mixed with 6 µL of either reducing (2% β-mercaptoethanol) or non-reducing 5 x SDS loading buffer. 3 µL of PageRuler™ Plus prestained Protein (Fermentas) served as a molecular marker. 10-14 µL of the samples were loaded on a precast NuPAGE® 10 or 12% Bis-Tris Gel (either 1.5 mm x 10 wells or 1.0 mm x 15 wells) (Invitrogen) and run at 180 V and 110 mA for 50 minutes. NuPAGE® MOPS SDS Running Buffer 20x (Invitrogen) diluted in millipore H_2O was used as the corresponding running buffer. Afterwards the gel was stained in coomassie blue staining solution for 30 minutes followed by destaining with the corresponding solution.

3.2.2 Protein Mass Spectrometry

Liquid chromatography-mass spectrometry (LC-MS) was performed on a Micromass Quattro API instrument (ESI-MS) coupled to a Waters Alliance 2795 HPLC using a MassPREP On-Line Desalting Cartridge 2.1 x 10 mm. Water:acetonitrile, 95:5 (solvent A) and acetonitrile (solvent B), with solvent A containing 0.1% formic acid, were used as the mobile phase at a flow rate of 0.3 mL/min. The gradient was programmed as follows: 95% A (0.5 minutes isocratic) to 80% B after 1.5 minutes, then isocratic for 1 minute, followed by 4 minutes to 95% A and finally isocratic for 6 minutes. The electrospray source was operated with a capillary voltage of 3.0 kV

and a cone voltage of 20 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/hr.

Proteins typically elute on a single peak between 3 and 4.5 minutes using this method. For conjugation analysis, the mass spectra corresponding to all protein in this peak were combined using MassLynx software (v. 4.0 from Waters). Mass spectra were calibrated using a calibration curve constructed from a minimum of 16 matched peaks from the multiply charged ion series of equine myoglobin, which was also obtained using the method described above. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.0 from Waters) according to manufacturer's instructions. The relative peak height that results from the reconstruction from total ion series is then used to calculate the relative amount of each protein and conjugation conversions. We assume that both modified and non-modified antibodies are ionized with similar efficiency. It has been demonstrated that the relative MS peak height correlates well with the relative amount of protein measured using independent methods in cases where a single site is modified.[7] In the case of the reactions reported here, the excess of reagent could be removed by dialysis or size exclusion chromatography and therefore did not interfere with LC-MS analysis.

3.2.3 Gel filtration analysis

The purified antibodies as well as the antibody drug conjugates (ADCs) were analysed by fast protein liquid chromatography (FPLC) on a Pharmacia ÄKTA-FPLC system (Amersham Pharmacia Biotech). A Superdex 200 HR 10/30 column was used to perform gel filtration. 100 µL of the protein were injected into the loop that was previously washed five times with millipore water. The column was run with PBS pH 7.4 at a flow rate of 0.5 mL/min and maximal pressure of 1.5 MPa. Proteins were detected at a wavelength of 280 nm.

3.2.4 Biacore

The immunoreactivity of the antibody and respective conjugate with CemCH_2-SH was studied by surface plasmon resonance measurements on a Biacore 3000 system (GE Healthcare). The recombinant 11A12 fibronectin fragment[6] was immobilized on a CM5 microsensor chip through a peptide bond between accessible amino groups of the protein and carboxylic acid residues on the chip previously activated with *N*-(3-

dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The antibody fragments and derivatives were injected in different concentrations at a flow rate of 20 µl /min on a high-density coated 11A12-chip (3000 RU). Because of the avidity of SIP preparations, no kinetic parameters were calculated but rather binding abilities were evaluated by comparing the sensorgram profiles of modified and non-modified antibody fragments.

3.2.5 ELISA

Enzyme-linked immunosorbent assay was used in order to determine the best producing clone during the process of growing stably transfected monoclonals as well as to confirm binding affinity of modified antibody conjugates for their antigen. The assay was essentially carried out as follows: StreptaWells (Roche) were coated with 100 µL of biotinylated EDA 10^{-5} M in PBS pH 7.4 and incubated at 37 °C for 30 minutes. After washing with PBS pH 7.4 a blocking step with 4% Milk PBS (MPBS) was carried out at room temperature for 45 minutes followed by washing with PBS pH 7.4. Afterwards 100 µL of sample were added. 100 µL of 2% MPBS served as a negative and 100 µL of known positive protein (around 10^{-7} M) served as a positive control, whereas the test samples consisted of 80 µL supernatant and 20 µL 10% MPBS. They were incubated at room temperature for 45 minutes. After washing three times with PBS pH 7.4, 100 µL of Protein A-HRP (GE-Healthcare, NA9120V), diluted 1:1000 in 4% MPBS, was added and incubated at room temperature for 45 minutes. Afterwards three washing steps with PBS pH 7.4 containing 0.1 % Tween followed by another three washing steps with PBS pH 7.4 were carried out. Detection was done by adding 100 µL of BM Blue POD substrate (Roche 11484281001) in each well. After incubating at room temperature for 1 minute, the development was stopped with 50 µL of 1 M H_2SO_4. The OD_{450} was measured with a Plate Reader (VersaMax tunable microplate reader, Molecular Devices, Software: SOFTmax PRO v3.1).

3.2.6 Production, purification and characterization of SIP(F8)GGC

The production and characterization of SIP(F8)GGC has been reported previously.[1]

Production: For SIP(F8)GGC production, 10^7 mycoplasma tested negatively monoclonal CHO-S cells expressing SIP(F8)GGC were taken into culture. Cells were transferred into a T75 flask together with 15 mL of supplemented Power CHO-2CD

medium and incubated overnight at 37 °C. On the next day, the cell suspension was diluted to 0.3 x 10^6 cells/mL in a sterile glass bottle and put on a shaking incubator at 37 °C, 170 rpm. Once a cell density of 4 – 6 x 10^6 cells/mL was reached, the cells were either split or incubated at 31 °C for protein production.

Purification: 1.2 L of supernatant were allowed to run overnight over a PD10 column and a protein A sepharose column. After washing, the protein was eluted using 0.1 M glycine (pH = 3) as the pI of SIP(F8)GGC was above 7. 3 fractions of 15 mL were collected in tubes that already contained 3 mL of 1 M Tris HCl pH 7.4 to ensure a rapid neutralization. All fractions were pooled and diluted to an OD_{280} below 1. The protein solution was dialyzed against 5 L PBS pH 7.4 overnight and against 5 L PBS pH 7.4 for four hours at 4 °C. The dialysed protein was filtered and the concentration was determined by measuring the OD_{280}. Aliquots of 2 mL each were made and stored at -80 °C.

SIP(F8)GGC	
Total yield	440 mg
Average yield	35 mg/L

Characterization: SIP(F8)GGC was characterized by LC-MS, SDS-PAGE and FPLC gel filtration and Biacore. See below.

Sequence of monomer: E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F S L F T M S W V R Q A P G K G L E W V S A I S G S G G S T Y Y A D S V K G R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K S T H L Y L F D Y W G Q G T L V T V S S G G G G S G G G G S G G G G E I V L T Q S P G T L S L S P G E R A T L S C R A S Q S V S M P F L A W Y Q Q K P G Q A P R L L I Y G A S S R A T G I P D R F S G S G S G T D F T L T I S R L E P E D F A V Y Y C Q Q M R G R P P T F G Q G T K V E I K S G G S G G P R A A P E V Y A F A T P E W P G S R D K R T L A C L I Q N F M P E D I S V Q W L H N E V Q L P D A R H S T T Q P R K T K G S G F F V F S R L E V T R A E W E Q K D E F I C R A V H E A A S P S Q T V Q R A V S V N P E S S R R G G C

Calculated mass of monomer: 38728

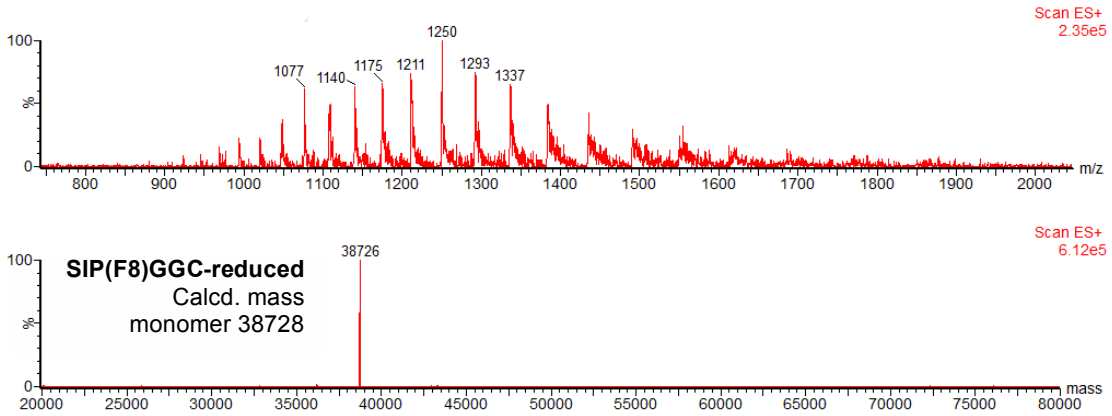


Figure 6. ESI-MS spectrum of SIP(F8)GGC after reduction.

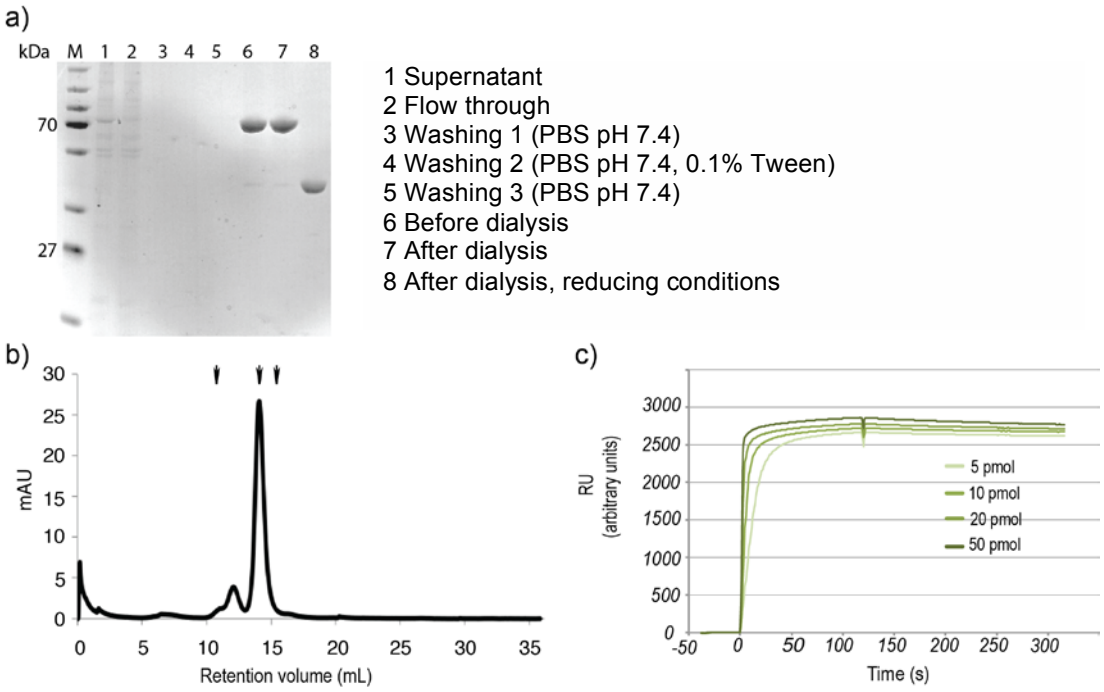


Figure 7. Characterization of SIP(F8)GGC. a) SDS-PAGE analysis. b) Gel filtration analysis of affinity-purified SIP(F8)GGC. The peak eluting at a retention volume of 14.1 mL corresponds to the homodimeric form of SIP(F8)GGC. Arrows indicate standard proteins (11 mL: Ferritin 440 kDa; 14.1 mL: BSA 67 kDa; 15.4 mL: β-lactoglobulin 35 kDa). c) Biacore analysis on an 11A12-coated chip.

3.2.7 Production, purification and characterization of SIP(KSF)GGC

The production and characterization of SIP(KSF)GGC has been reported previously.[1]

Production and purification of SIP(KSF)GGC was performed in complete analogy to the production of SIP(F8)GGC. Purification of SIP(KSF)GGC was performed in analogy to the purification of SIP(F8)GGC, but maximal 500 mL of supernatant were loaded on a protein A sepharose column and 4 fractions of 15 mL were collected in

15 mL tubes. In cases when protein precipitation was observed during the elution step, protein was filtered before and after dialysis.

SIP(KSF)	
Total yield	386 mg
Average yield	54 mg/L

Characterization: SIP(KSF)GGC was characterized by LC-MS, SDS-PAGE and FPLC gel filtration. See below.

Sequence of monomer: E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F S S Y A M S W V R Q A P G K G L E W V S A I S G S G G S T Y Y A D S V K G R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K S P K V S L F D Y W G Q G T L V T V S S G G G G S G G G G S G G G G S S E L T Q D P A V S V A L G Q T V R I T C Q G D S L R S Y Y A S W Y Q Q K P G Q A P V L V I Y G K N N R P S G I P D R F S G S S S G N T A S L T I T G A Q A E D E A D Y Y C N S S P L N R L A V V F G G G T K L T V L G S G G S G G P R A A P E V Y A F A T P E W P G S R D K R T L A C L I Q N F M P E D I S V Q W L H N E V Q L P D A R H S T T Q P R K T K G S G F F V F S R L E V T R A E W E Q K D E F I C R A V H E A A S P S Q T V Q R A V S V N P E S S R R G G C

Calculated mass of monomer: 38330

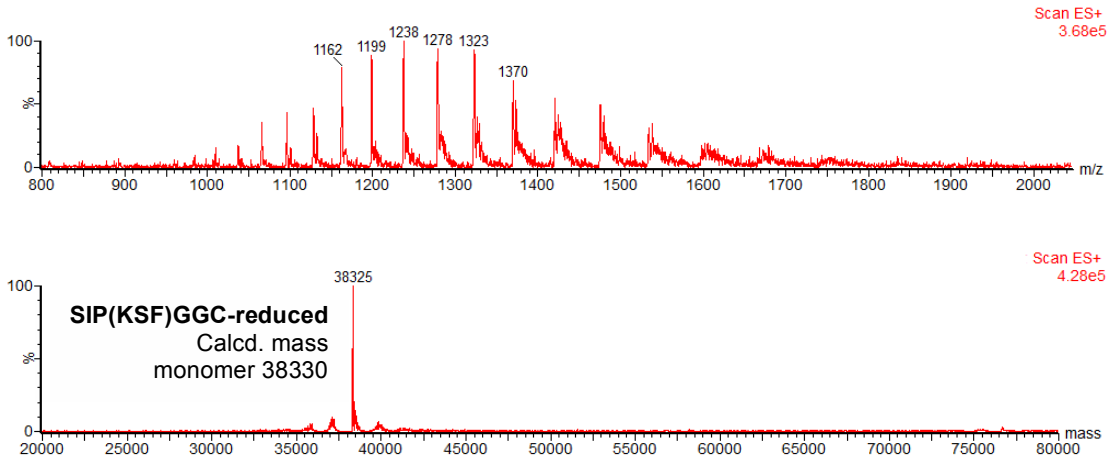


Figure 8. ESI-MS spectrum of SIP(KSF)GGC after reduction.

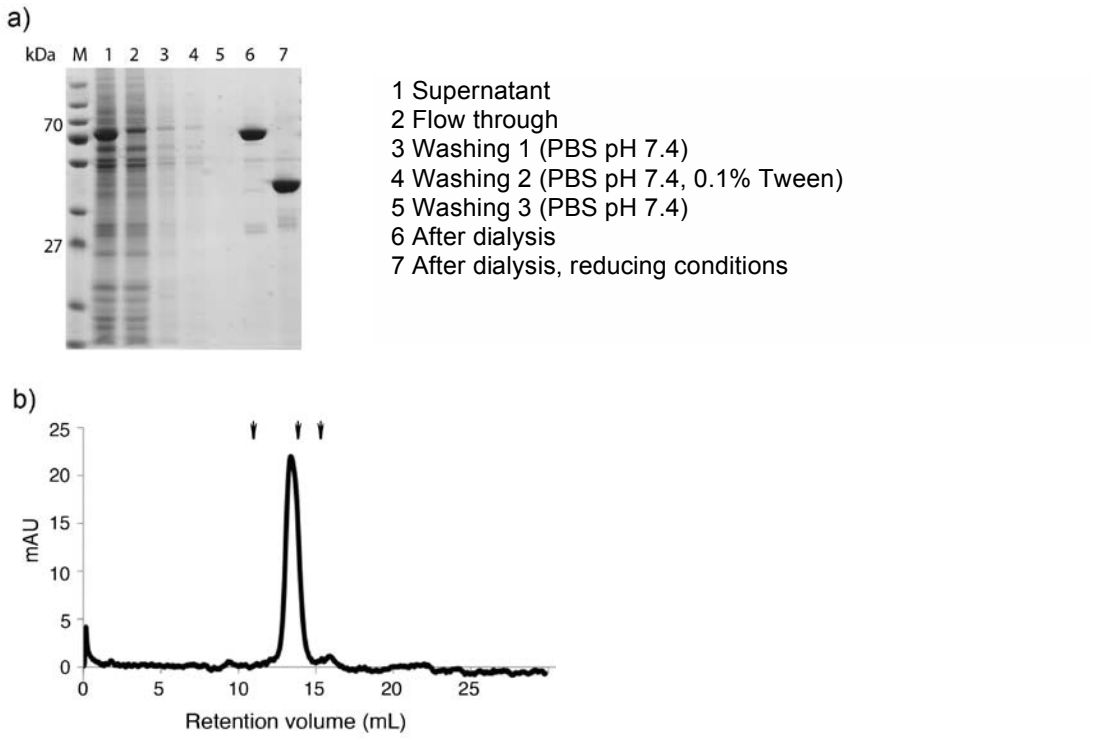


Figure 9. Characterization of SIP(KSF)GGC. (*A*) SDS-PAGE analysis. (*B*) Gel filtration analysis of affinity-purified SIP(KSF)GGC. The peak eluting at a retention volume of 13.4 mL corresponds to the homodimeric form of SIP(KSF)GGC. Arrows indicate standard proteins (11 mL: Ferritin 440 kDa; 14.1 mL: BSA 67 kDa; 15.4 mL: β-lactoglobulin 35 kDa).

3.2.8 Production and purification of Db(F8)GGC

Production and purification of Db(F8)GGC was performed in complete analogy to the production of SIP(F8)GGC and as previously described.[8]

3.2.9 Production, purification and characterization of SIP(F8)GGAGGC and SIP(F8)GGAGAGGC

For the cloning of SIP(F8)GGAGGC the gene codifying for the SIP(F8)GGC with the cleavable N-terminal secretory sequence was PCR amplified with the primers 5'-CCCAAGCTTGTCGACCATGGGCTGGAGCCTGATCC-3' (forward) and 5'-GAGTCATCGAGGAGGGGTGGCGCAGGAGGG-3' (reverse), introducing the extra amino acids. The resulting PCR product was used as a template for a second PCR with the same forward primer and the reverse primer 5'-ATAGTTTAGCGGCCGCCTAGCACCCTCCTGCGCCACCCCTCCTCG-3', introducing the stop codon and the NotI restriction site.

Supporting Information

For the cloning of SIP(F8)GGAGAGGC the gene codifying for the SIP(F8)GGC with the cleavable N-terminal secretory sequence was PCR amplified with the primers 5'-CCCAAGCTTGTCGACCATGGGCTGGAGCCTGATCC-3' (forward) and 5'-GAGTCATCGAGGAGGGGTGGCGCAGGCGCAGGAGGG-3' (reverse), introducing the extra amino acids. The resulting PCR product was used as a template for a second PCR with the same forward primer and the reverse primer 5'-GGAGGGGTGGCGCAGGCGCAGGAGGGTGCTAGGCGGCCGCTAAACTAT-3', introducing the stop codon and the NotI restriction site.

The PCR products were were digested with with HindIII and NotI (NEB) and ligated (NEB, T4 ligase) into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen). The resulting gene was verified by sequencing (GATC Biotech).

Expression, purification and characterization of SIP(F8)GGAGGC, SIP(F8)GGAGAGGC and Db(F8)GGC was performed in analogy to SIP(F8)GGC. For Db(F8)GGC, the monoclonal cell cultures of stably transfected CHO cells were selected by FACS cell sorting methods as described by Zuberbühler et al. [9]

	SIP(F8)GGAGGC	SIP(F8)GGAGAGGC
Average yield	20 mg/L	20 mg/L

DNA-sequence of SIP(F8)GGAGGC: HindIII - Secretory sequence - scFv(F8) - BspE1 - EC_H4 – GGAGGC - **NotI**

CCCAAGCTTGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCT
CGTCGCTGTGGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGA
GGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTT
CTCTCCACAGGTGTGCACTCGGAGGTGCAGCTGTTGGAGTCTGG
GGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTG
CAGCCTCTGGATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCC
GCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGT
GGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCG
GTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGC
AAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGT
GCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGGCCAGGGA
ACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTTCAGGCGGAGG

Supporting Information

TGGCTCTGGCGGTGGCGGAGAAATTGTGTTGACGCAGTCTCCAG
GCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCA
GGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGGTACCAG
CAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATC
CAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGT
CTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAA
GATTTTGCAGTGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCG
ACGTTCGGCCAAGGGACCAAGGTGGAAATCAAATCCGGAGGCTC
TGGGGGCCCGCGTGCTGCCCCGGAAGTCTATGCGTTTGCGACGC
CGGAGTGGCCGGGGAGCCGGGACAAGCGCACCCTCGCCTGCCTG
ATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCA
CAACGAGGTGCAGCTCCCGGACGCCCGGCACAGCACGACGCAGC
CCCGCAAGACCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGCCTG
GAGGTGACCAGGGCCGAATGGGAGCAGAAAGATGAGTTCATCTG
CCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGACCGTCCAGC
GAGCGGTGTCTGTAAATCCCGAGTCATCGAGGAGGGGTGGCGCA
GGAGGGTGCTAGGCGGCCGCTAAACTAT

DNA-sequence of SIP(F8)GGAGAGGC: HindIII **-** Secretory sequence **-** scFv(F8)- BspE1 - EC_H4 – GGAGAGGC - **NotI**

CCCAAGCTTGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCT
CGTCGCTGTGGCTACAGGTAAGGGGCTCACAGTAGCAGGCTTGA
GGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTT
CTCTCCACAGGTGTGCACTCGGAGGTGCAGCTGTTGGAGTCTGG
GGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTG
CAGCCTCTGGATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCC
GCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGT
GGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCG
GTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGC
AAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGT
GCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGGCCAGGGA
ACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTTCAGGCGGAGG
TGGCTCTGGCGGTGGCGGAGAAATTGTGTTGACGCAGTCTCCAG
GCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCA
GGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGGTACCAG
CAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATC
CAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGT
CTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAA

Supporting Information

GATTTTGCAGTGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCG
ACGTTCGGCCAAGGGACCAAGGTGGAAATCAAATCCGGAGGCTC
TGGGGGCCCGCGTGCTGCCCCGGAAGTCTATGCGTTTGCGACGC
CGGAGTGGCCGGGGGAGCCGGGACAAGCGCACCCTCGCCTGCCTG
ATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCA
CAACGAGGTGCAGCTCCCGGACGCCCGGCACAGCACGACGCAGC
CCCGCAAGACCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGCCTG
GAGGTGACCAGGGCCGAATGGGAGCAGAAAGATGAGTTCATCTG
CCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGACCGTCCAGC
GAGCGGTGTCTGTAAATCCCGAGTCATCGAGGAGGGGTGGCGCA
GGCGCAGGAGGGTGCTAGGCGGGCCGCTAAACTAT

The protein sequence is analogous to that of SIP(F8)GGC shown in 3.2.6 p. 15, but with modified C-terminal sequences GGAGGC and GGAGAGGC instead of GGC for SIP(F8)GGAGGC and SIP(F8)GGAGAGGC, respectively.

Supporting Information

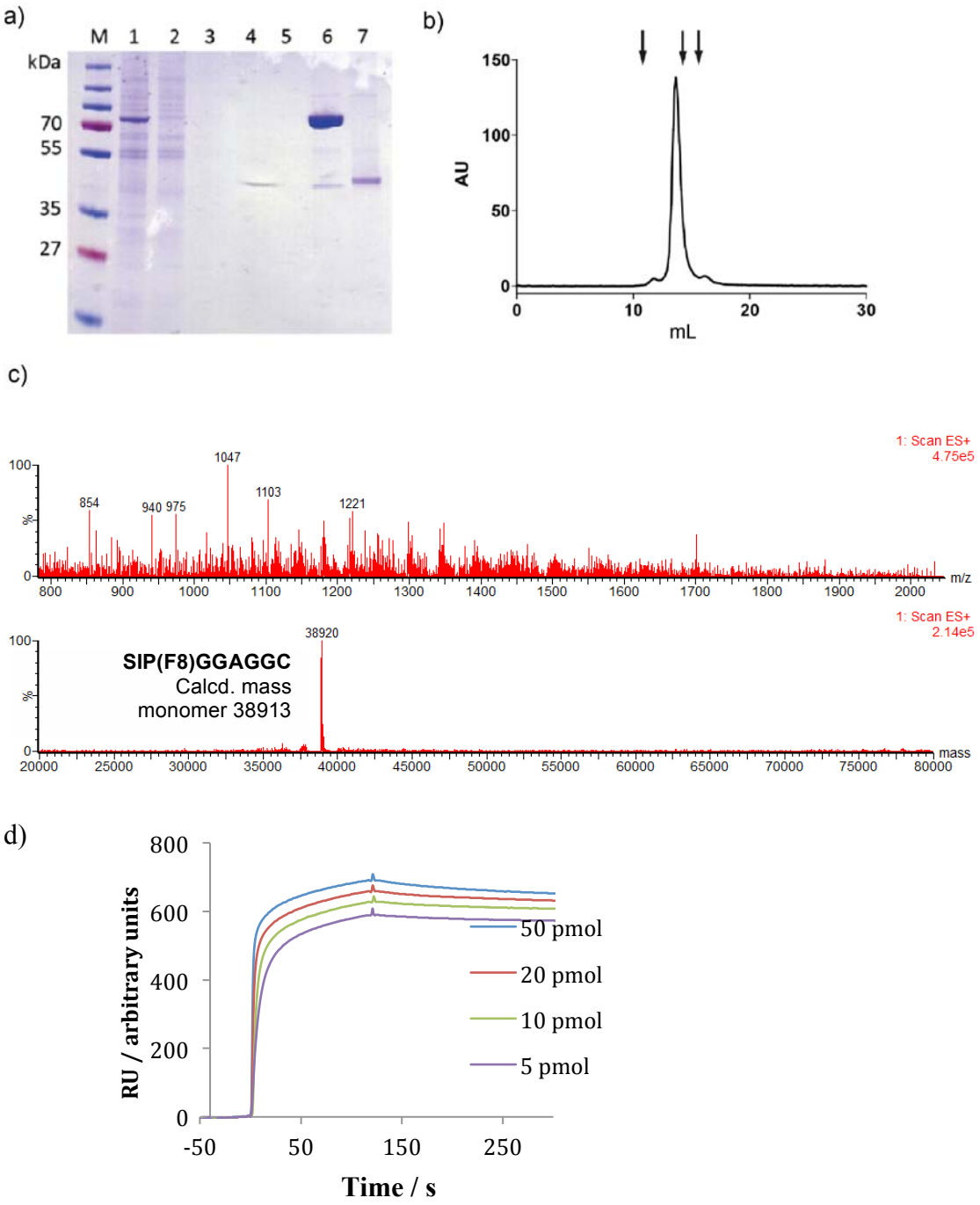


Figure 10. a) Analytical SDS-PAGE for SIP(F8)GGAGGC protein purification; samples were taken after every step of protein purification described in section, M: molecular weight marker, 1: Supernatant, 2: Flow through, 3: Washing 1 (PBS), 4: Washing 2 (PBS, 1% Tween 20), 5: Washing 3 (PBS), 6+7: After dialysis; Samples 1-6 were run under non-reducing conditions, sample 7 under reducing conditions, b) Gel filtration profile of SIP(F8)GGAGGC on Sephadex 200, SIP(F8)GGAGGC eluting as a homodimer after 13.7 mL, arrows indicate standard proteins (11 mL: Ferritin 440 kDa; 14.1 mL: BSA 67 kDa; 15.4 mL: β-lactoglobulin 35 kDa). c) Deconvoluted ESI-MS spectrum of SIP(F8)GGAGGC reduced with TCEP HCl, corresponding ion series in small. Calculated mass

SIP(F8)GGAGGC: 389130 Da, observed mass: 38920 Da. d) Biacore of SIP(F8)GGAGGC on an 11A12 coated CM5 chip; apparent K_D = 0.937 nM.

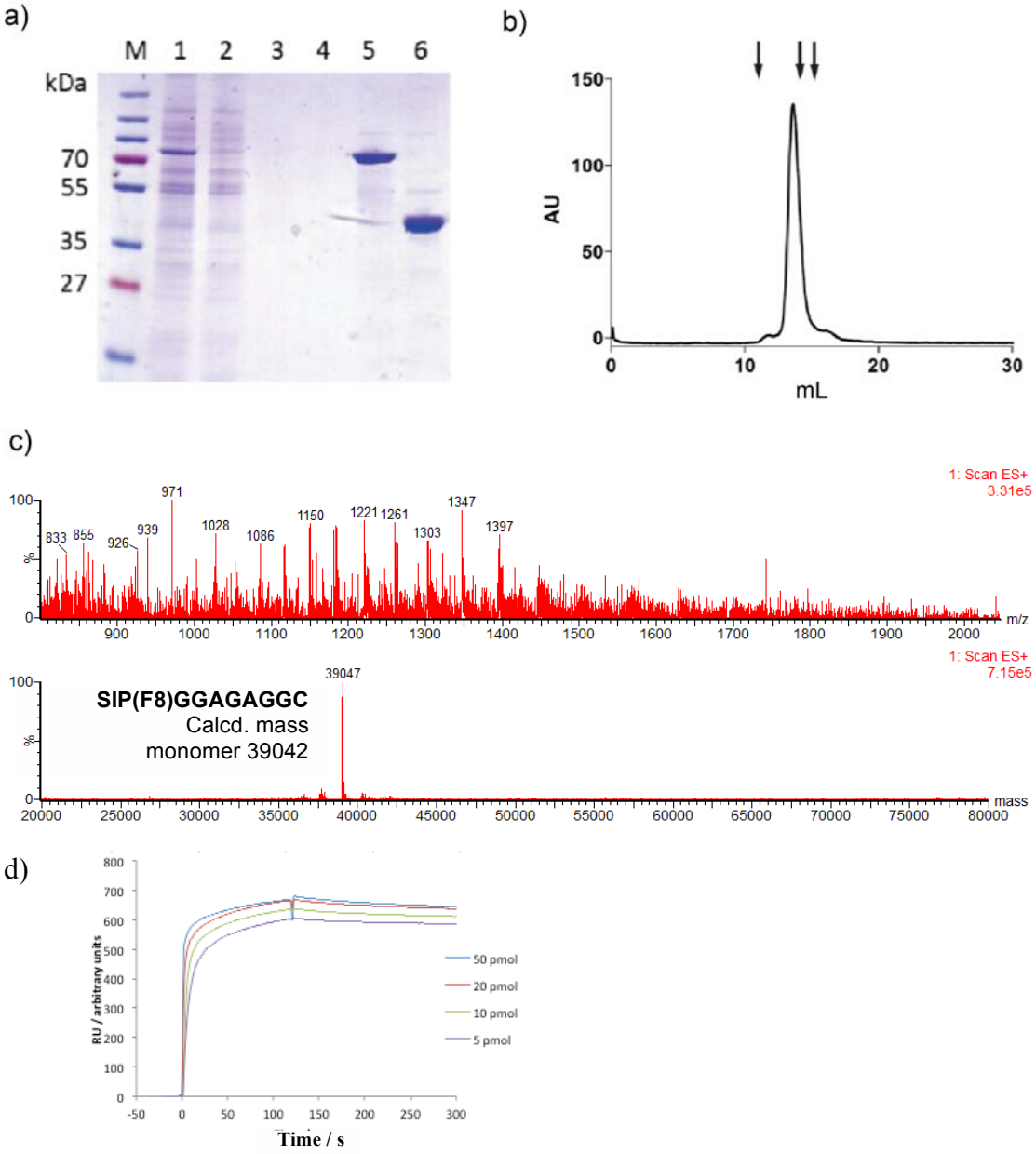


Figure 11. a) analytical SDS-PAGE for SIP(F8)GGAGAGGC protein purification; Samples were taken after every step of protein purification described in section, M: molecular weight marker, 1: Supernatant, 2: Flow through, 3: Washing 1 (PBS), 4: Washing 2 (PBS, 1% Tween 20), 5+6: After dialysis; Samples 1-5 were run under non-reducing conditions, sample 6 under reducing conditions, b) Gel filtration profile of SIP(F8)GGAGAGGC on Sephadex 200, SIP(F8)GGAGAGGC forming a homodimer and eluting after 13.7 mL, arrows indicate standard proteins (11 mL: Ferritin 440 kDa; 14.1 mL: BSA 67 kDa; 15.4 mL: β-lactoglobulin 35 kDa). c) Deconvoluted ESI-MS spectrum of SIP(F8)GGAGAGGC reduced with TCEP HCl, corresponding ion series in small. Calculated mass SIP(F8)GGAGAGGC: 39041 Da, observed mass: 39047 Da. d) Biacore of SIP(F8)GGAGAGGC on an 11A12 coated CM5 chip; apparent K_D = 0.524 nM.

4 Mixed disulfide conjugation and preparation of ADCs for therapy

4.1 Conjugation analysis by LC-MS

A typical analysis of a conjugation reaction by LC-MS is described below. The total ion chromatogram, combined ion series and deconvoluted spectra are shown for the product of the reaction of SIP(F8)GGC-Ellman's with CemCH$_2$-SH. Identical analyses were carried out for all the conjugation reactions performed in this work.

SIP(F8)GGC-SS-CH$_2$Cem

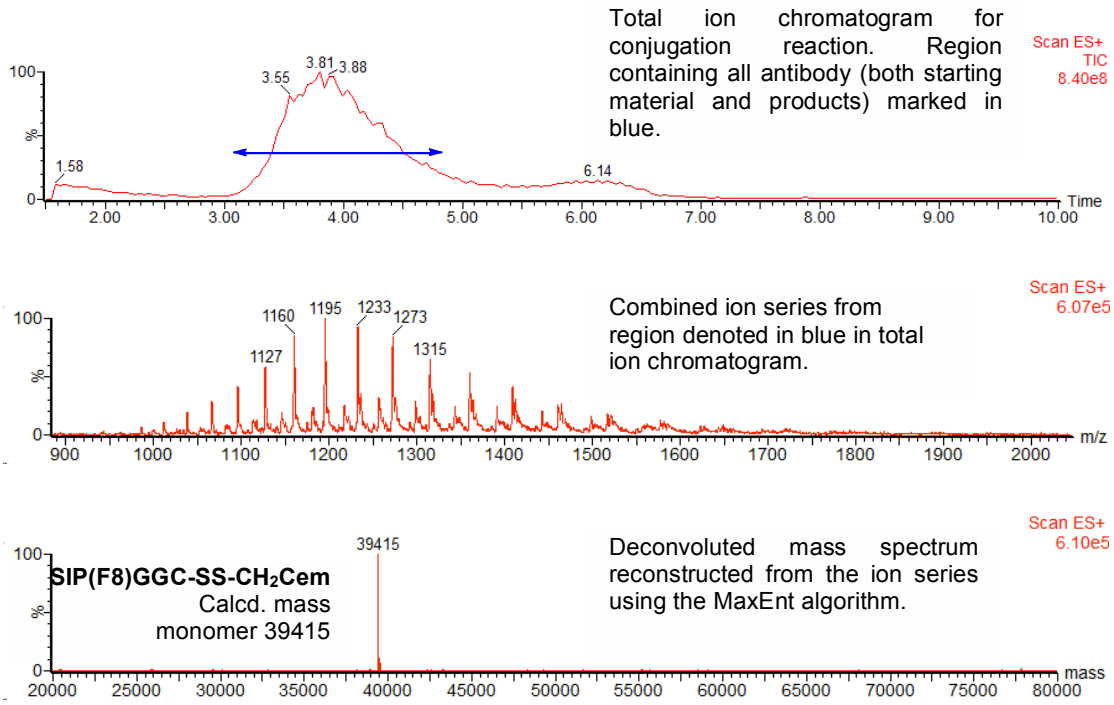


Figure 12. LC-MS analysis of the conjugation reaction of CemCH$_2$-SH with SIP(F8)GGC-Ellman's.

4.2 Mixed disulfide-linked ADCs

4.2.1 Electrophilic Approach

General conjugation procedure using Ellman's reagent

Reaction with Ellman's reagent

Typically, a solution of TCEP•HCl is prepared by dissolving TCEP•HCl in PBS pH 7.4. 20 μL of this solution (corresponding to 30 equiv. to the Db(F8)GGC or SIP(F8)XXC monomers, i.e. SIP(F8)GGC, SIP(F8)GGAGGC and SIP(F8)GGAGAGGC) is added to Db(F8)GGC or SIP(F8)XXC (2 mL, *c* =

0.65 mg/mL) in PBS pH 7.4. The mixture is vortexed for 30 seconds and left standing for 3 hours at room temperature or overnight at 4 °C. Ellman's reagent (500 equiv. excess to the Db(F8)GGC or SIP(F8)GGC monomers) is dissolved in 200 μL of PBS pH 7.4 and degassed. Previously reduced and degassed Db(F8)GGC or SIP(F8)XXC is added to the Ellman's reagent solution and the mixture is stirred for an additional 30 minute period under argon atmosphere. A 50 μL sample is taken for LC-MS and SDS-PAGE* analysis. Purification of the modified antibody is performed by FPLC (Amersham Pharmacia) using a HiTrap desalting column (GE Healthcare) of 5 mL volume equilibrated in PBS pH 7.4 and applying a flow of 1 mL/min. The fractions containing the modified antibody are collected, the concentration determined by measuring absorbance at 280 nm, and an aliquot is analysed by LC-MS and SDS-PAGE. Typically, a final concentration of 0.41 mg/mL is obtained after reaction and purification.

Reaction of Db(F8)GGC-Ellman's and SIP(F8)CCG-Ellman's with thiol drugs

The thiol drug, in this case $CemCH_2$-SH, or any other thiol nucleophile (e.g., reduced glutathione) (10 equiv.) is weighed as a solid in a 2 mL plastic tube. To this tube, purified Db(F8)GGC-Ellman's or SIP(F8)XXC-Ellman's (2 mL, c = 0.41 mg/mL) is added and the resulting mixture stirred at room temperature for 10 minutes. The solution becomes slightly yellow immediately after addition to a thiol. LC-MS analysis is used to check for complete to the corresponding mixed disulfide conjugate. Purification of the modified antibody is performed by FPLC (Amersham Pharmacia) using two HiTrap desalting columns in series (GE Healthcare) of 5 mL volume equilibrated in PBS pH 7.4 and applying a flow of 1 mL/min. The fractions containing the modified antibody are collected, the concentration determined by measuring absorbance at 280 nm, and an aliquot is analysed by LC-MS and SDS-PAGE. Typically, a final concentration of 0.35 mg/mL is obtained after the two-step reactions and purifications, corresponding to an average yield of 50 %. The fractions containing the modified antibody can be collected and concentrated using Vivaspin

* A weak, minor band is often observed by SDS-PAGE for the reactions of Db(F8)GGC and SIP(F8)XXC. This band could correspond to re-oxidation of terminal cysteines to form a covalent dimer. However, and after carefully analysis by LC-MS where a single peak is always detected indicating mixed disulfide formation, the observed band is likely a result of the SDS-PAGE running conditions.

15R (MWCO 10 kDa, Hydrosart-membrane; Sartorius stedim biotech, France) at 3800 rpm (Megafue 1.0R, Heraeus Instruments, Kendro Laboratory Products) to the desired concentration. In the case of Db(F8)GGC, up to 2 mg/mL and of SIP(F8)XXC up to 5 mg/mL without observing any precipitation.

General conjugation procedure using phenylselenenyl bromide

Typically, a solution of TCEP•HCl is prepared by dissolving TCEP•HCl in PBS pH 7.4. 20 μL of this solution (corresponding to 30 equiv. to the Db(F8)GGC or SIP(F8)GGC monomers) is added to Db(F8)GGC or SIP(F8)GGC (2 mL, *c* = 0.65 mg/mL) in PBS pH 7.4. The mixture is vortexed for 30 seconds and left standing for 3 hours at room temperature or overnight at 4 °C. Phenylselenyl bromide (150 equiv. excess to the Db(F8)GGC or SIP(F8)GGC monomers) is dissolved in DMF (6 % total reaction volume) and added to previously reduced and degassed Db(F8)GGC or SIP(F8)GGC. The mixture is stirred for an additional 3 hours under argon atmosphere. A 50 μL sample is taken for LC-MS and SDS-PAGE analysis. Purification of the modified antibody is performed by FPLC (Amersham Pharmacia) using a HiTrap desalting column (GE Healthcare) of 5 mL volume equilibrated in PBS pH 7.4 and applying a flow of 1 mL/min. The fractions containing the modified antibody are collected, the concentration determined by measuring absorbance at 280 nm, and an aliquot is analysed by LC-MS and SDS-PAGE. Typically, a final concentration of 0.25 mg/mL is obtained after reaction and purification.

Reaction of Db(F8)GGC-SSePh and SIP(F8)GGC-SSePh with thiol drugs

The thiol drug, in this case CemCH_2-SH, or any other thiol nucleophile (e.g., reduced glutathione) (20 equiv.) is weighed as a solid in a 2 mL plastic tube. To this tube, purified Db(F8)GGC-SSePh or SIP(F8)GGC-SSePh (2 mL, *c* = 0.41 mg/mL) is added and the resulting mixture stirred at room temperature for 20 minutes. LC-MS analysis is used to check for complete to the corresponding mixed disulfide conjugate. Purification of the modified antibody is performed by FPLC (Amersham Pharmacia) using two HiTrap desalting columns in series (GE Healthcare) of 5 mL volume equilibrated in PBS pH 7.4 and applying a flow of 1 mL/min. The fractions containing the modified antibody are collected, the concentration determined by measuring absorbance at 280 nm, and an aliquot is analysed by LC-MS and SDS-

PAGE. Typically, a final concentration of 0.2 mg/mL is obtained after the two-step reactions and purifications, corresponding to an average yield of 30 %.

4.2.2 Nucleophilic approach

General conjugation procedure using CemCH$_2$-SSPy

Typically, a solution of TCEP•HCl is prepared by dissolving TCEP•HCl in PBS pH 7.4. 20 µL of this solution (corresponding to 30 equiv. to the Db(F8)GGC or SIP(F8)GGC monomers) is added to Db(F8)GGC or SIP(F8)GGC (2 mL, *c* = 0.65 mg/mL) in PBS pH 7.4. The mixture is vortexed for 30 seconds and left standing for 3 hours at room temperature or overnight at 4 °C. Removal of the reducing agent is carried out by using a PD-10 column (GE Healthcare) previously conditioned with degassed PBS pH 7.4. The fraction containing the reduced Db(F8)GGC or SIP(F8)GGC is immediately added to a 2 mL plastic tube containing CemCH$_2$-SSPy (20 equiv. excess to the Db(F8)GGC or SIP(F8)GGC monomers), prior dissolved in 20 µl of PBS pH 7.4 and degassed. The reaction mixture is shortly degassed and stirred for 2 hours at room temperature. A 50 µL sample is taken for LC-MS and SDS-PAGE analysis. Purification of the modified antibody is performed by FPLC (Amersham Pharmacia) using two HiTrap desalting columns in series (GE Healthcare) of 5 mL volume equilibrated in PBS pH 7.4 and applying a flow of 1 mL/min. The fractions containing the modified antibody are collected, the concentration determined by measuring absorbance at 280 nm, and an aliquot is analysed by LC-MS and SDS-PAGE. Typically, a final concentration of 0.25 mg/mL is obtained after the two-step reactions and purifications, corresponding to an average yield of 35 %. The fractions containing the modified antibody can be collected and concentrated using Vivaspin 15R (MWCO 10 kDa, Hydrosart-membrane; Sartorius stedim biotech, France) at 3800 rpm (Megafue 1.0R, Heraeus Instruments, Kendro Laboratory Products) to the desired concentration.

4.3 Characterization of conjugates

Db(F8)GGC-Ellman's

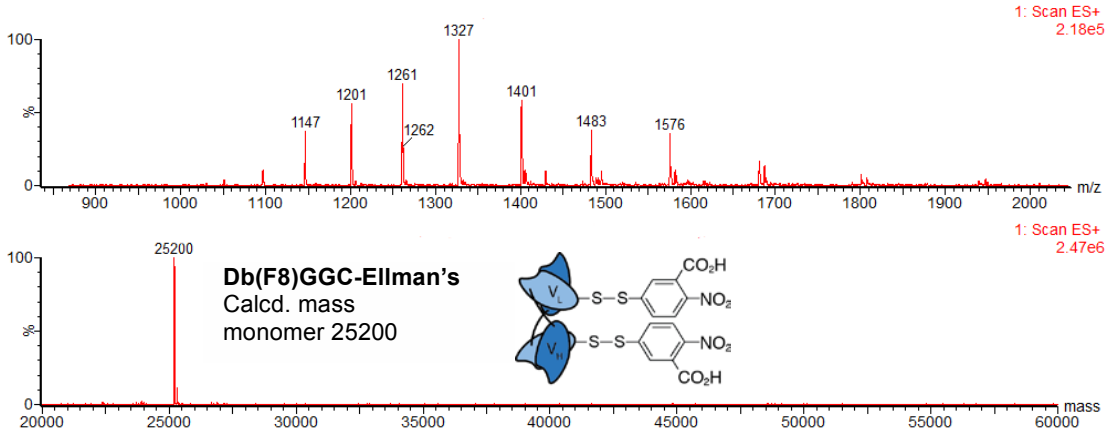
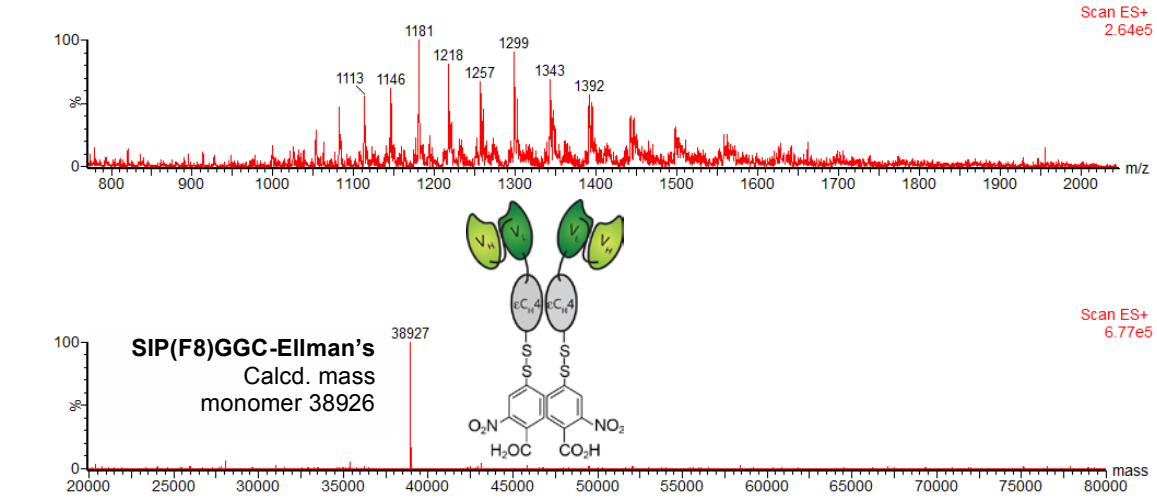


Figure 13. ESI-MS spectrum analysis of the reaction of Db(F8) with Ellman's reagent.

SIP(F8)GGC-Ellman's



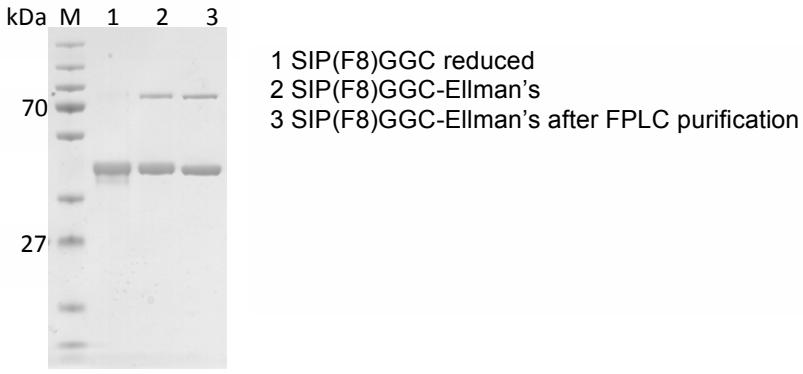
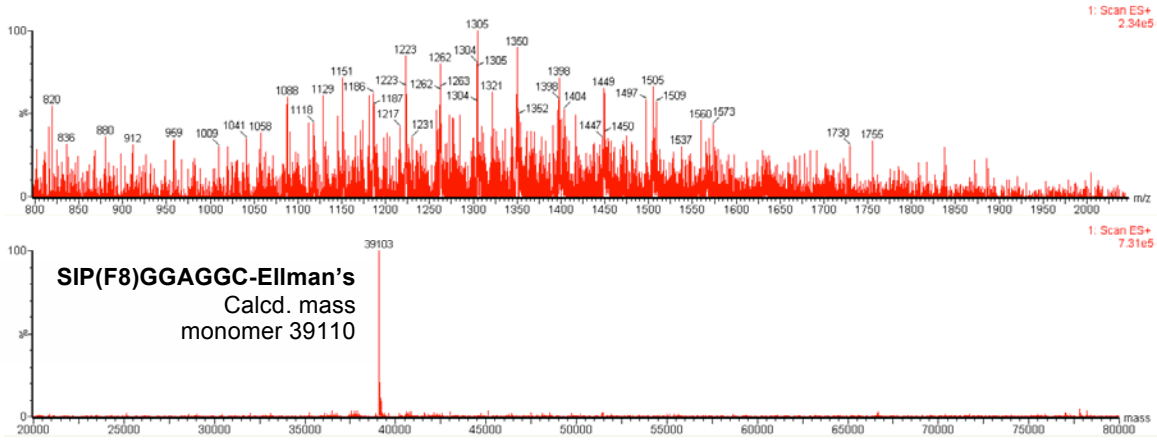


Figure 14. ESI-MS spectrum and SDS-PAGE analysis of the reaction of SIP(F8)GGC with Ellman's reagent.

SIP(F8)GGAGGC-Ellman's



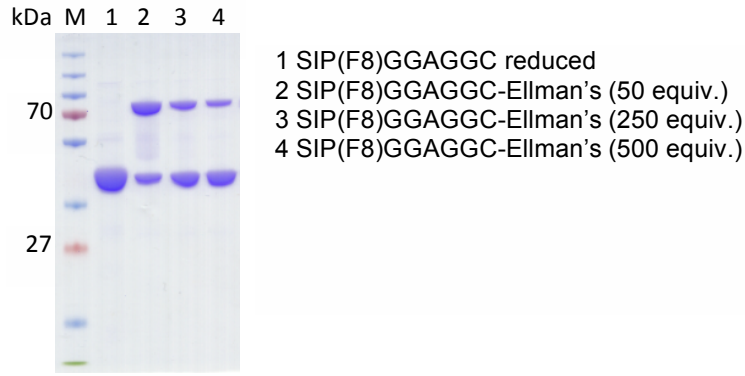
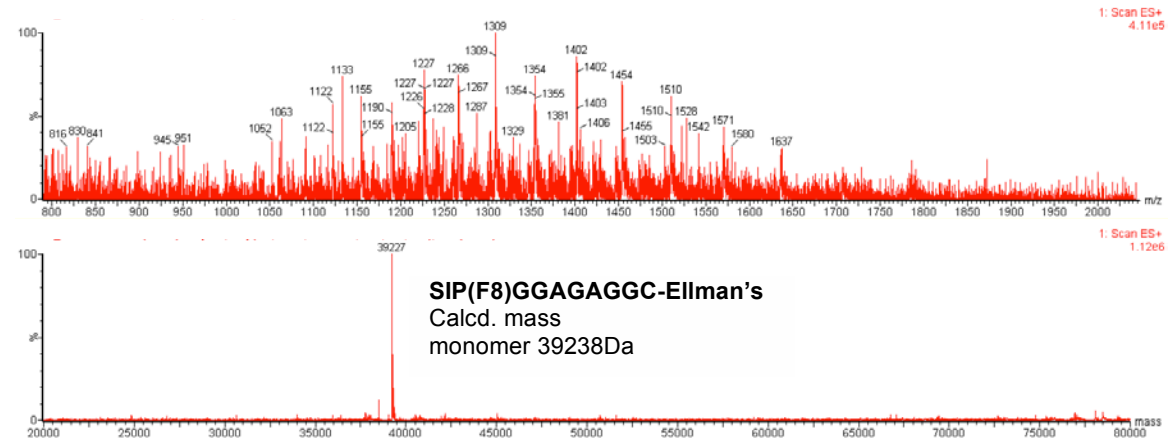


Figure 15. ESI-MS spectrum and SDS-PAGE analysis of the reaction of SIP(F8)GGAGGC with Ellman's reagent.

Supporting Information

SIP(F8)GGAGAGGC-Ellman's



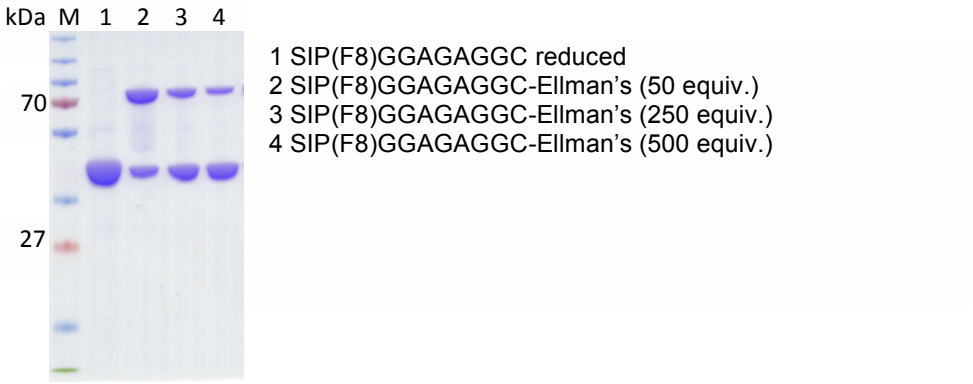


Figure 16. ESI-MS spectrum and SDS-PAGE analysis of the reaction of SIP(F8)GGAGAGGC with Ellman's reagent.

Db(F8)GGC-SSePh

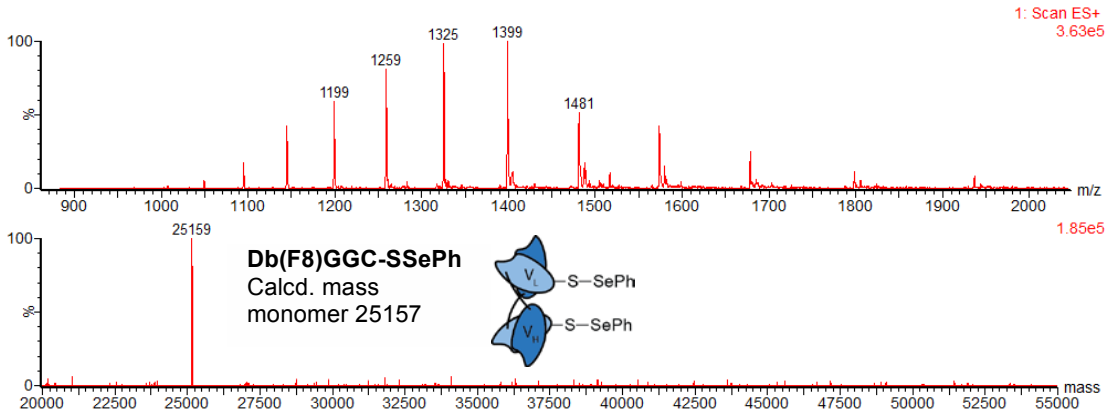


Figure 17. ESI-MS spectrum analysis of the reaction of Db(F8)GGC with phenylselenyl bromide.

SIP(F8)GGC-SSePh

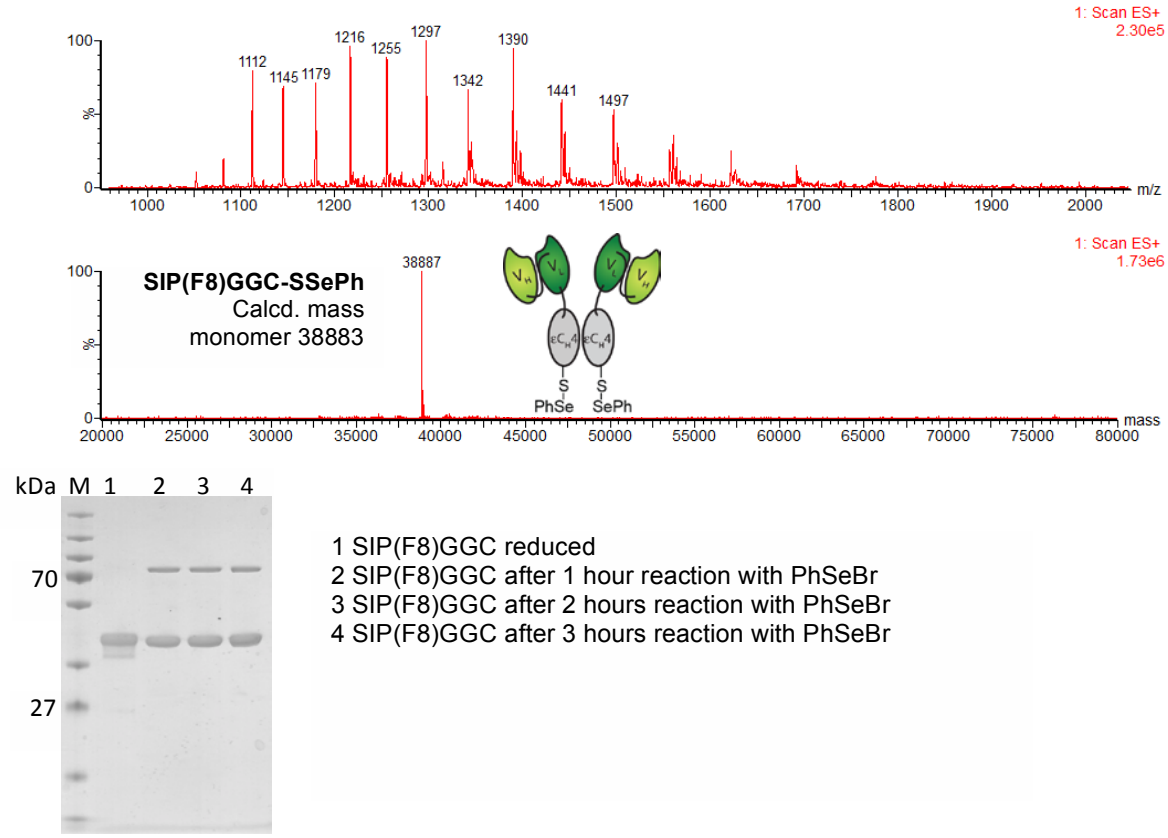


Figure 18. ESI-MS spectrum and SDS-PAGE analysis of the reaction of SIP(F8)GGC with phenylselenyl bromide.

Db(F8)GGC-SS-CH_2Cem

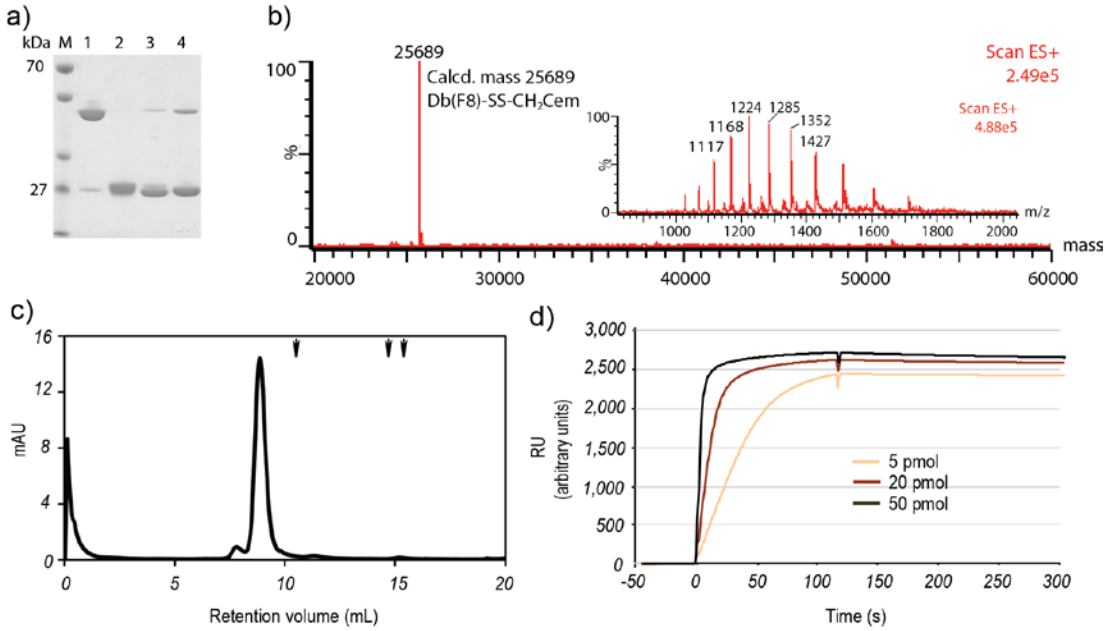


Figure 19. Characterization of Db(F8)GGC-SS-CH_2Cem. a) SDS-PAGE analysis of site-specific conjugation of CemCH_2-SH to Db(F8)GGC-Ellman's: M, molecular marker; 1, Db(F8)GGC non-reducing; 2, Db(F8)GGC reduced with TCEP; 3, purified Db(F8)GGC-Ellman's; 4, purified Db(F8)GGC-SS-CH_2Cem. b) ESI-MS spectrum of purified Db(F8)GGC-SS-CH_2Cem. c) Gel filtration analysis of purified Db(F8)GGC-SS-CH_2Cem. The peak eluting at a retention volume of 8.9 mL corresponds to the noncovalent homodimeric form of Db(F8)GGC-SS-CH_2Cem. Arrows indicate standard proteins (11 mL: Ferritin 440 kDa; 14.1 mL: BSA 67 kDa; 15.4 mL: β-lactoglobulin 35 kDa). d) Biacore analysis of purified Db(F8)GGC -SS-CH_2Cem towards recombinant 11A12 fibronectin. Identical data was obtained for the products of the reactions of Db(F8)GGC-SSePh with CemCH_2-SH and of Db(F8)GGC with CemCH_2-SSPy.

SIP(F8)GGC-SS-CH_2Cem, SIP(F8)GGAGGC-SS-CH_2Cem and SIP(F8)GGAGAGGC-SS-CH_2Cem

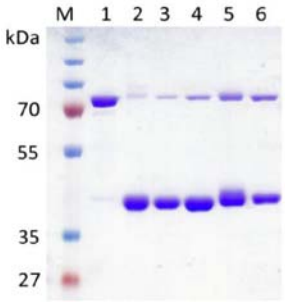


Figure 20. SDS-PAGE analysis of site-specific conjugation of CemCH_2-SH to SIP(F8)GGC-Ellman's: M: molecular weight marker, 1: SIP(F8)GGC non-reducing, 2: SIP(F8)GGC reduced with TCEP, 3: SIP(F8)GGC-Ellman's reaction mixture, 4: SIP(F8)GGC-Ellman's after purification, 5: SIP(F8)GGC-Ellman's + CemCH_2-SH, after 10 min at RT, 6: SIP(F8)GGC-SS-CH_2Cem purified. ESI-MS, gel filtration and Biacore data can be found in the manuscript. Identical data was obtained for the products of the reactions of SIP(F8)GGC-SSePh with CemCH_2-SH and of SIP(F8)GGC with CemCH_2-SSPy.

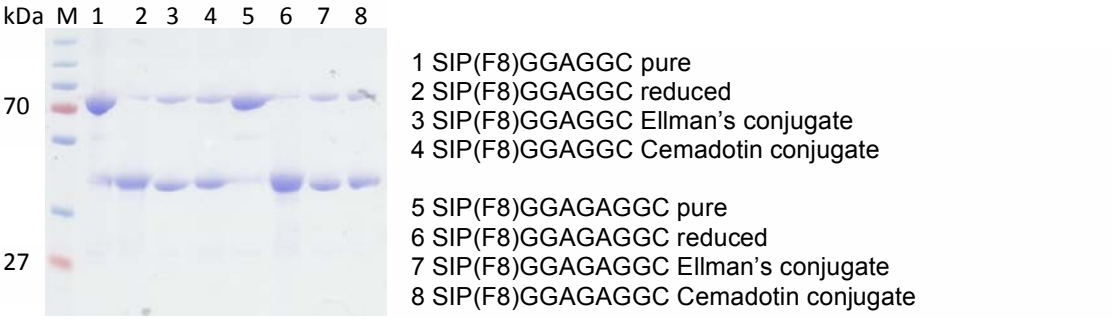


Figure 21. SDS-PAGE analysis of site-specific conjugation of $CemCH_2$-SH to SIP(F8)GGAGGC-Ellman's and SIP(F8)GGAGAGGC-Ellman's. ESI-MS, gel filtration and Biacore data can be found in the manuscript.

4.4 Stability assessment of SIP(F8)GGC-SS-CH_2Cem

The stability of the antibody-drug conjugates (1 mg/mL or 4.8 mg/mL) was assessed as follows: aliquots of 50 µL in 0.5 mL plastic tubes were stored at three different temperatures: -80 ºC, 4 °C and 37 °C. Stability was measured on the day of production and after 1, 3, 7 and 10 days. 45 µL were taken for LC-MS, measured immediately after, and 5 µL were used for SDS-PAGE analysis. SDS samples were stored at -20 °C until the last day of the assay.

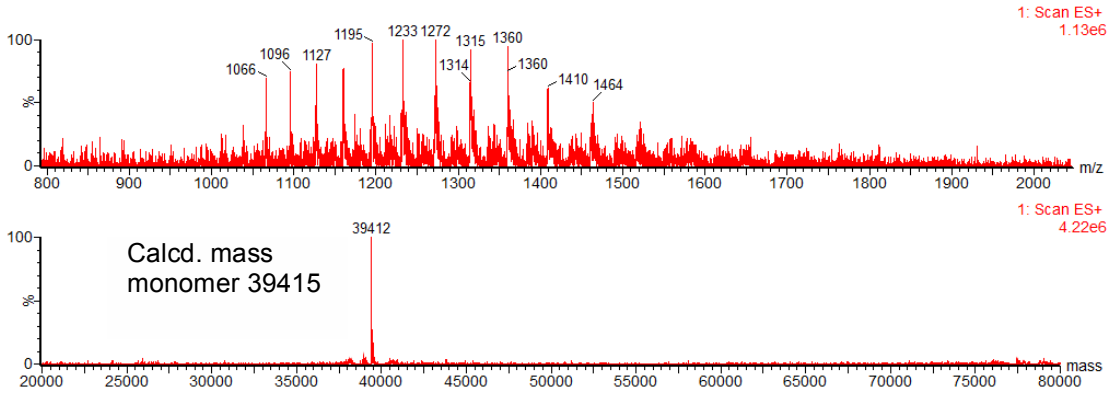
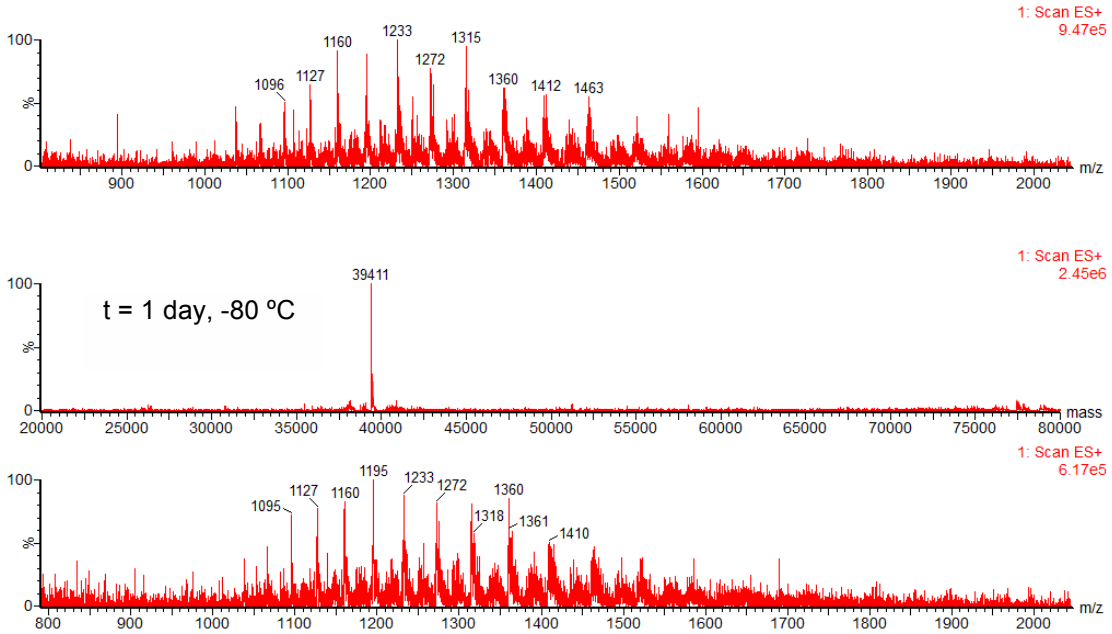
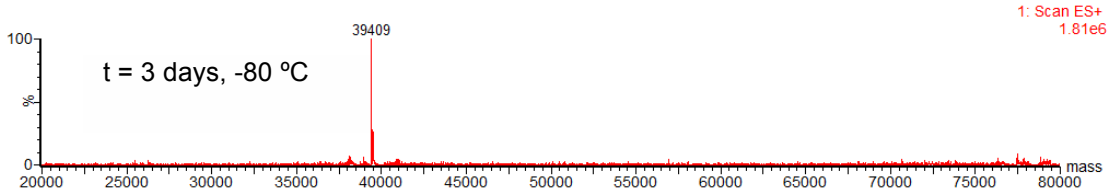


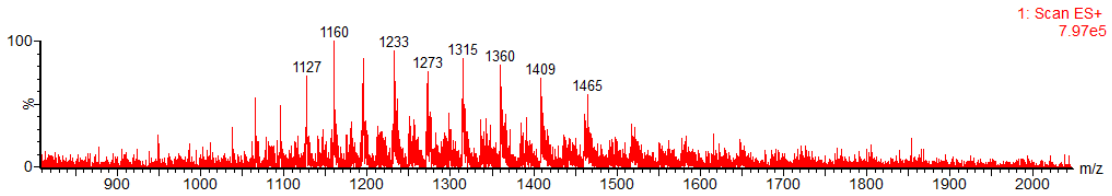
Figure 22. ESI-MS spectrum of SIP(F8)GGC-SS-CH_2Cem at 4.8 mg/mL.

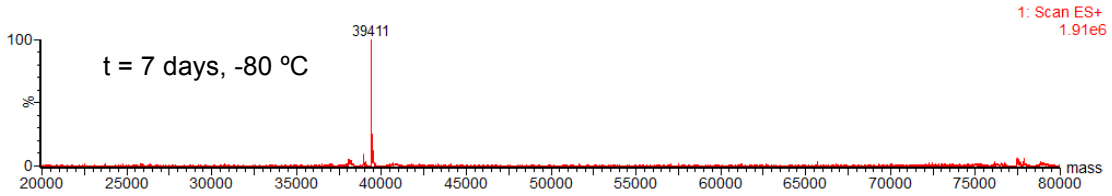
-80 ºC

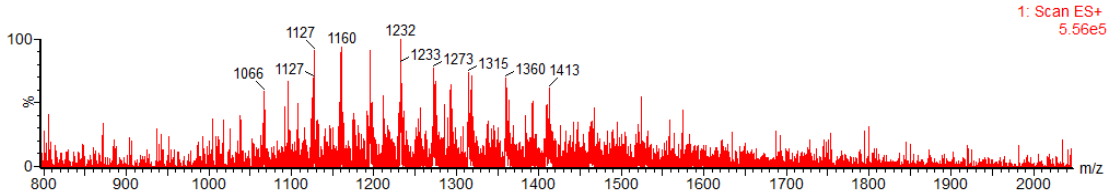


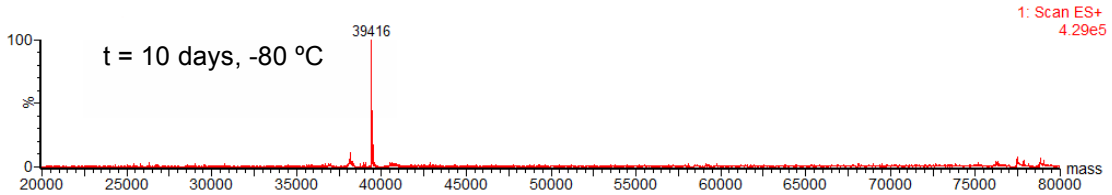
Supporting Information



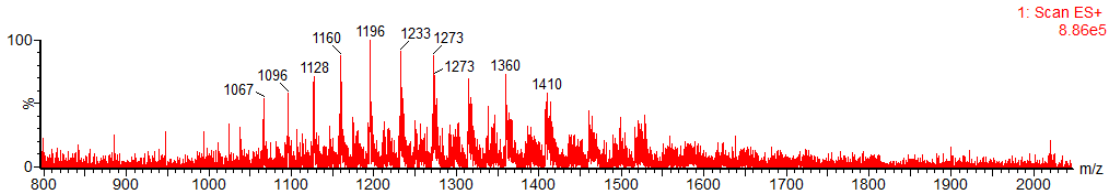


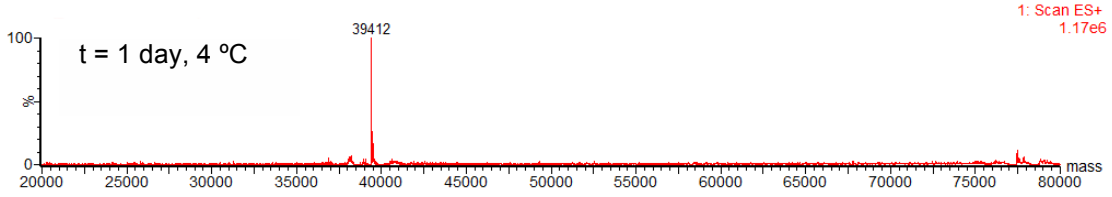




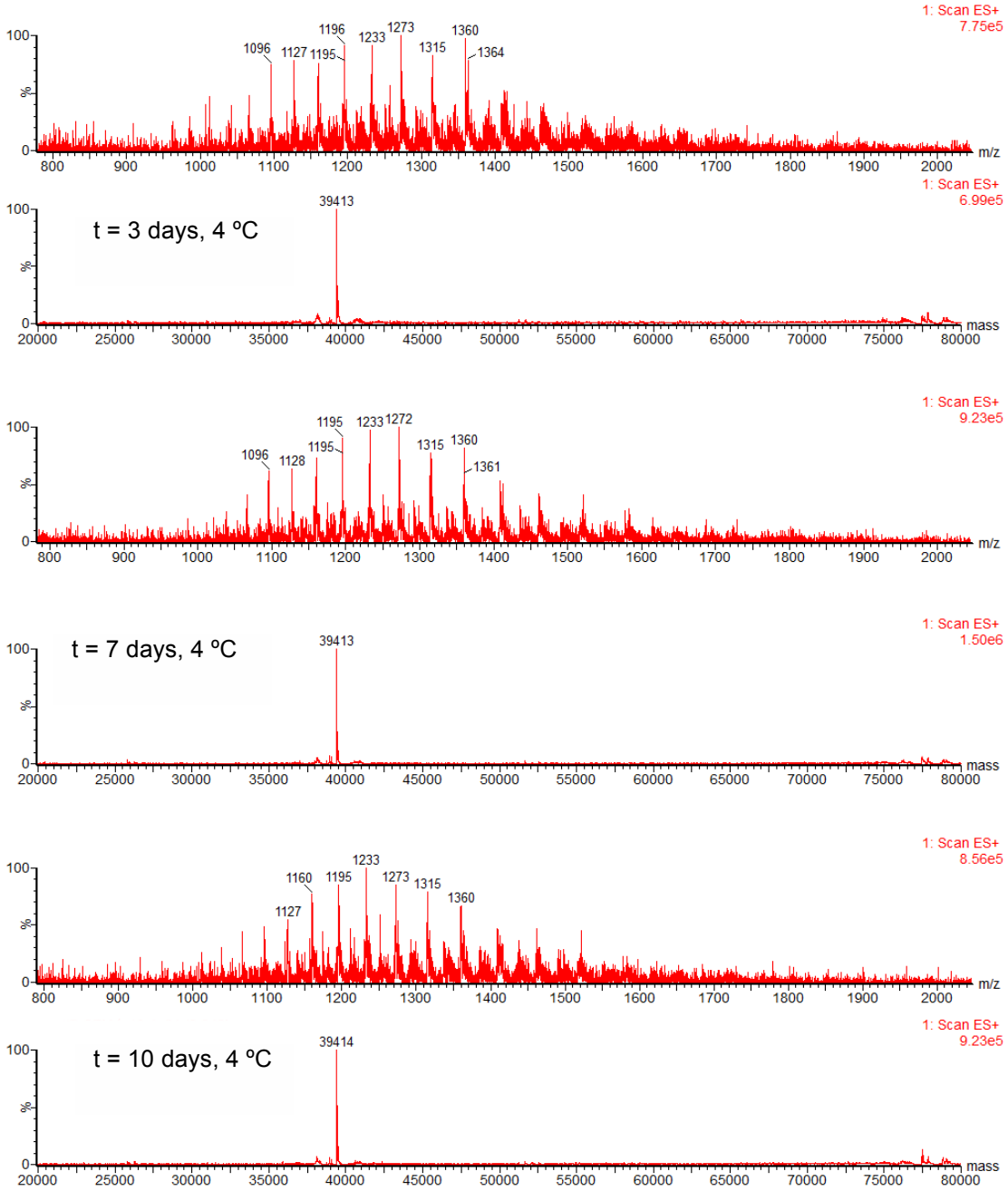


4 ºC





Supporting Information



37 ºC

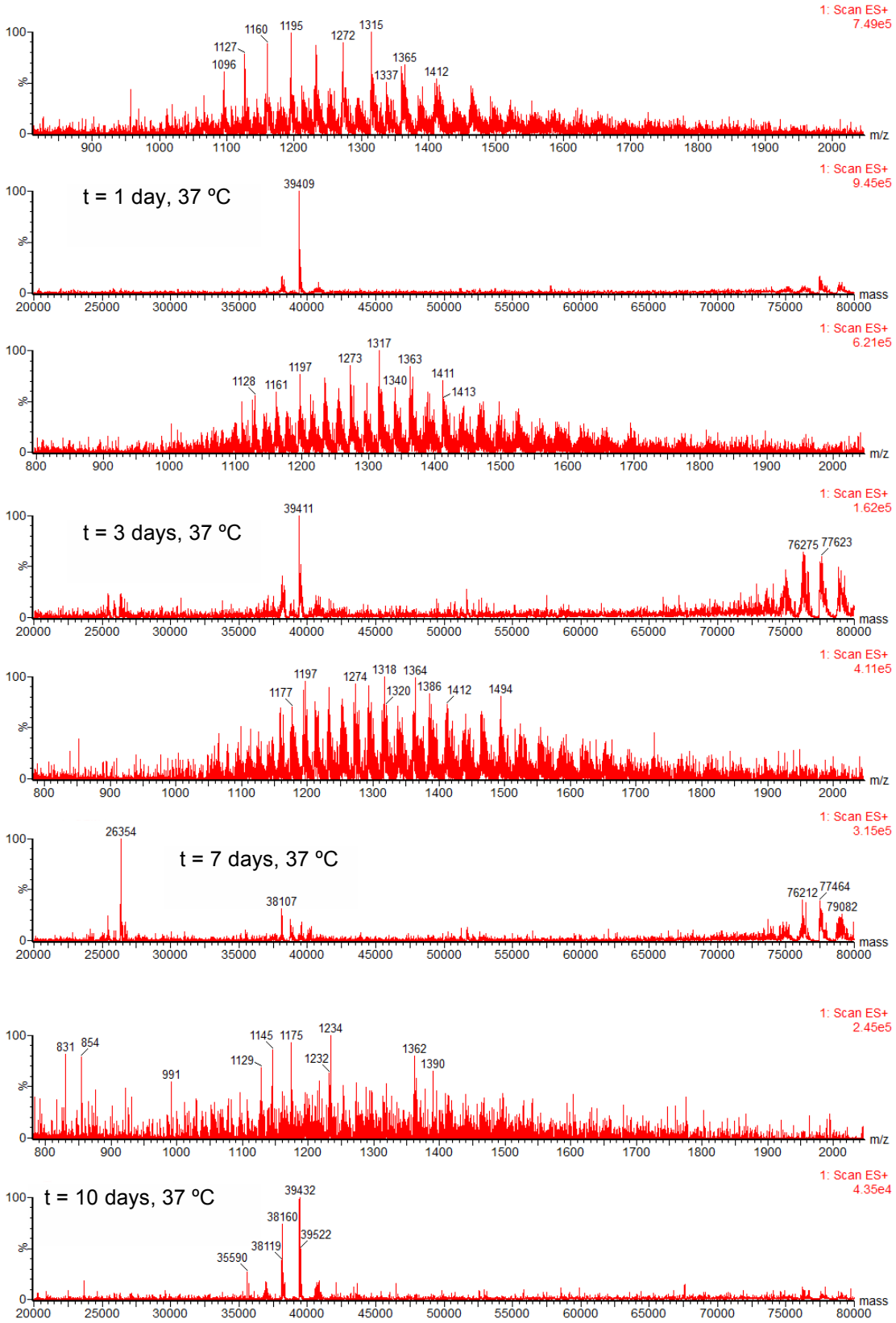


Figure 23. ESI-MS spectra of stability assessment of SIP(F8)GGC-SS-CH_2Cem at 4.8 mg/mL.

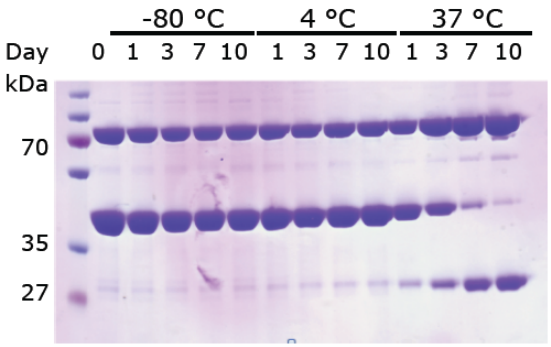


Figure 24. SDS-PAGE of stability of SIP(F8)GGC-SS-CH_2Cem at 4.8 mg/mL

4.5 Stability assessment of SIP(F8)GGAGGC-SS-CH_2Cem

The stability was determined as described under 4.4 p. 35.

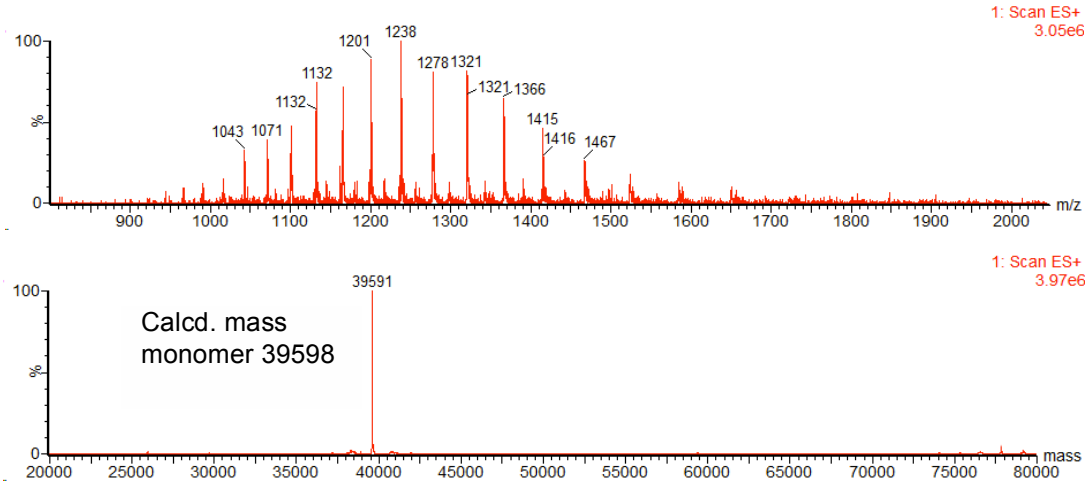
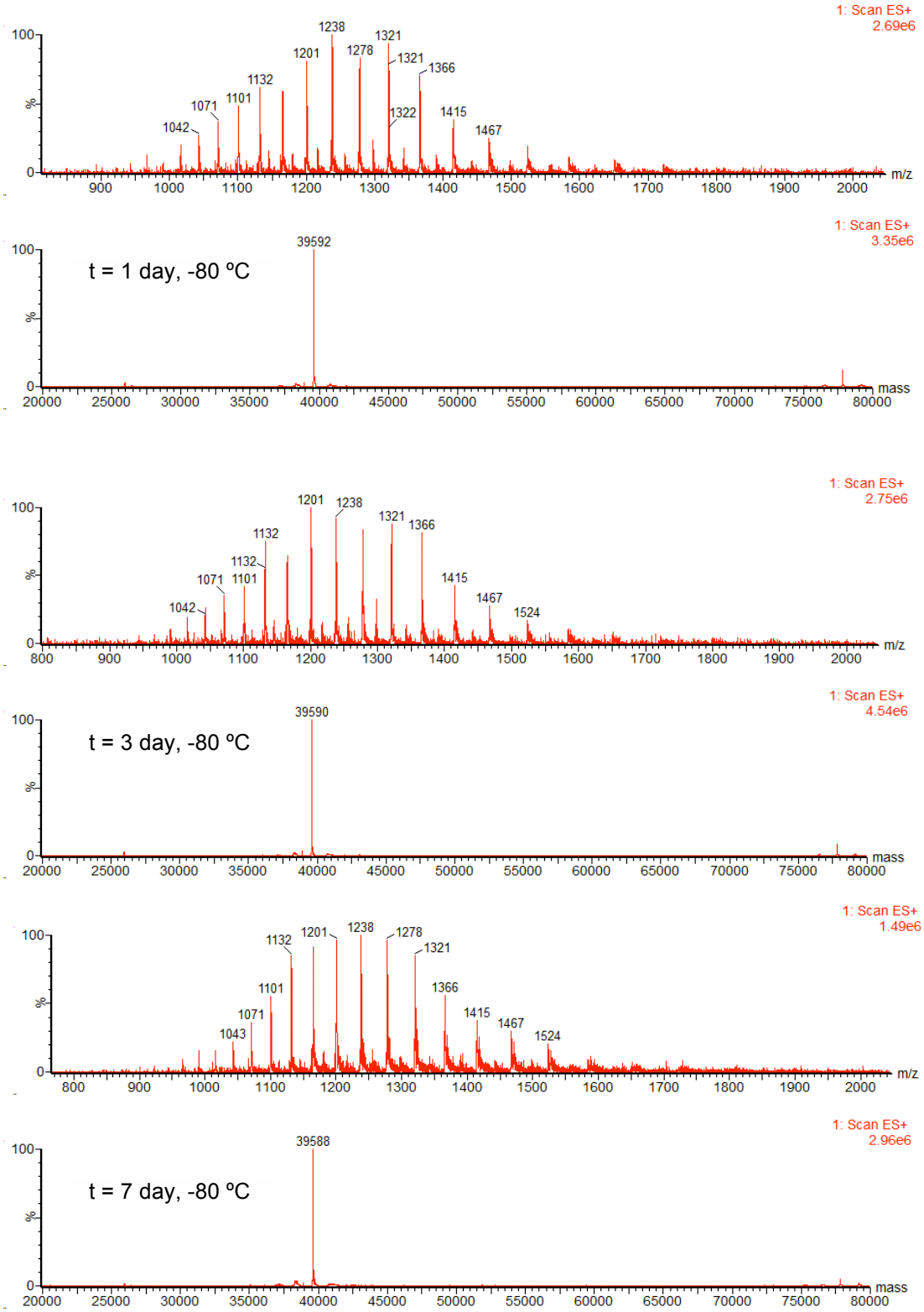


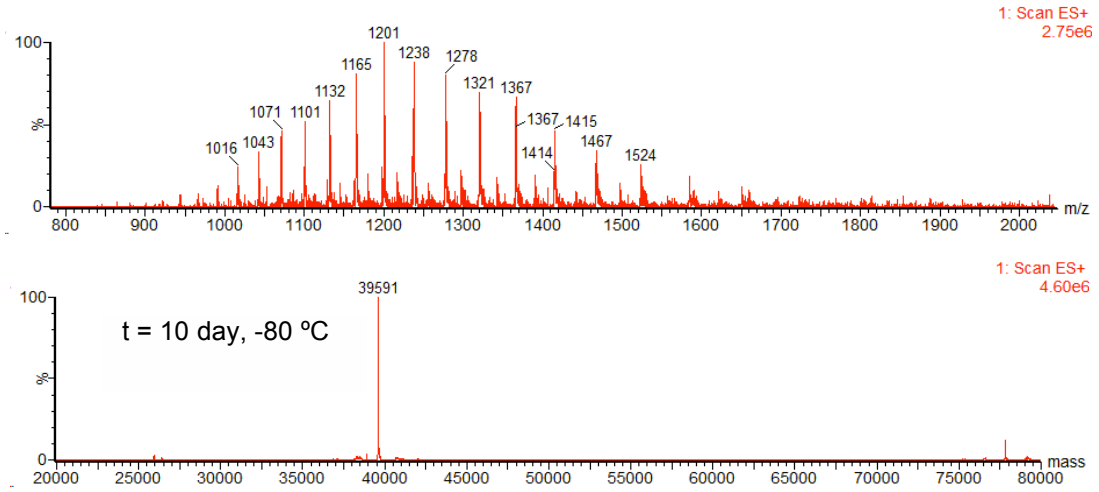
Figure 25. ESI-MS spectrum of SIP(F8)GGAGGC-SS-CH_2Cem at 1 mg/mL.

Supporting Information

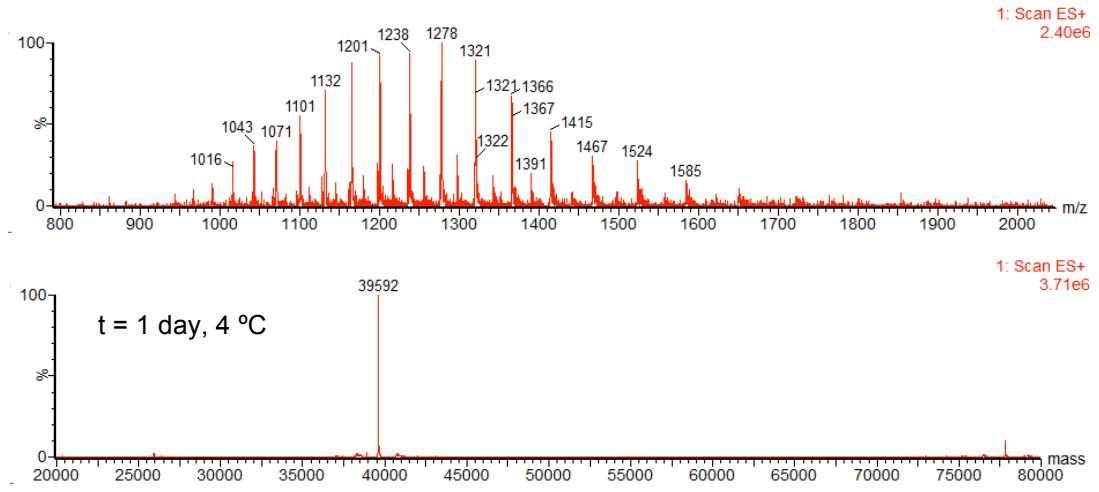
-80 ºC

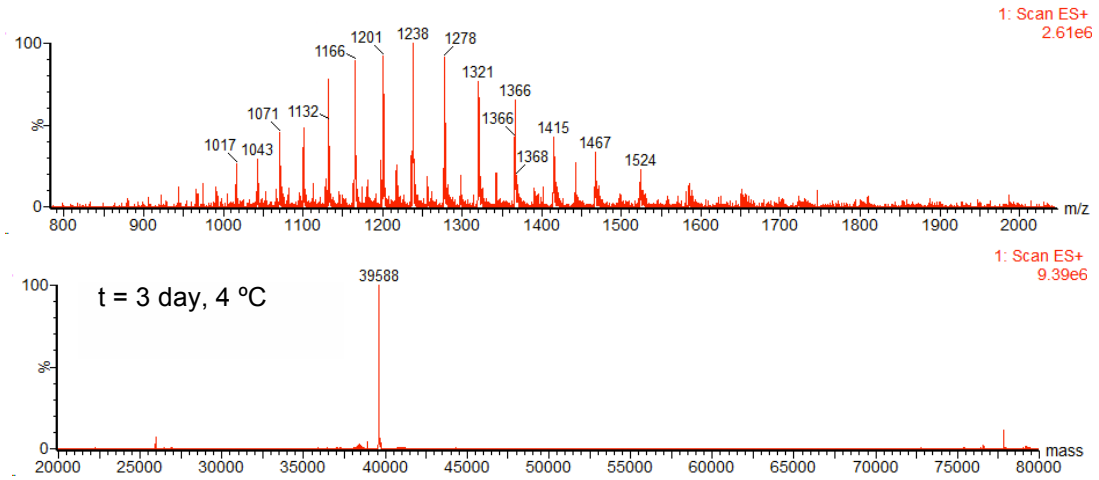


Supporting Information

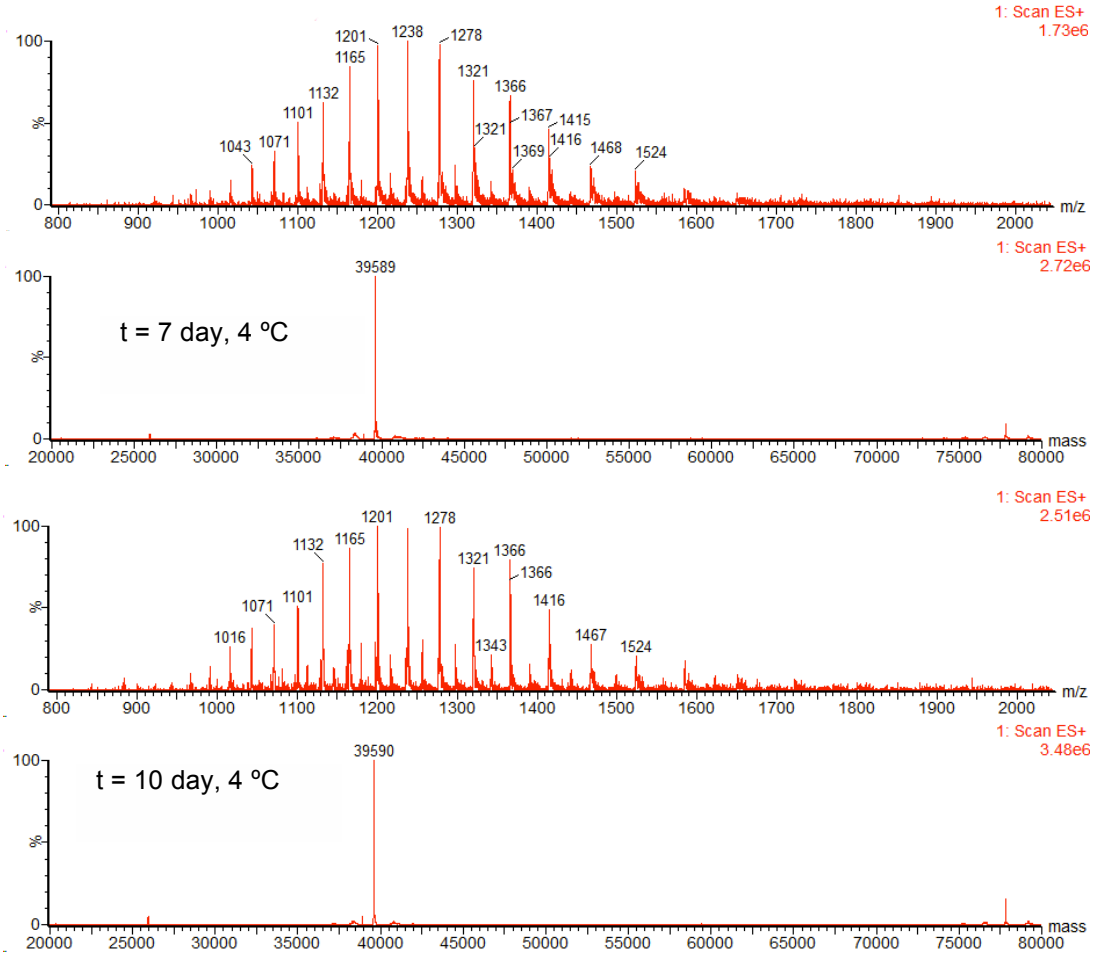


4 ºC

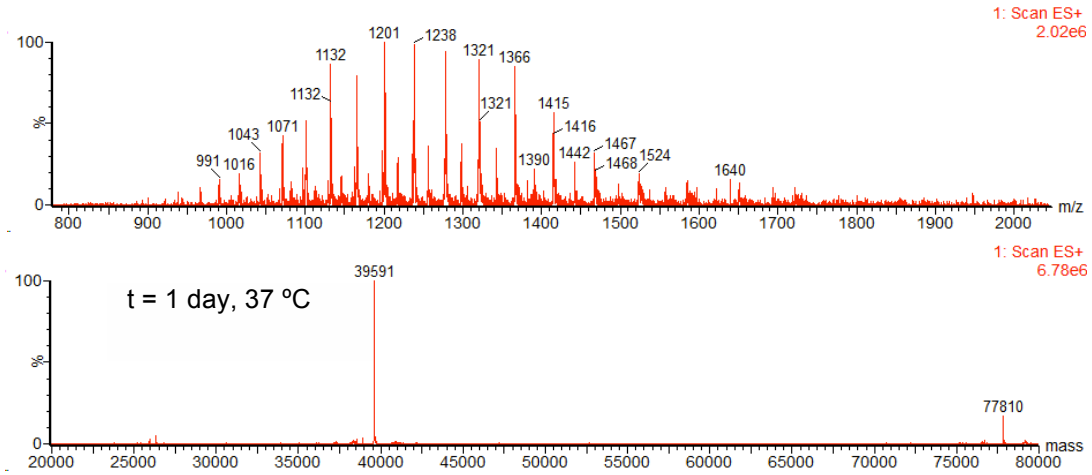




Supporting Information



37 ºC



Supporting Information

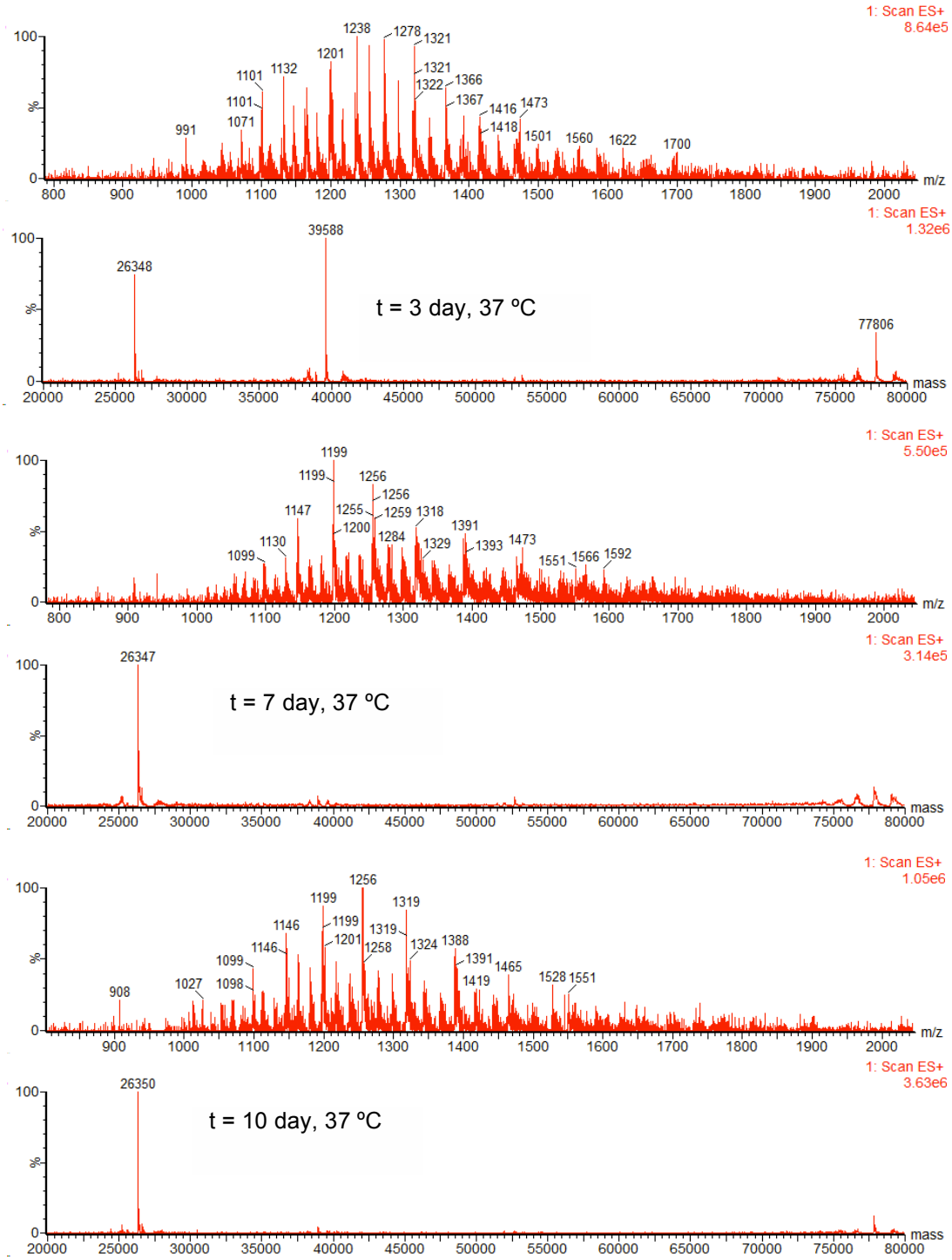


Figure 26. ESI-MS spectra of stability assessment of SIP(F8)GGAGGC-SS-CH_2Cem (1 mg/mL) at -80 ºC, 4 ºC and 37 ºC.

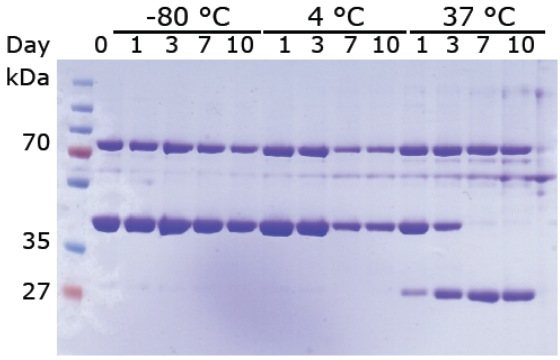


Figure 27. SDS-PAGE of stability of SIP(F8)GGAGGC-SS-CH_2Cem at 1 mg/mL

4.6 Stability assessment of and SIP(F8)GGAGAGGC-SS-CH_2Cem

The stability was determined as described under 4.4 p. 35

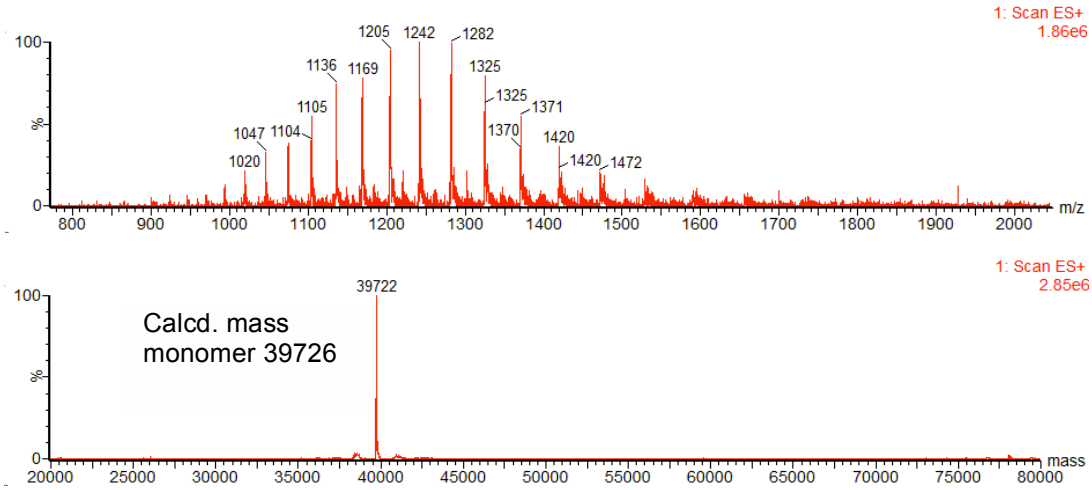
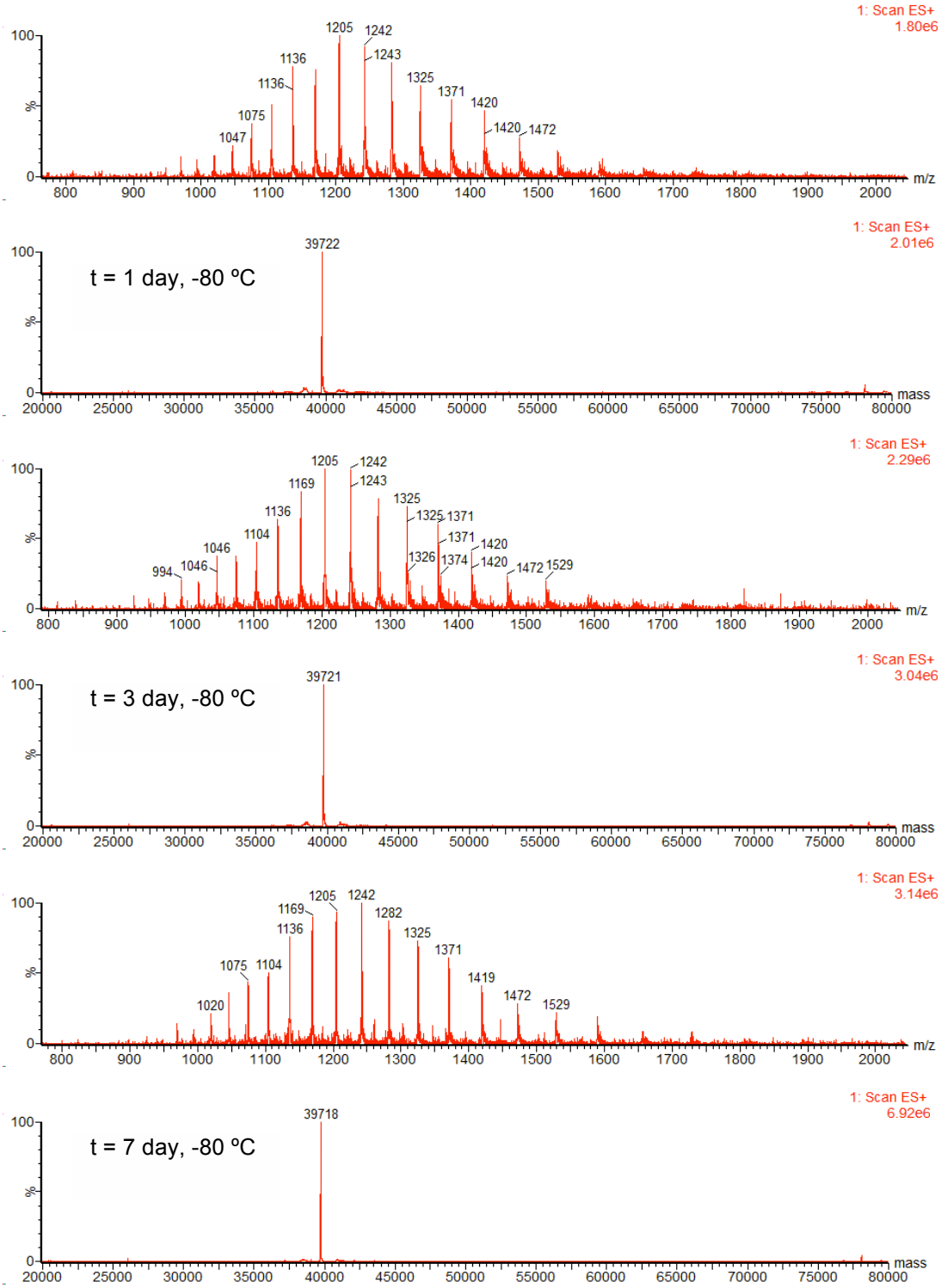
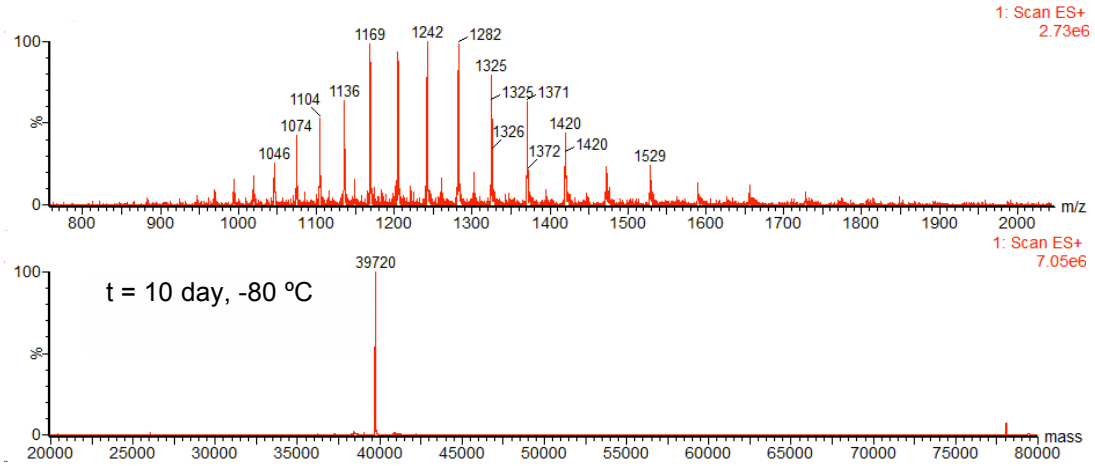


Figure 28. ESI-MS spectrum of SIP(F8)GGAGAGGC-SS-CH_2Cem at 1 mg/mL.

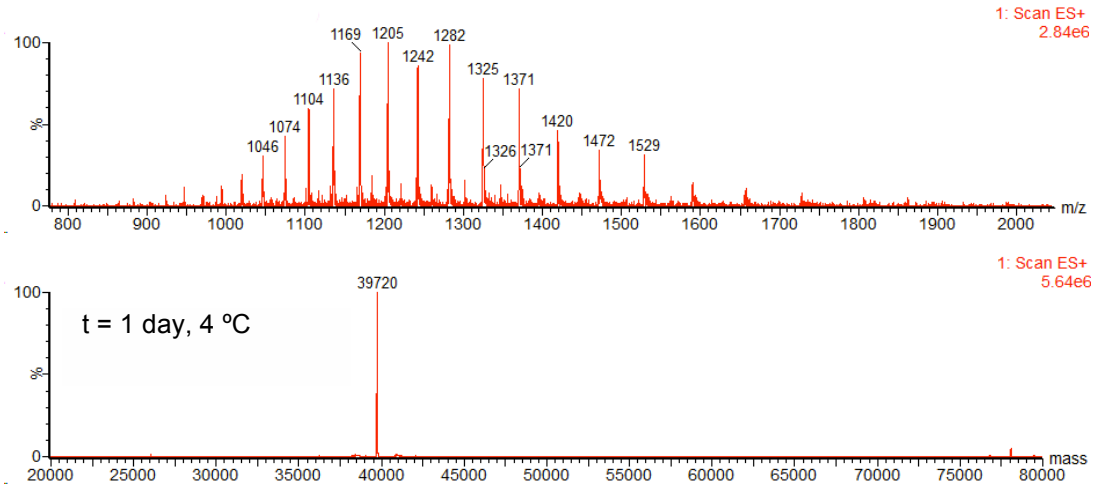
Supporting Information

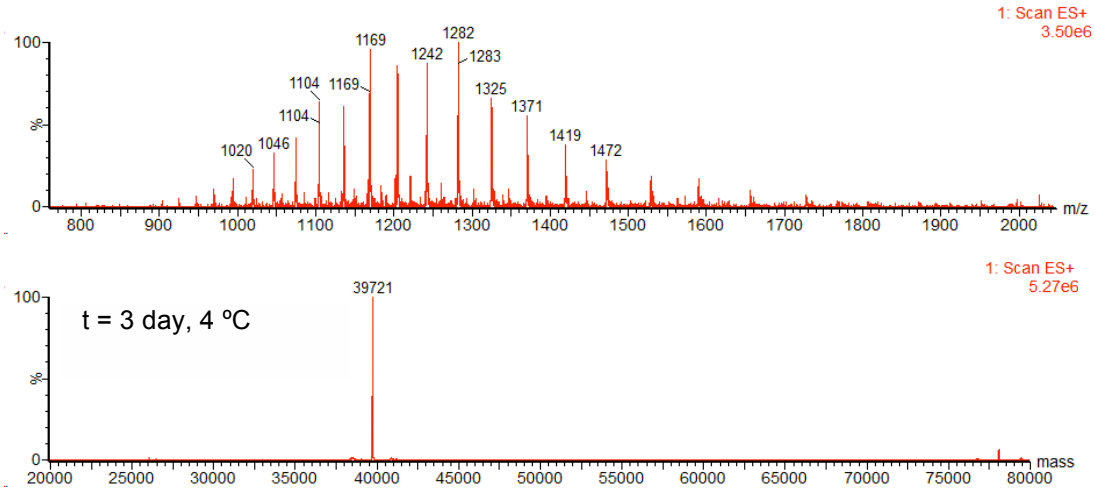
-80 ºC



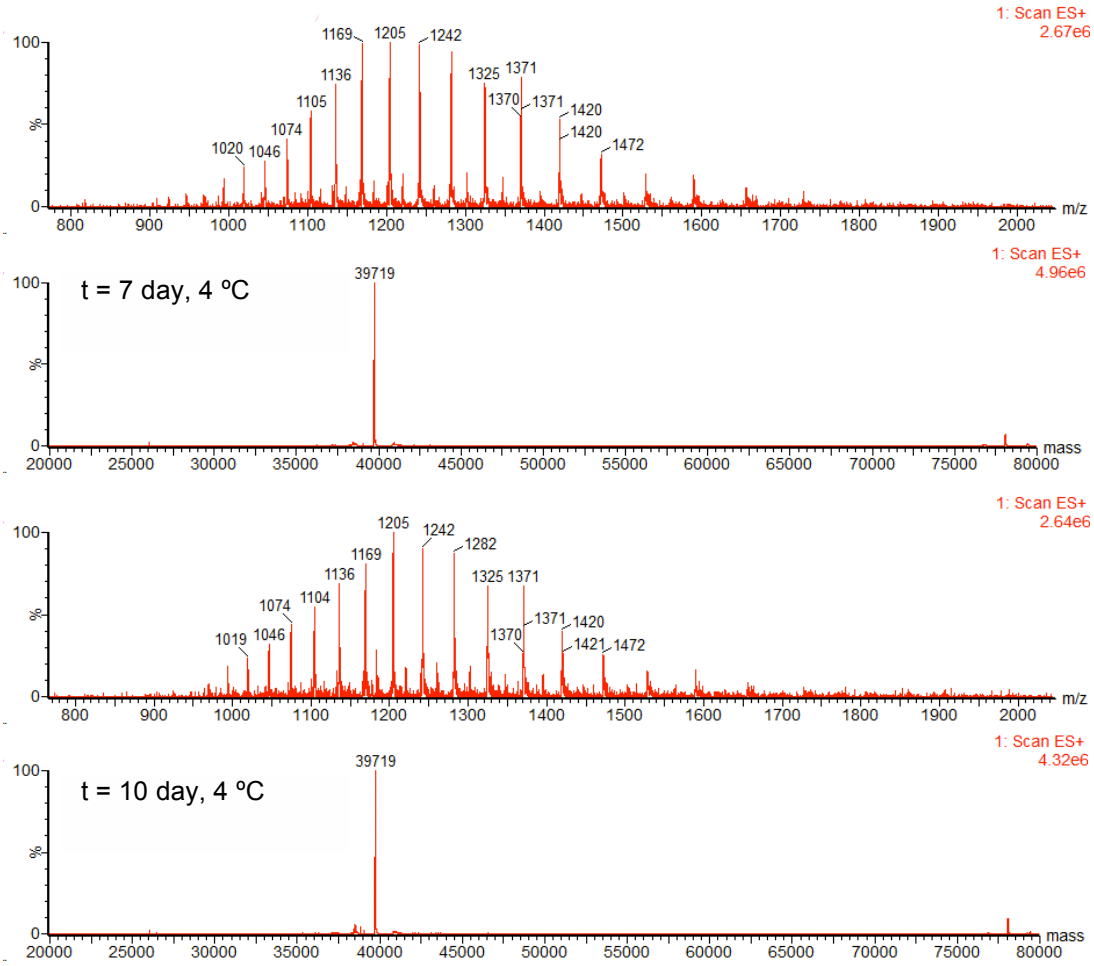


4 ºC



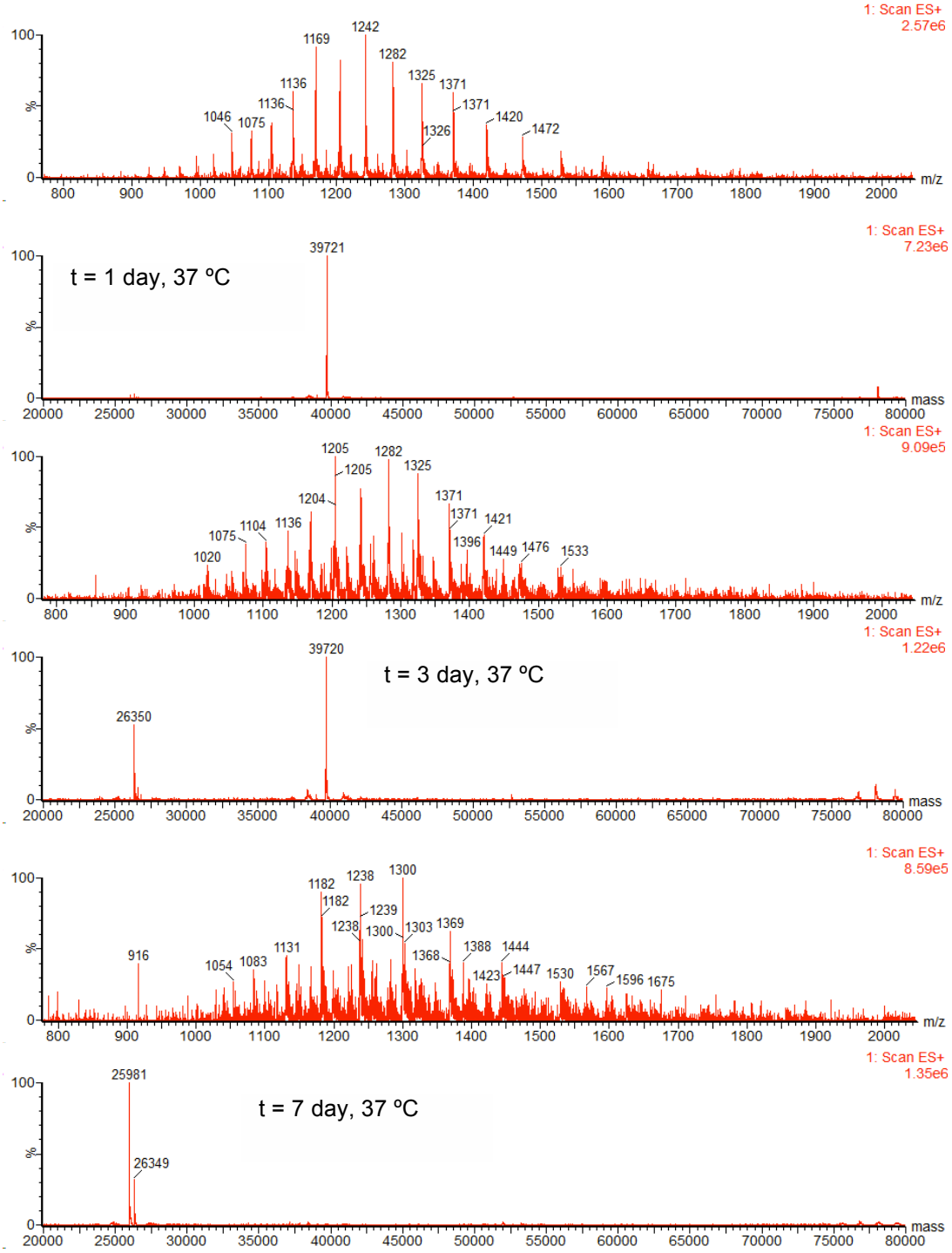


Supporting Information



Supporting Information

37 ºC



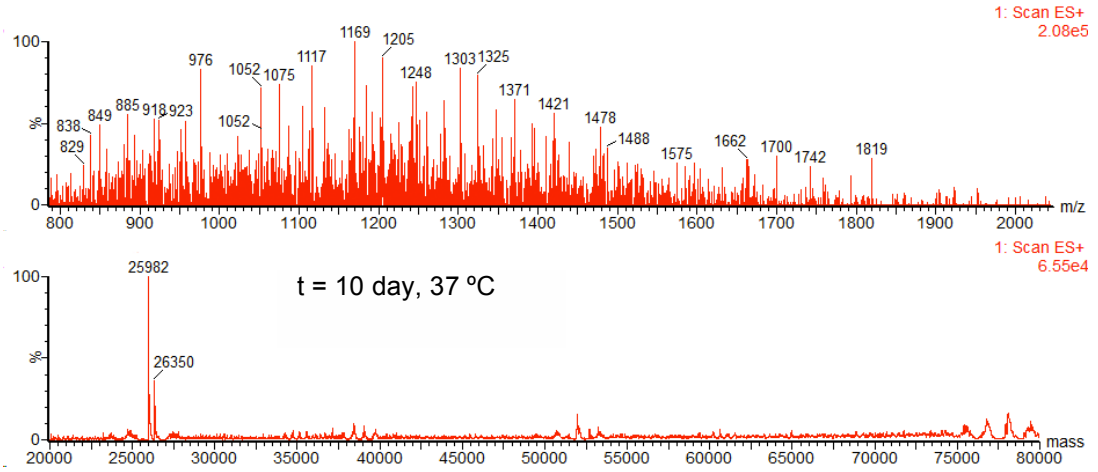


Figure 29. ESI-MS spectra of stability assessment of SIP(F8)GGAGAGGC-SS-CH_2Cem (1 mg/mL) at -80 ºC, 4 ºC and 37 ºC.

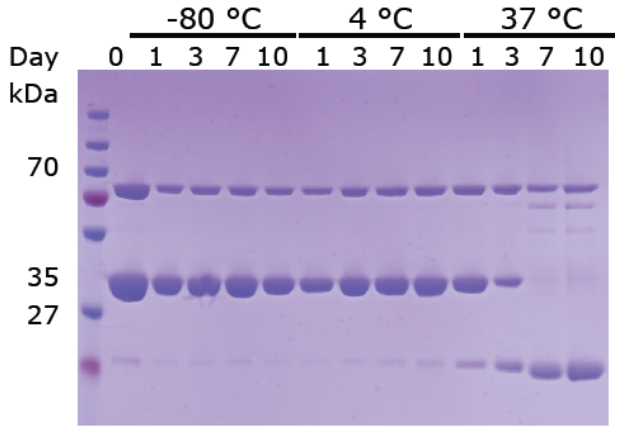


Figure 30. SDS-PAGE of stability of SIP(F8)GGAGAGGC-SS-CH_2Cem at 1 mg/mL

4.7 Assessment of release of Ellman's reagent from SIP(F8)GGC-SS-Ellman's, SIP(F8)GGAGGC-SS-Ellman's, SIP(F8)GGAGAGGC-SS-Ellman's in the presence of DTT

Antibody-Ellman's conjugates were prepared as described above, but the FPLC was run with PBS pH 6.0 rather than pH 7.4. 80 µL of Ellman's conjugates were mixed with 8 µL of a freshly prepared DTT solution in PBS pH 6.0. The amount of the free Ellman's reagent was measured by following the absorption at 412 nm (Cary300 UV-visible spectrophotometer, Varian) (**Figure 31** b, c & d). Data from 10 to 30 sec upon DTT addition were fitted linearly (GraphPad Prism, version 5). The slope of the

corresponding linear equation was plotted depending on the ratio of DTT to conjugate (**Figure 31** a).

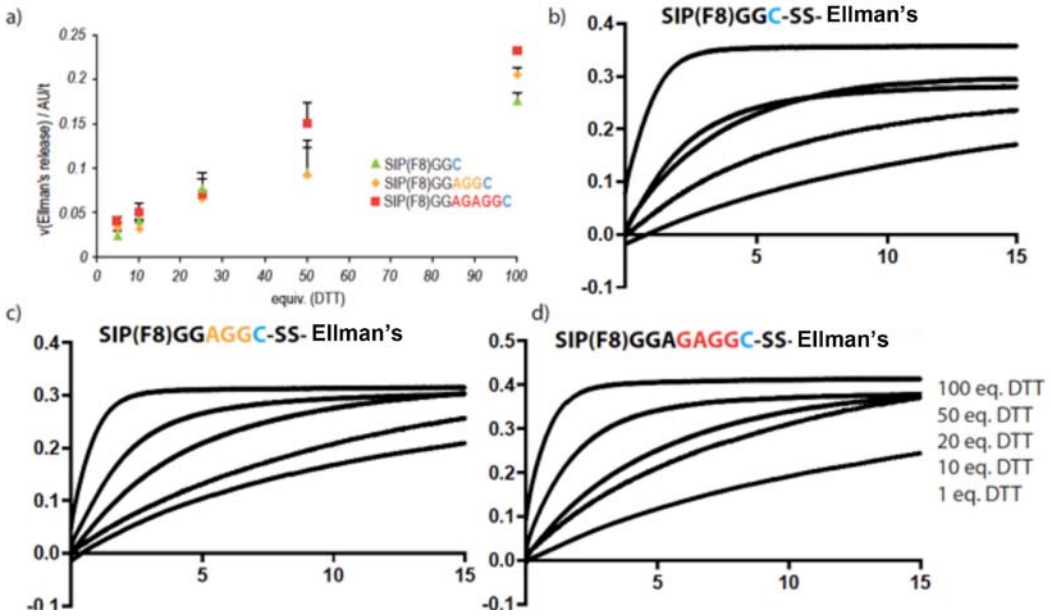


Figure 31. Release of Ellman's reagent from antibody-Ellman's conjugates. Absorption at 412 nm over time was measured for 1, 10, 20, 50 and 100 molar equivalents of DTT over b) SIP(F8)GGC-SS-Ellman's, c) SIP(F8)GGAGGC-SS-Ellman's and d) SIP(F8)GGAGAGGC-SS-Ellman's. a) The slope of the initial velocity was plotted versus the equivalents of DTT.

4.8 Assessment of the reactivity of SIP(F8)GGC-SS-CH_2Cem, SIP(F8)GGAGGC-SS-CH_2Cem, SIP(F8)GGAGAGGC-SS-CH_2Cem in the presence of reduced glutathione

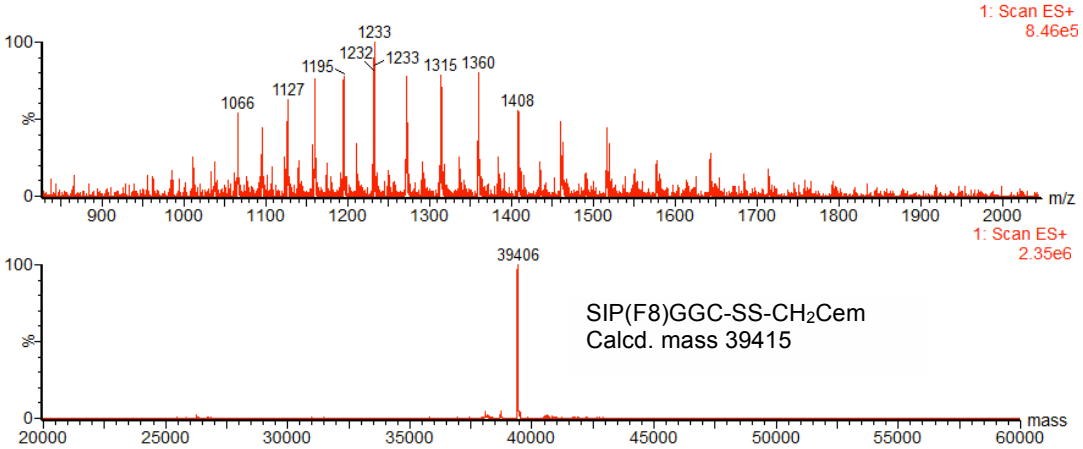
Reactivity of the ADCs (5 mg/mL) in the presence of different amounts of physiological reducing agent glutathione (GSH) was assessed as follows: aliquots of 25 µL in 0.5 mL plastic tubes were incubated at 37 ºC for 10 minutes with 10 µL of reduced glutathione, that was at a final concentration of: 0 mm, 0.9 mm, 1.8 mm, 2.7 mm, 3.6 mm, 4.5 mm and 9 mm. 35 µL aliquots were analysed immediately after the incubation by LC-MS.

Supporting Information

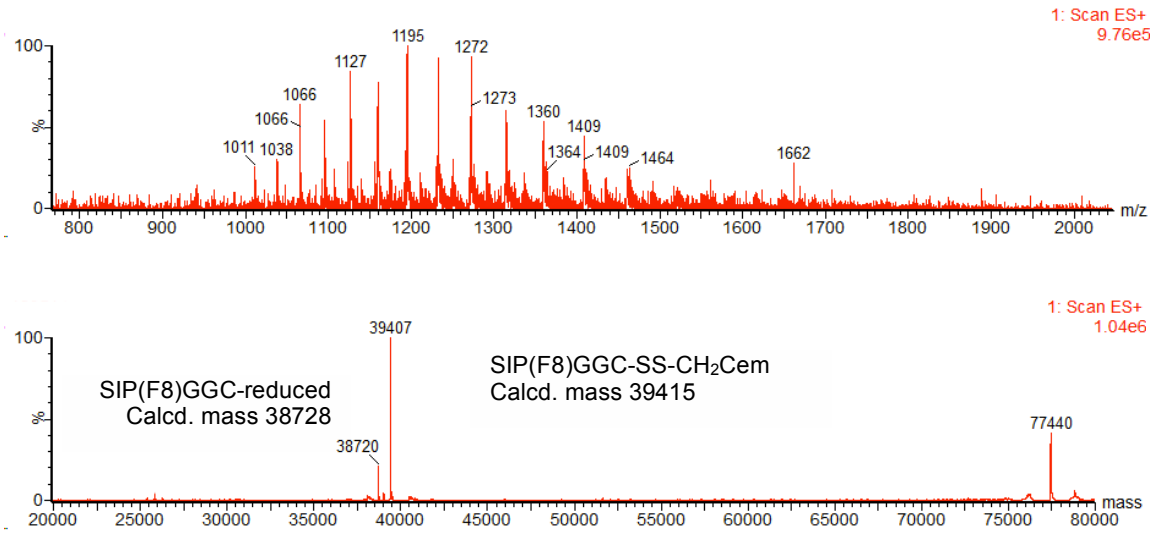
[GSH]	Non-reduced ADC (%)		
	SIP(F8)GGC	SIP(F8)GGAGGC	SIP(F8)GGAGAGGC
0	100	100	100
0.9	83	80	60
1.8	71	67	62
2.7	67	56	33
3.6	56	37	30
4.5	39	36	23
9	0	0	0

SIP(F8)GGC-SS-CH_2Cem incubated at 37 ºC for 10 minutes with:

0 mm GSH

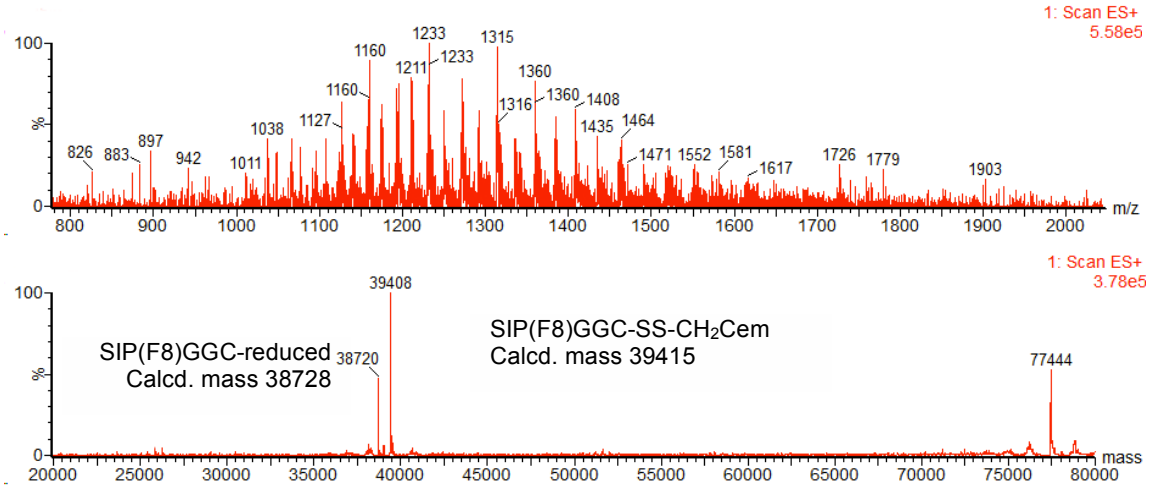


0.9 mm GSH

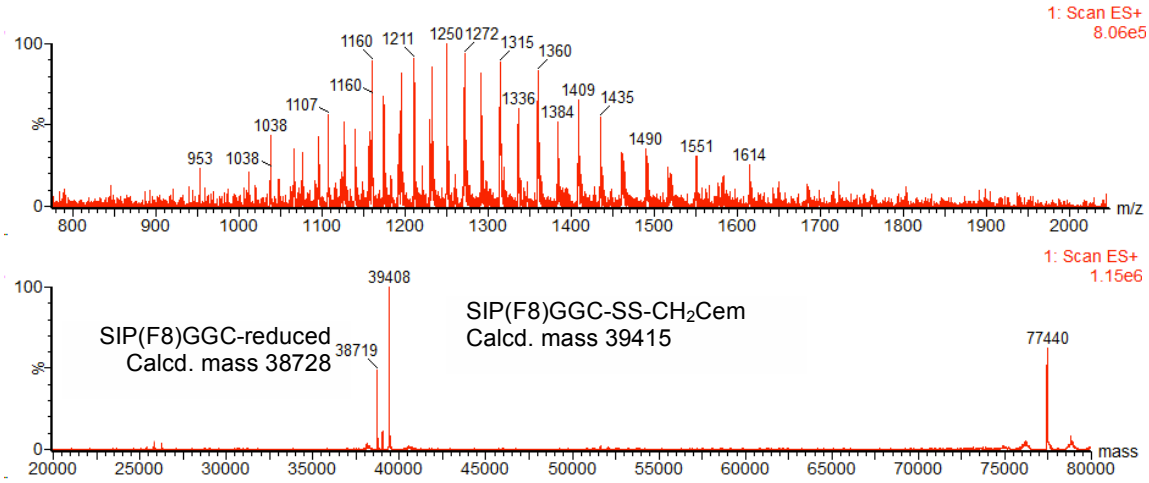


Supporting Information

1.8 mm GSH



2.7 mm GSH



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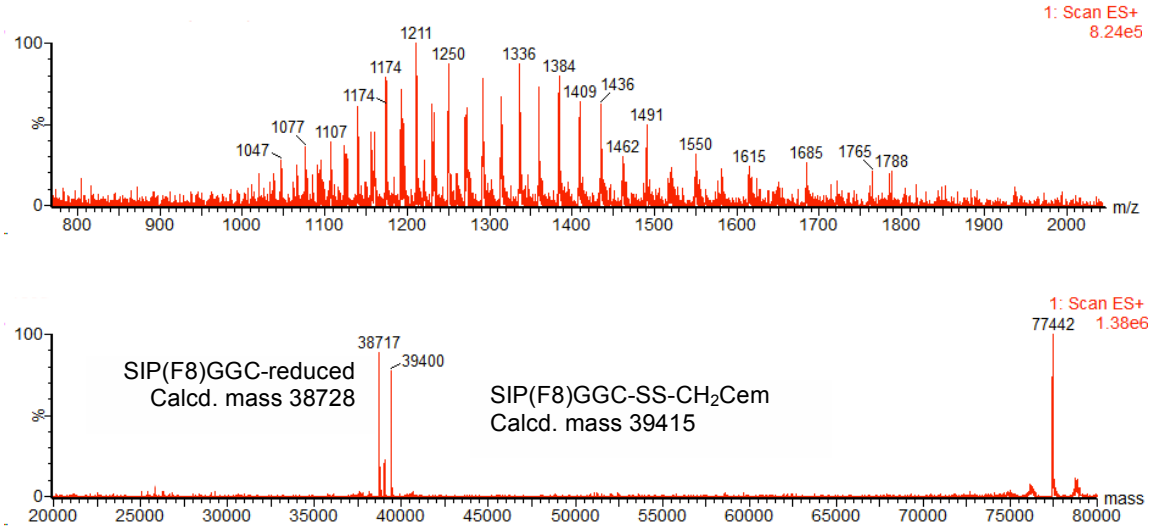
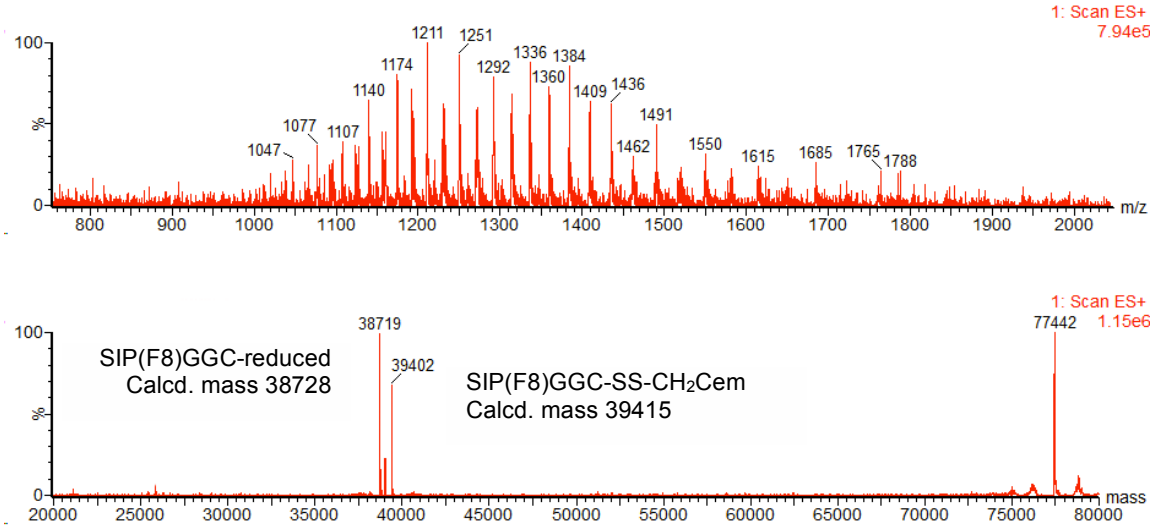


4.5 mm GSH



Supporting Information

9 mm GSH

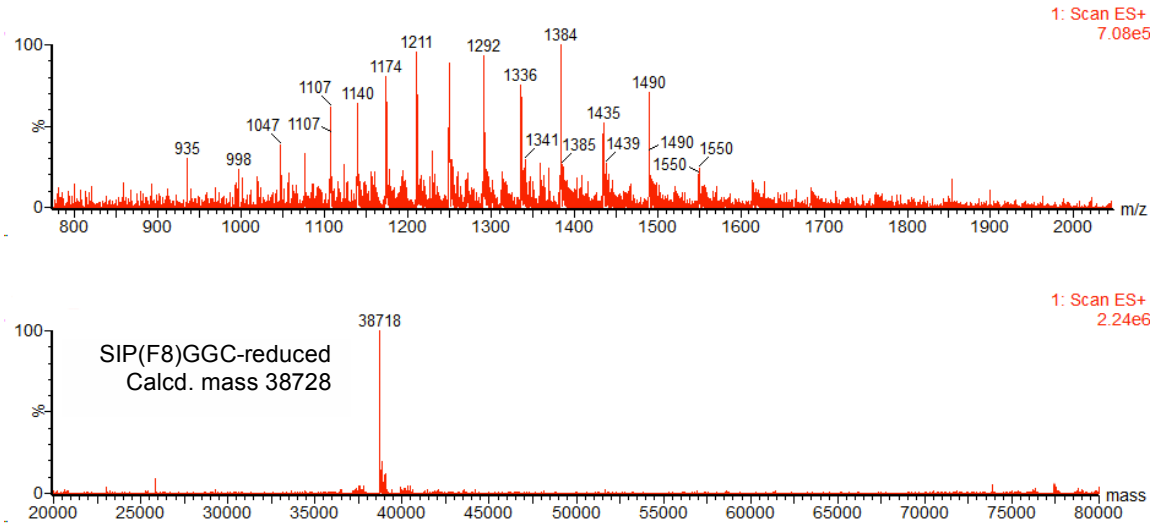
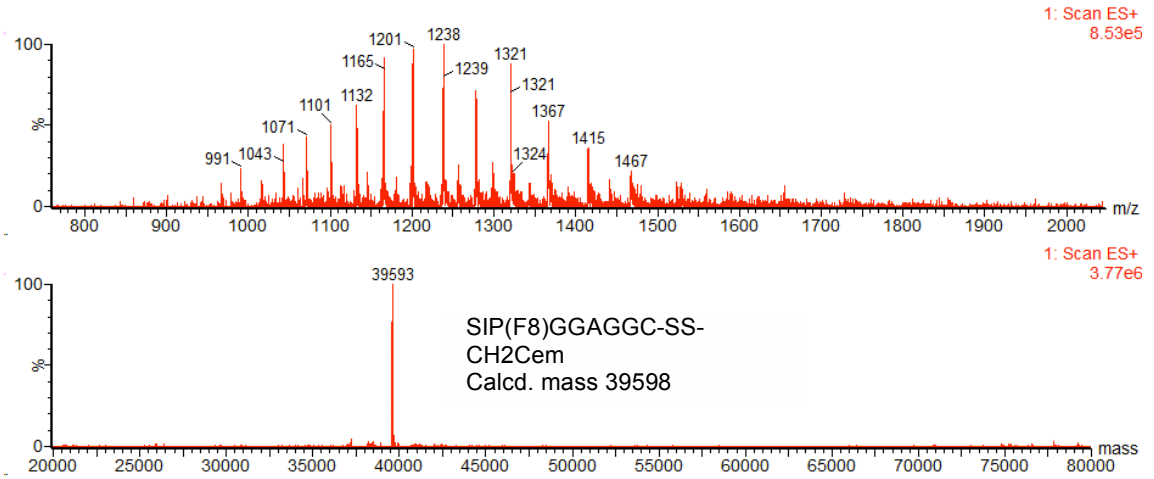


Figure 32. ESI-MS spectra of SIP(F8)GGC-SS-CH_2Cem at 5 mg/mL after incubation at 37 ºC for 10 minutes with different concentrations of reduced glutathione.

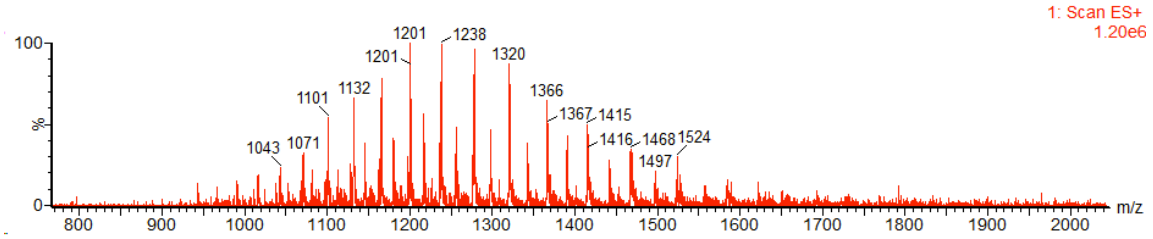
SIP(F8)GGAGGC-SS-CH_2Cem incubated at 37 ºC for 10 minutes with:

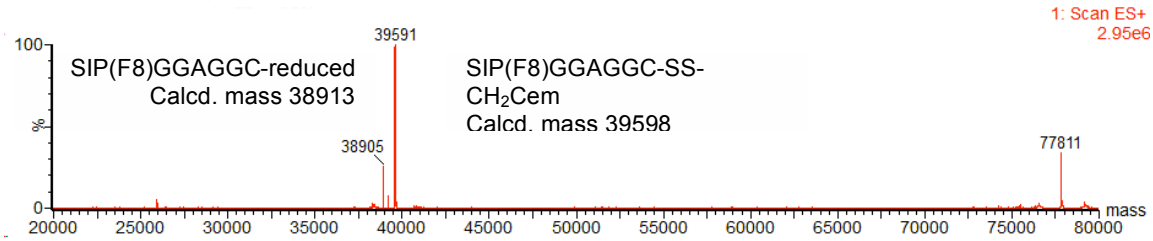
0 mm GSH



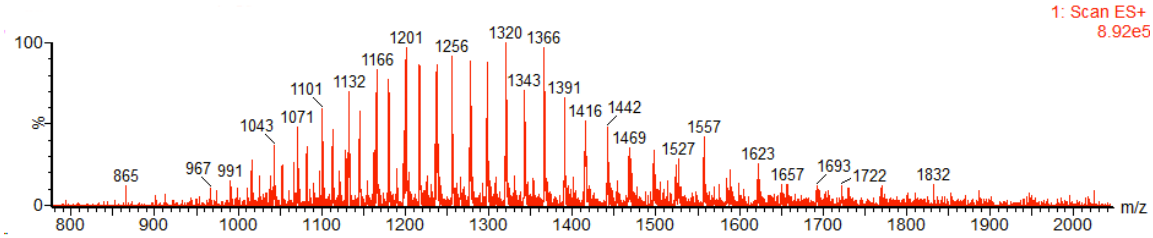
Supporting Information

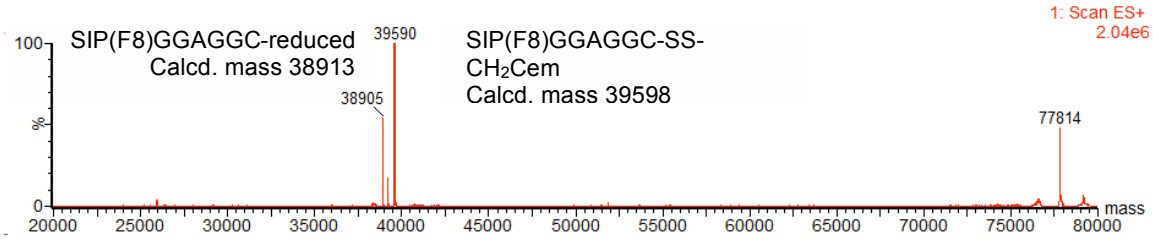
0.9 mm GSH



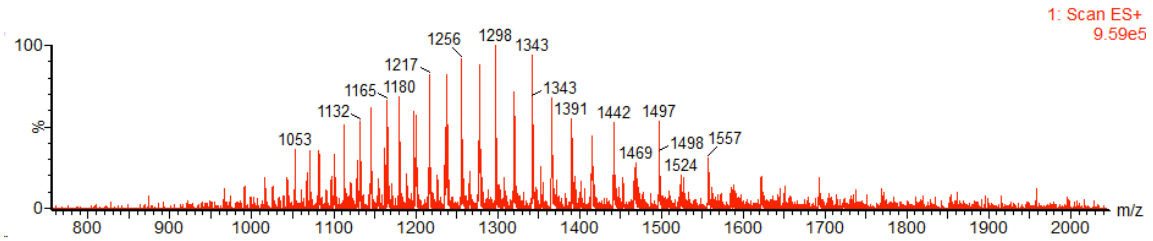


1.8 mm GSH





2.7 mm GSH



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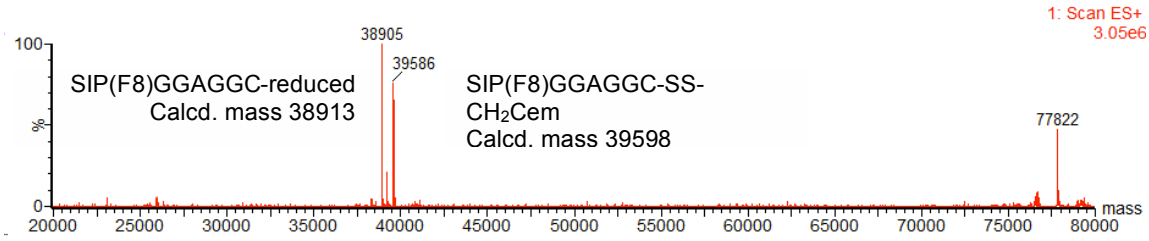
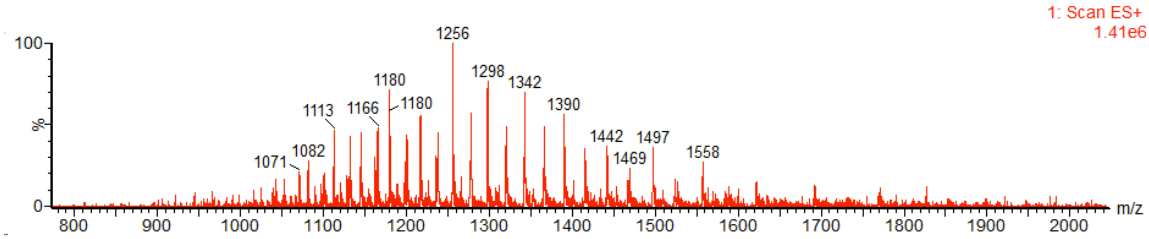
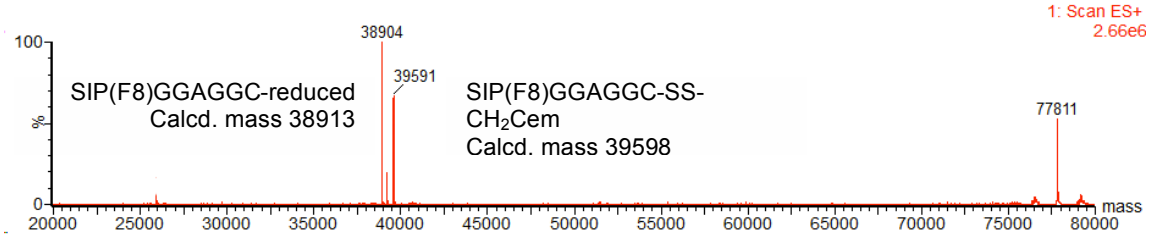
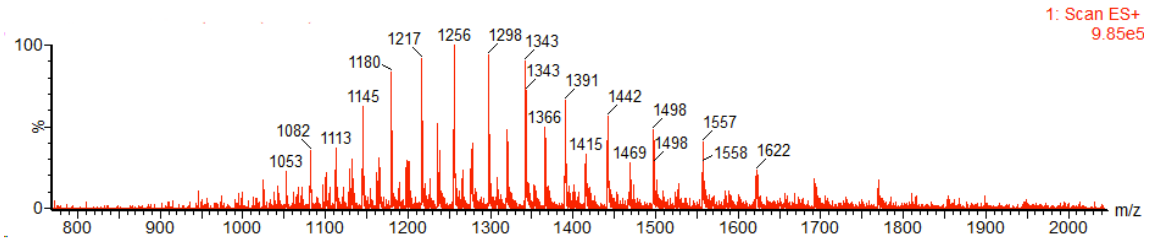


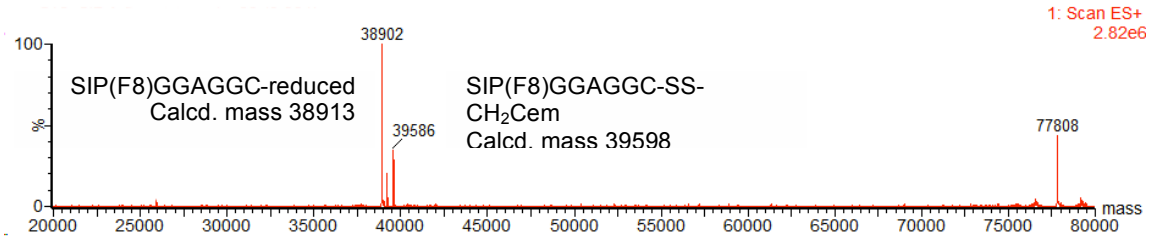
3.6 mm GSH





4.5 mm GSH





Supporting Information

9 mm GSH

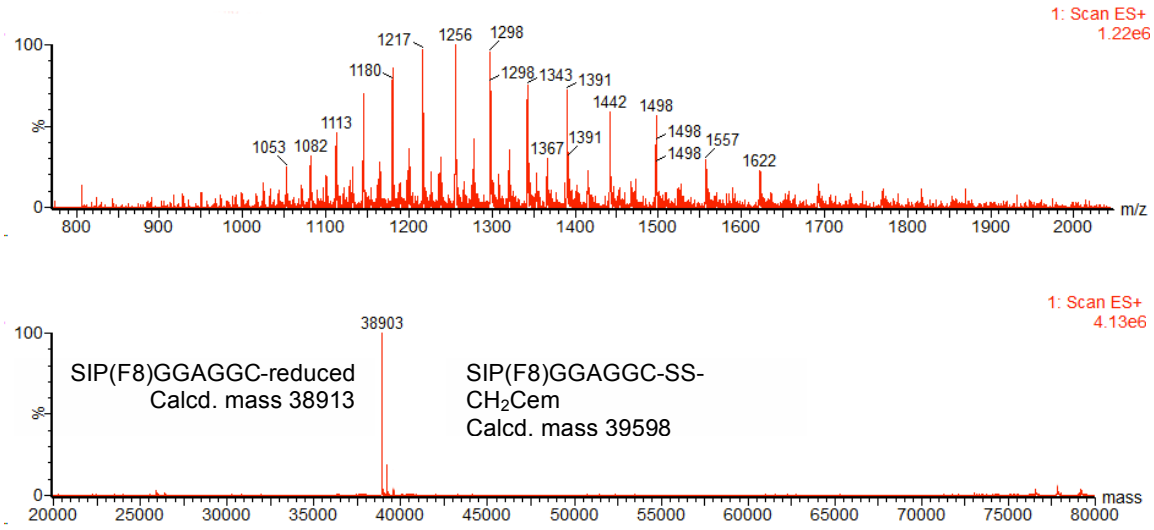
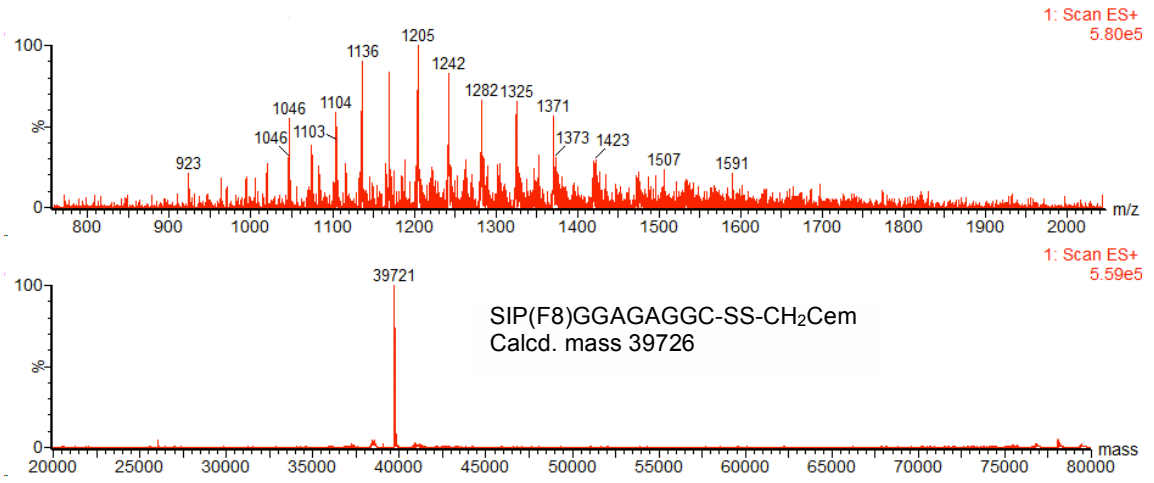


Figure 33. ESI-MS spectra of SIP(F8)GGAGGC-SS-CH_2Cem at 5 mg/mL after incubation at 37 ºC for 10 minutes with different concentrations of reduced glutathione.

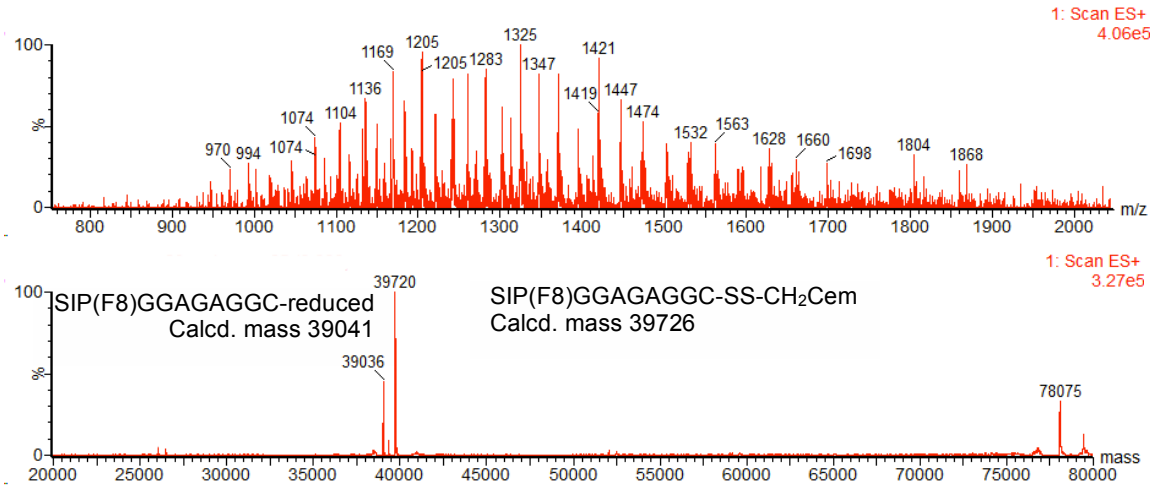
SIP(F8)GGAGAGGC-SS-CH_2Cem incubated at 37 ºC for 10 minutes with:

0 mm GSH

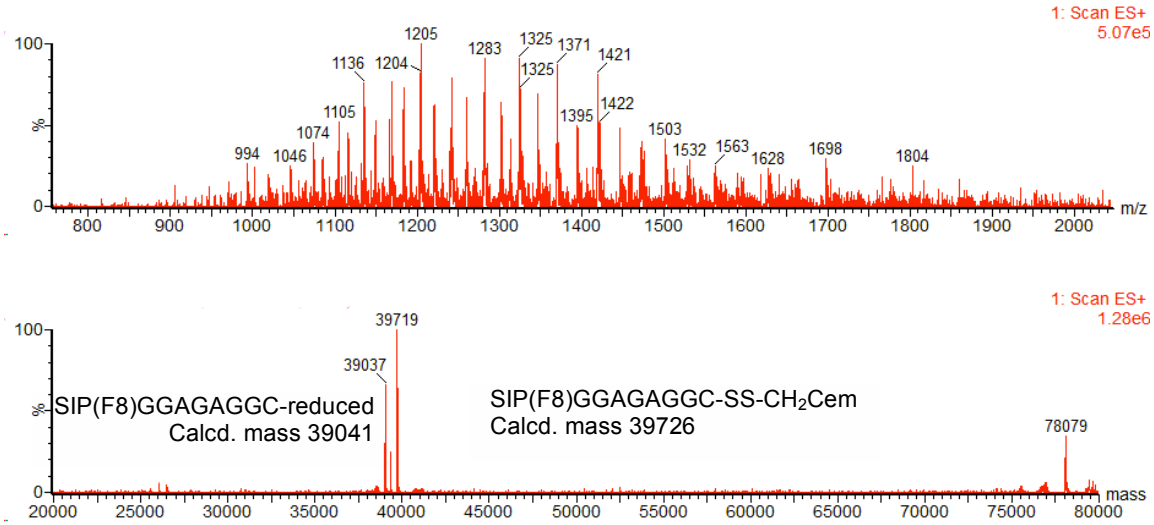


Supporting Information

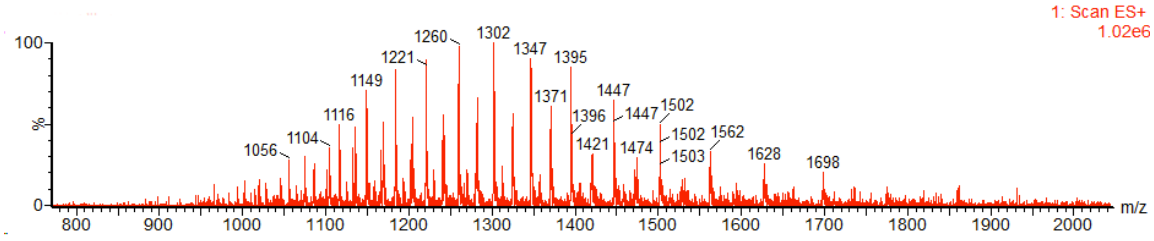
0.9 mm GSH



1.8 mm GSH



2.7 mm GSH



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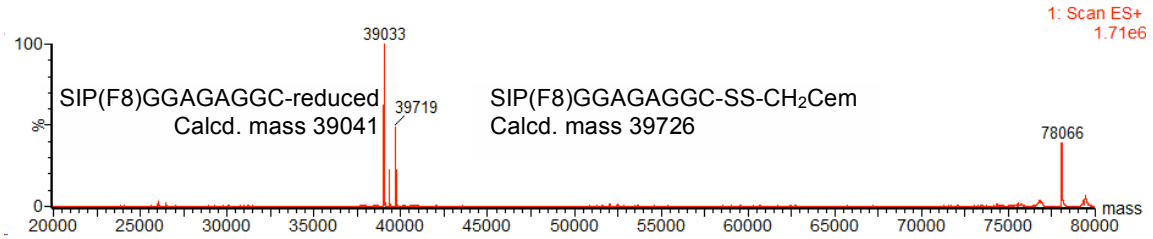
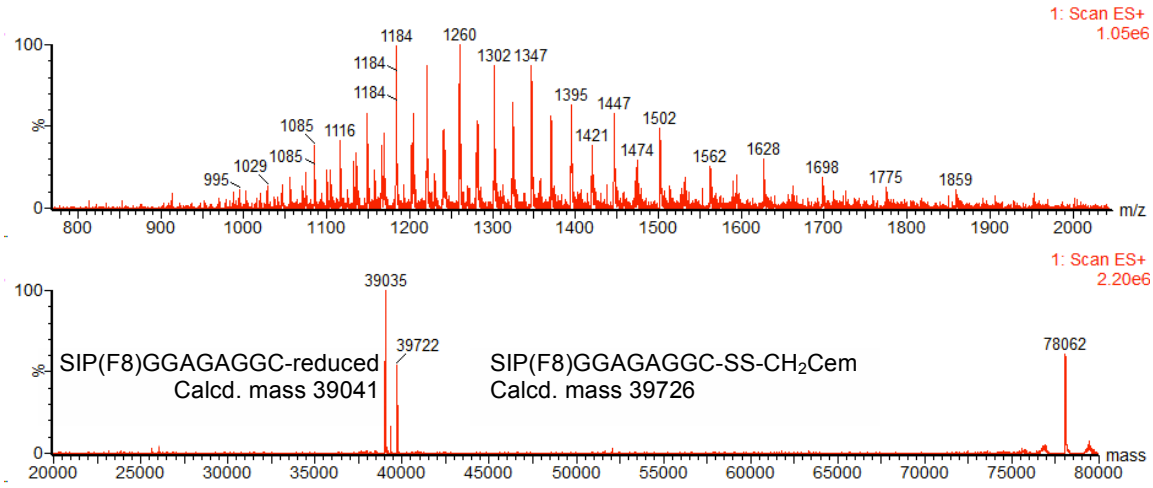
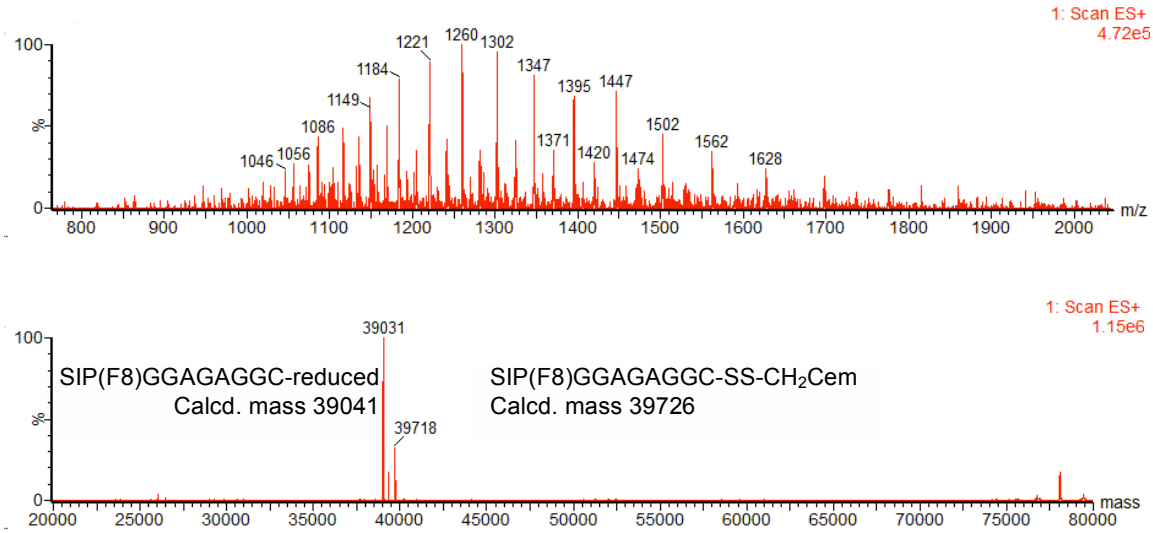


3.6 mm GSH



4.5 mm GSH



9 mm GSH

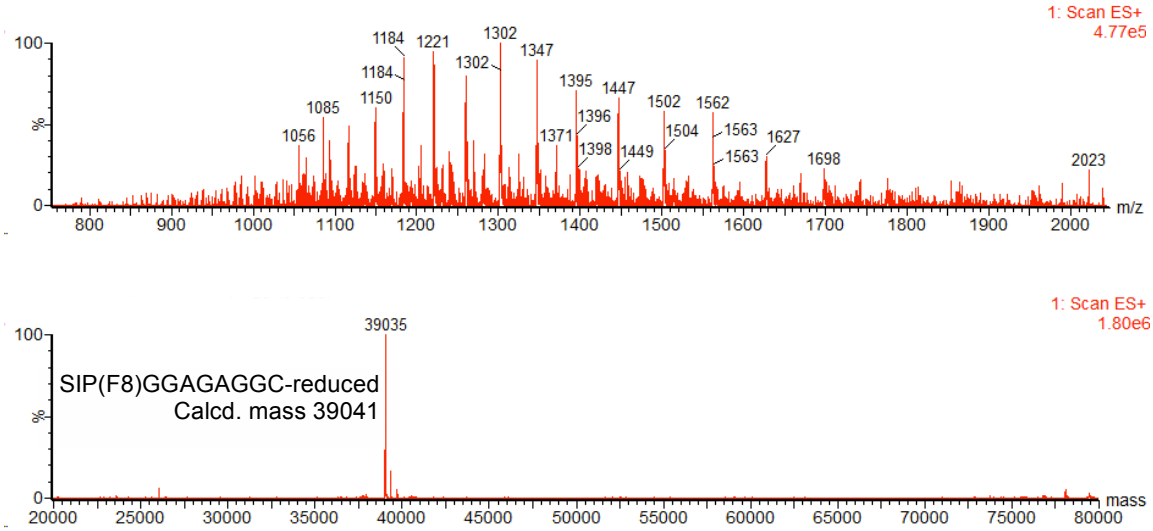


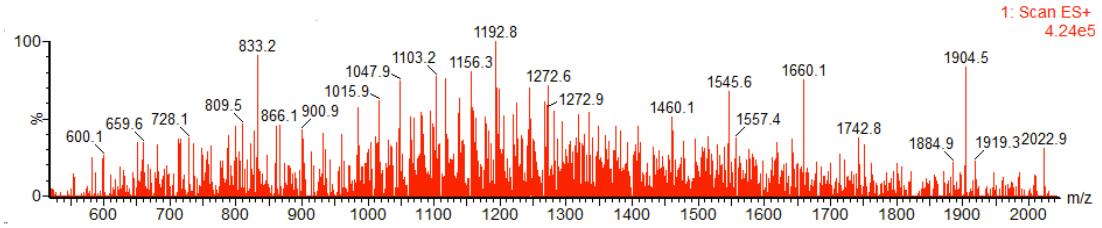
Figure 34. ESI-MS spectra of SIP(F8)GGAGAGGC-SS-CH_2Cem at 5 mg/mL after incubation at 37 ºC for 10 minutes with different concentrations of reduced glutathione.

4.9 Stability assessment of ADCs (1 mg/mL) in mouse plasma

Blood sampling was performed by puncture of the left posterior vena cava and collection using an EDTA Microvette CB300K2E. Cells were removed by centrifugation at 500 *g* for 10 min (4 ºC), and the resulting plasma was isolated. Stability of the ADCs in mouse plasma was assessed as follows: 20 µL of ADC (1 mg/mL) was incubated with 20 µL of mouse plasma (experiments were performed in replicates with plasma from two different mice) for 1, 12 and 24 hours. The mixture was diluted with 60 µL of PBS pH 7.4, centrifuged for 30 seconds and a 50 µL aliquot taken for LC-MS analysis.

SIP(F8)GGC-SS-CH_2Cem

1 hour at 37 ºC



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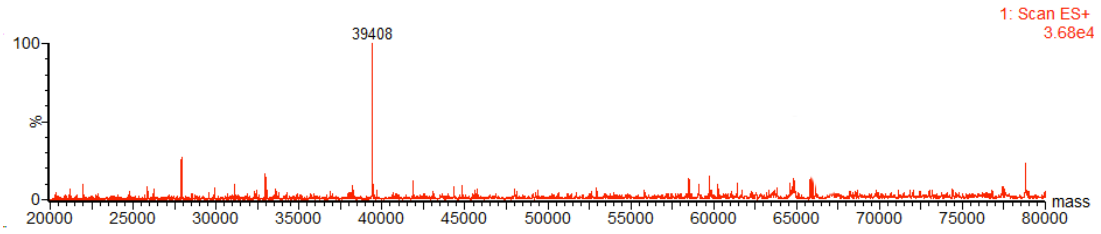
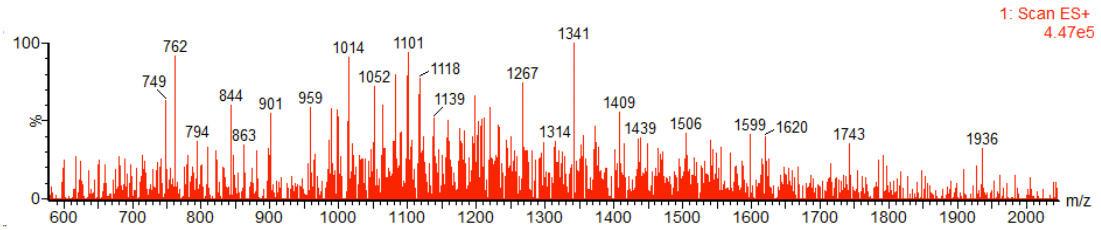
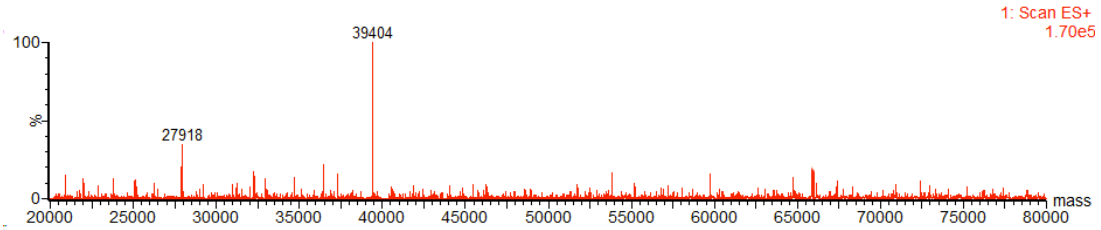
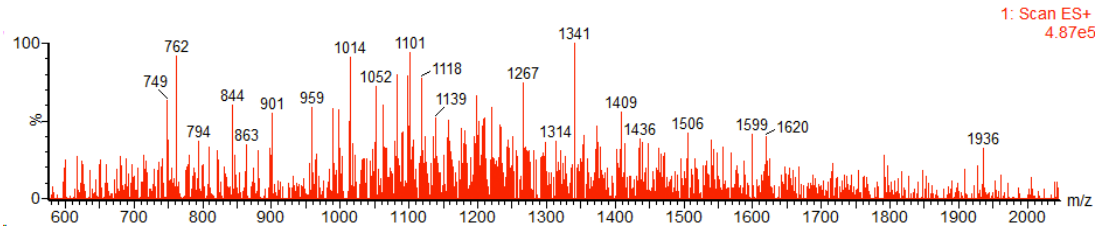


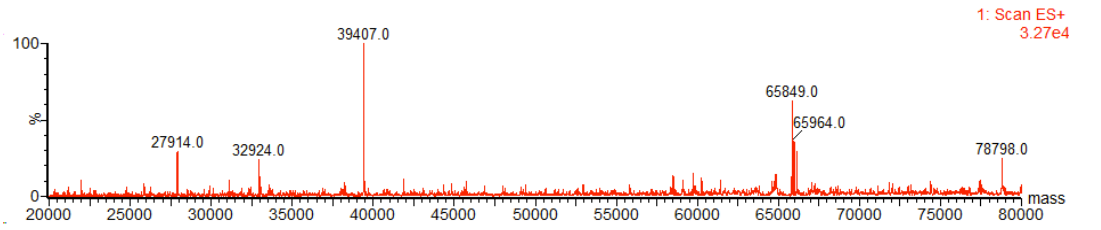
12 hours at 37 ºC





24 hours at 37 ºC

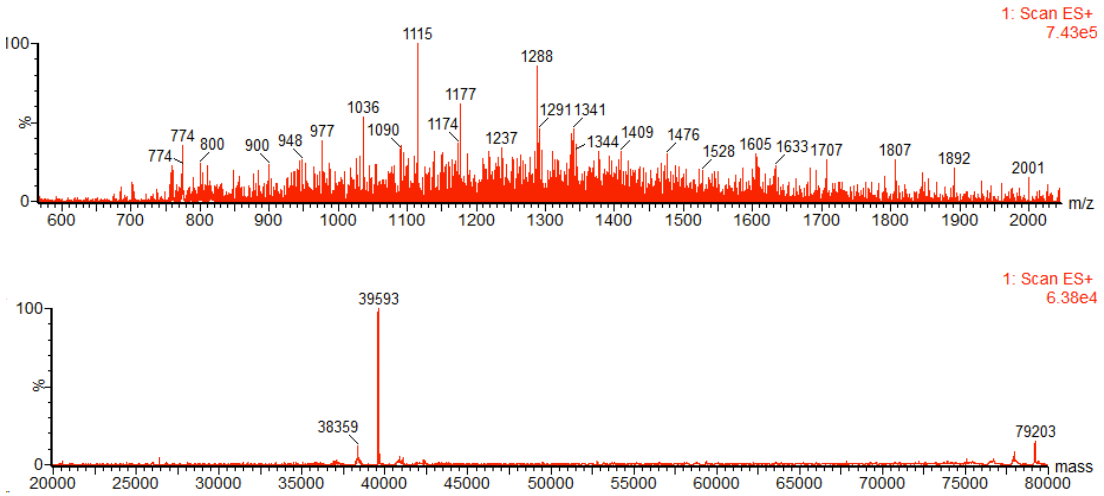




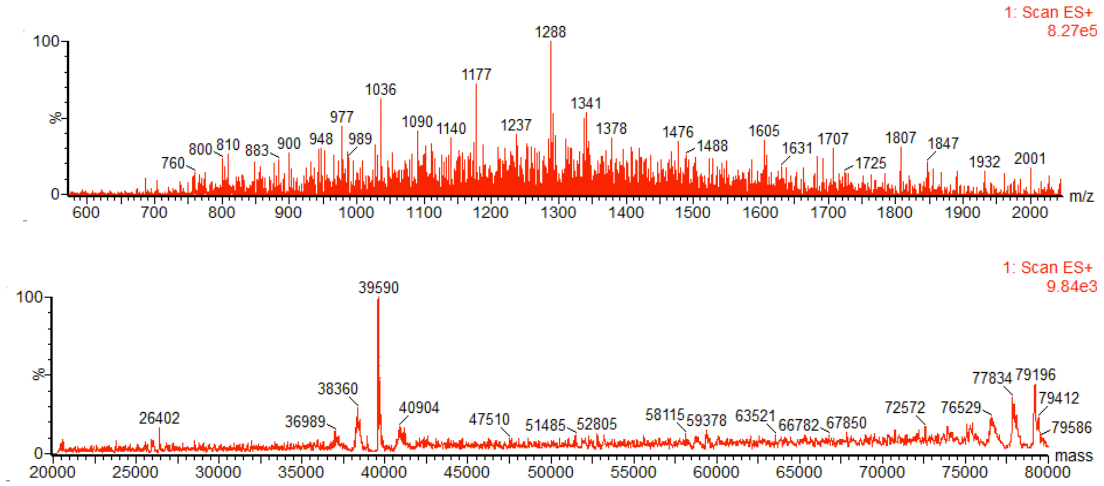
Supporting Information

SIP(F8)GGAGGC-SS-CH_2Cem

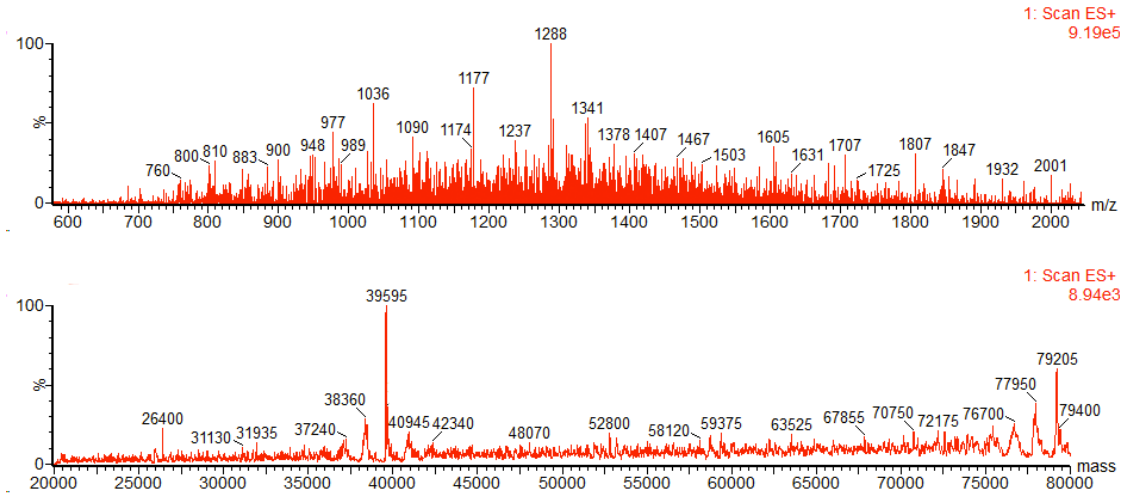
1 hour at 37 ºC



12 hours at 37 ºC



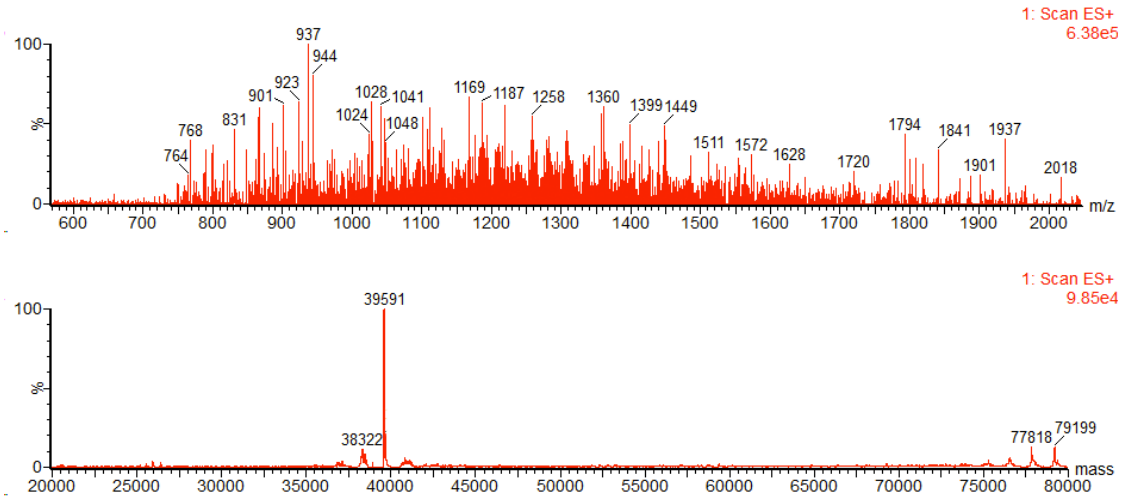
24 hours at 37 ºC



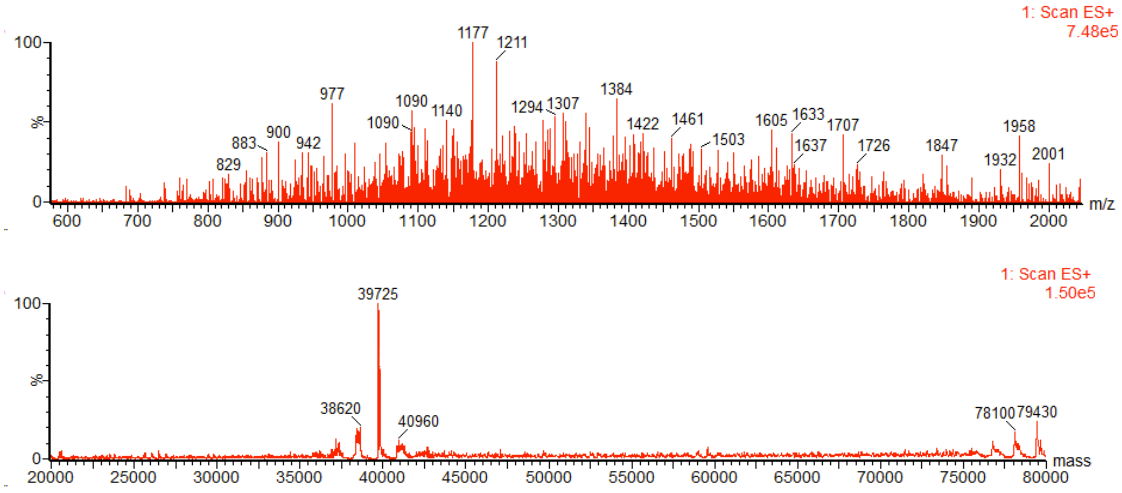
Supporting Information

SIP(F8)GGAGAGGC-SS-CH_2Cem

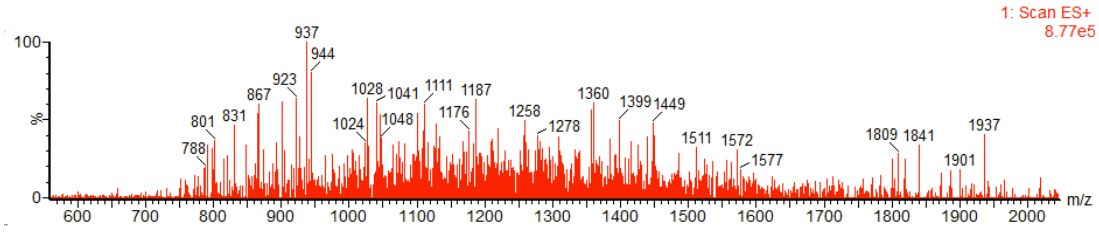
1 hour at 37 ºC



12 hours at 37 ºC



24 hours at 37 ºC



Supporting Information

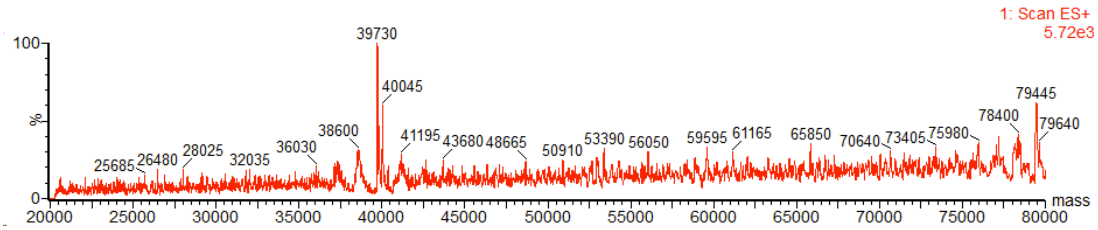


Figure 35. ESI-MS spectra of ADCs at 1 mg/mL after incubation with mouse plasma for 1, 12 and 24 hours.

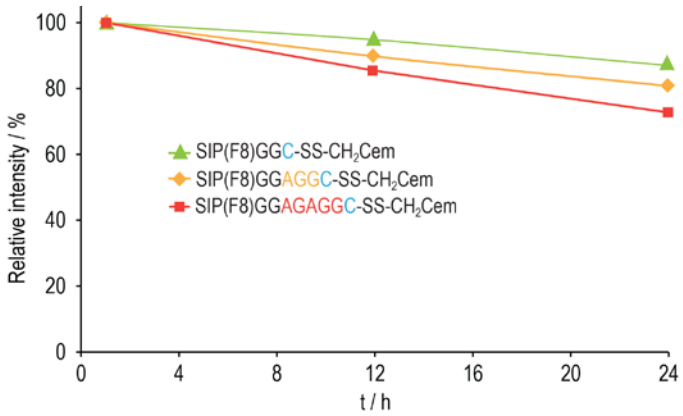


Figure 36. Stability of ADCs (1 mg/mL) in mouse plasma measured by LC–MS. The peak intensities for each substrate were measured and the intensity/total ion count (TIC) calculated. The intensity/total ion count ratio was normalized considering the initial time point at 1 hour to give the relative intensity (%), i.e. (intensity/total ion count ratio at a given time)/(intensity/total ion count ratio at 1 hour).

5 Therapy Studies

5.1 Tumor cell lines

CT26 cell were cultured with DMEM (Gibco) supplemented with 10% FCS, A20 cell were cultured in RPMI supplemented with 10% FCS, 5mM Ultraglutamine and 50µM β-Mercaptoethanol. Cells were incubated at 37 °C and 5% CO2.

5.2 Syngeneic tumor mouse model in immunocompetent mice

Tumor bearing mice were obtained by subcutaneous injection of A20 reticulum cell sarcoma cells (3×10^7) or CT26 colon carcinoma cells (5×10^7) into the left flank of 12-week-old female female BALB/c (Charles River Laboratories, Sulzfeld, Germany). The tumors were allowed to grow for 4 or 5 days (A20 and CT26, respectively) to a size of typically 80 – 120 mm^3. All animal experiments were carried out according to Swiss regulations under project licenses granted by the Veterinäramt des Kanton Zürich (169/2008 and 42/2012).

5.3 Therapy experiments

Therapy experiments were assessed in A20 tumor bearing BALB/c mice starting 4 days and in CT26 starting 5 days after subcutaneous tumor implantation when the tumors typically exhibited a size of 80 – 120 mm^3. Mice were randomly grouped ($n = 5$). Mice were injected daily into the lateral tail vein for a period of 5 days with targeting and nontargeting antibody-drug conjugates, CemCH_2-SH, Cem, and PBS pH 7.4 as the vehicle. The antibody-drug conjugates SIP(F8)GGC-SS-CH_2Cem, SIP(F8)GGAGGC-SS-CH_2Cem, SIP(F8)GGAGAGGC-SS-CH_2Cem and SIP(KSF)GGC-SS-CH_2Cem were prepared at a concentration of 4.8 mg/mL corresponding to a daily dose of 960 µg of antibody. 18 µg of CemCH_2-SH (25.7 nmol) that correspond to the amount of conjugated drug was injected daily. Mice were monitored daily, tumor volumes were measured daily with a digital caliper and calculated using the formula: volume = length × $width^2$ × 0.5. Animals were sacrificed when tumor volumes reached a value between 1800 mm^3. Mice were photographed using a digital camera (Sony).

6 DSC experimental section

DSC experiments were performed on a VP-DSC calorimeter (MicroCal Inc.) equipped with twin coin-shaped cells of 0.52 mL volume. Details on the instrument's performance are given elsewhere.[10] The heating rate was 1 °C min^{-1}. Protein and complex samples were dialyzed for 18-24 hours against the same batch of buffer used to establish the baseline. Reversibility was checked by 4 cycles of heating and cooling in the temperature range 20 – 40 °C. The raw experimental data were corrected for the instrumental buffer-buffer baseline. Data handling and analysis were carried out using the subroutines for Origin provided by MicroCal. Because complete denaturation proceeded with subsequent aggregation in all samples considered, a cubic baseline was subtracted from the heat capacity data after estimating the heat capacity change at the transition temperature based on the elementary contribution of 58 J K^{-1} $(mol\ res)^{-1}$.[11]

7 References

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