Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Minimal Hybridization Chain Reaction (HCR) System using Peptide Nucleic Acid

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1. Materials and methods

1.1 General information

PNAs were purified by HPLC and lyophilized. LC-MS spectra were recorded by using a DIONEX Ultimate 3000 UHPLC coupled with a Thermo LCQ Fleet Mass Spectrometer System (electrospray ionization (ESI)) operated in positive. A Bruker Daltonics Autoflex spectrometer was used for MALDI-TOF mass results.

Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific) was used to determine the DNA and PNA concentrations. The absorbance at 260 nm of the sample was measured. For quantification of oligonucleotides, 13700, 6600, 11700, 8800, 13700, 7200 M^{-1} were used as extinction coefficient at 260 nm for A, T, G, C, FITC, Dabcyl, respectively.

The hairpin samples were heated at 98 °C for 6 min and cooled down on ice for 10 min before use (snap-cooling).

Procedures and methods for synthesis and purification of PNA strands was carried out based on our previous report.[1]

1.2 Evaluation of PNA HCR by gel electrophoresis

For 5 % native PAGE analysis, 2~4 μM (based on final concentration) stocks of H1, H2, I were prepared in buffer pH 7.5 0.1×PBS, 0.02 % tween-20. The stocks were heated to 98 °C for 6 min and cooled down in ice for 10 min before use. H1, H2, I (5 μL each) were mixed and buffer was added to the mixture to reach the desired dilution (20 μL final sample volume). The HCR reaction was carried out for 2 h at r.t. followed by addition of 2 μL of glycerol. The sample was loaded and analyzed on 5% polyacrylamide gel (1×TBE buffer, 100~250 V) at r.t. The FITC band was visualized on a Fusion FX7.

For SDS-PAGE of PNAs, TruPAGE Precast Gel 4-12 % (12 well, PCG2003, Sigma-Aldrich) and TruPAGE™ Tris-MOPS SDS Express Running Buffer, 20× were used. 15 μL of final volume and 1 μM (0.5 μM for γ-serine modified PNAs) of reaction components were used for each reaction. The HCR reactions (pH 7.5 0.1×PBS, 0.02 % tween-20) were carried out for 2 h at r.t. 5 μL of 4×loading buffer were added to each sample for gel loading. Afterwards, the samples were loaded on gel without heating. The fluorescence of Cy3 in the gel was monitored by Fusion FX7.

1.3 Streptavidin bead fluorescent pulldown assay for estimation of HCR elongation

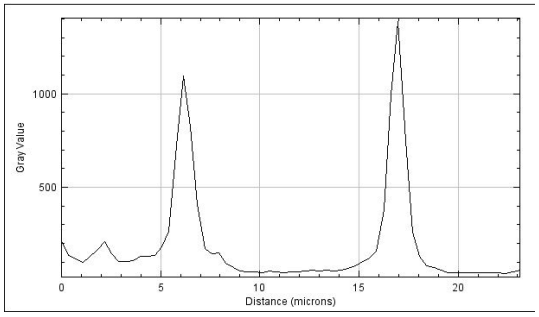
For Fig. S2, 4 μL of High Capacity Streptavidin Agarose beads (Thermo Scientific) in 200 μL eppendorf were washed with reaction buffer. 30 μL of 0.1 μM **BCy3serI** (3 pmol) were added to the beads and reacted for 1 h. in a rotating shaker. After initiator loading, the supernatant was taken for Cy3 fluorescence measurement to calculate the yield of initiator loaded on beads. After removing the supernatant by

micropipette, the beads were treated with 30 μL of annealed 1 μM of **FserH1** and **FserH2** (30 pmol) for HCR reaction. After 2 h. of reaction, the fluorescence intensity of the supernatant of the reaction mixture was monitored by plate reader (cuvette) to calculate elongation efficiency of γ-serine-modifed PNA HCR.

For Fig. 4A, 4 μL of High Capacity Streptavidin Agarose beads (Thermo Scientific) in 200 μL eppendorf were washed with reaction buffer. 150 μL of 0.1 μM **BpegI** (15 pmol) were added to the beads and reacted for 1 h. in a rotating shaker. After removing the supernatant by micropipette, the beads were treated with 75 μL of annealed 2 μM of **pegH1** and **Cy3pegH2** (150 pmol for each) for HCR reaction. After 4 h. of reaction, the fluorescence intensity of the supernatant was monitored by plate reader (cuvette) to calculate elongation efficiency.

1.4 Fluorescence microscopy analysis for estimation of HCR elongation

Streptavidin beads (3 μL) were washed with water and 0.1 μM, 10~100 μL (1~10 pmol, 10 pmol for FITC-labelled I) of biotinylated-I in pH 7 1×SSC buffer, 0.02 % tween-20 was incubated with beads for 1 h. After removing the supernatant, HCR was initiated by adding 0.5 μM of mixture of H1 and H2 hairpins (pH 7 1×SSC buffer, 0.02 % tween-20). After 4 h. of reaction at r.t., the beads were washed with 1×SSC buffer 3 times and moved to microscopy plate using 100 μL of 1×SSC buffer. Fluorescence image of the beads were taken by Leica SP5 by using the parameters: 495 nm of excitation and 510~530 nm of emission, 20 % laser power (Argon white laser) for FITC imaging, 550 nm of excitation and 570~600 nm of emission, 3 % laser power for Cy3 imaging. Average values of fluorescence peaks were obtained from Plot profiles of surface of beads. All images were analysed in ImageJ.



1.5 Size exclusion chromatography (SEC) of HCR product

As reference samples, 100 μL of 5 μM **Ref1+Ref2** (20-mer γ-peg-modifed PNA duplex), **Ref3+Ref4** (10-mer), or **Ref5+Ref6** (6-mer) were prepared in pH 7.5 0.1×PBS. Each sample was spiked with 0.5 μL of 360 μM of Caffeine as an internal standard. The samples were analyzed by HPLC using size exclusion column (Agilent Bio SEC-3 Columns, 100 Å, 300 mm * 4.6 mm, 0.35 mL/min., r.t., 0.5×PBS buffer). 2 μM stock sample of each pegH1, pegH2, and pegI (2 μM, 500 μL) were prepared and annealed. Reaction

samples are prepared as described in the figures of the main text (100 μL of final volume) and injected into HPLC after 2 h. of reaction time for analysis. Retention time of reference PNAs were utilized to draw a standard curve for size-estimation.

1.6 Kinetics analysis of HCR

The kinetics of HCR was monitored in buffer solution (pH 7 1×SSC buffer, 0.02 % tween-20). To measure the k_{obs} of the first step by using pseudo first order kinetics, 10 pmol of **pegIF** in 800 μL of buffer was prepared. To this solution, 200 pmol of **DpegH2** in 200 μL (1 μM in 1×SSC buffer) was added and the mixture was mixed very quickly. Fluorescence at 530 nm was measured every 10 seconds before adding **DpegH2** (λ_{exc}: 485 nm, cutoff: 515 nm, λ_{emi}: 530 nm, PMT gain: medium). To measure the the k_{obs} of the second step, 20 μL of 0.5 μM **pegIF** (10 pmol) in 1×SSC buffer was mixed with 20 μL of 1 μM **pegH2** (20 pmol) and incubated. After 10 min. of incubation, 760 μL of 1×SSC buffer was added to the sample. During the fluorescence measurement of the **pegIF+pegH2** mixture, 200 pmol of **DpegH1** in 200 μL (1 μM in 1×SSC buffer) were added to monitor fluorescence changes. The fluorescence intensity after addition was corrected by dilution effect (×0.8).

Determination of K_d was done by measuring fluorescence change (λ_{exc}: 485 nm, cutoff: 515 nm, λ_{emi}: 530 nm, PMT gain: medium) of 2 nM of **pegIF** (total 1 mL) for the first step or 10 nM of **pegIF+pegH2** mixture for the second step in 1×SSC buffer at r.t.; every data point was taken after 1 min from addition to allow the solution to equilibrate.

1.7 Cell culture

HT-29 cell lines were obtained from the American Type Culture Collection (ATCC) and expanded following their instructions. Cells were grown in McCoy's 5A (modified) medium (Gibco) containing 10% FCS and 1% pen-strep at 37 °C under 5% CO_2 in a humidified incubator. Cells were regularly tested for mycoplasma contamination by staining with Hoechst 33342.

Experiments under hypoxic conditions were conducted in an Eppendorf® Galaxy® 48R CO_2 incubator at 37 °C with 1% O_2, 5% CO_2 and 94% N_2 settings.

1.8 HCR reaction on hypoxic/normoxic HT-29 cells

HT-29 cells ($2x10^5$) were seeded into 3.5 cm glass bottom dishes with 10 mm microwell (Mattek); cells were incubated in McCoy's 5A medium at 37 °C under 5% CO_2 in a humidified incubator for 48 hours. Medium was replaced with fresh one and cells were transferred into the hypoxia incubator (1% O_2) for 24 hours.

Initiator-CA IX ligand: cells were treated with Initiator-CA IX ligand (LpegI, 100 nM; 30 min) under hypoxia; cells were washed with HBSS and imaged with a Leica SP8 microscope.

Initiator-CA IX ligand + Hairpins: cells were treated with Initiator-CA IX ligand (LpegI, 100 nM; 30 min) under hypoxia; cells were washed with HBSS and incubated with hairpins (pegH1+Cy3pegH2, 1 μM) under hypoxia for 1 hour. Then cells were washed with HBSS and imaged with a Leica SP8 microscope.

Hairpins: cells were washed with HBSS and incubated with hairpins (pegH1+Cy3pegH2, 1 μM) under hypoxia for 1 hour. Then cells were washed with HBSS and imaged with a Leica SP8 microscope.

Normoxia: cells were treated with Initiator-CA IX ligand (LpegI, 100 nM; 30 min) under normal conditions (37 °C, 5% CO_2); cells were washed with HBSS and incubated with hairpins (pegH1+Cy3pegH2, 1 μM) for 1 hour. Then cells were washed with HBSS and imaged with a Leica SP8 microscope.
All images were analysed in ImageJ.

1.9 Information of sequences

Table S1 PNA sequences investigated in this study

Name	Sequence (N- to C-terminal for PNA)
F34H1	FITC-peg-ACA-GCCG-TAT-CGGC
F34H2	FITC-peg-CGGC-TGT-GCCG-ATA
F34I	FITC-peg-TAT-CGGC
F45H1	FITC-peg-GAAT-TGCCG-ACTA-CGGCA
F45H2	FITC-peg-CGGCA-ATTC-TGCCG-TAGT
F45I	FITC-peg-ACTA-CGGCA
FserH1	FITC-peg-GAATG-TGCCG-ACTAG-CGGCA
FserH2	FITC-peg-CGGCA-CATTC-TGCCG-CTAGT
FserI	FITC-peg-ACTAG-CGGCA
serH1	NH_2-peg-GAATG-TGCCG-ACTAG-CGGCA
serH2	NH_2-peg-CGGCA-CATTC-TGCCG-CTAGT
BCy3serI	Biotin-peg-Lys(Cy3)-peg-ACTAG-CGGCA
FpegH1	FITC-peg-GAATG-TGCCG-ACTAG-CGGCA (peg)
FpegH2	FITC-peg-CGGCA-CATTC-TGCCG-CTAGT (peg)
FpegI	FITC-peg-ACTAG-CGGCA-Lys-Ac (peg)
pegH1	NH_2-peg-GAATG-TGCCG-ACTAG-CGGCA (peg)
pegH2	NH_2-peg-CGGCA-CATTC-TGCCG-CTAGT (peg)
Cy3pegI	Cy3-peg-ACTAG-CGGCA (peg)
Cy3pegH2	cy3-peg-CGGCA-CATTC-TGCCG-CTAGT (peg)
pegI	NH_2-peg-ACTAG-CGGCA (peg)
BpegI	Biotin-peg-peg-peg-peg-peg-ACTAG-CGGCA (peg)
FBpegI	Biotin-peg-peg-Lys(FITC)-peg-ACTAG-CGGCA (peg)
Ref1	NH_2-peg-AGTCTCAGTGTCAACGTACG (peg)
Ref2	NH_2-peg-CGTACGTTGACACTGAGACT (peg)

Ref3	NH_2-peg-AGTCTCAGTG (peg)
Ref4	NH_2-peg-CACTGAGACT (peg)
Ref5	NH_2-peg-GATGCT (peg)
Ref6	NH_2-peg-AGCATC (peg)
DpegH1	Dabcyl-peg-GAATG-TGCCG-ACTAG-CGGCA (peg)
DpegH2	Dabcyl-peg-CGGCA-CATTC-TGCCG-CTAGT (peg)
pegIF	Ac-ACTAG-CGGCA-Lys-peg(FITC) (peg)
LpegI	Ligand-Lys(Cy3)-peg-ACTAG-CGGCA (peg)

[a] L-serine- or peg-modified PNA is marked with underline; (peg) = peg-modified sequence

2. Results

Figure S1 Evaluation of hairpin metastability and hybridization yield of I and hairpin H2 by native polyacrylamide gel electrophoresis.

FITC-labelled H1, H2 and I PNA sequences are used for PNA mobility in gel electrophoresis. Among three designs, 5,5-system, using 5-mer stem and 5-mer loop hairpins, showed the strongest (>90%) hybridization between H2 and I (H2+I). Hairpin H1 and H2 were not hybridized in the absence of I (H1+H2) for all three cases, which proves metastability of the hairpins. Underlined letters represent γ-serine backbone modification. Conditions for reaction: pH 7.5 0.1×PBS, 0.02 % tween-20, 1 μM of hairpins, 20 μL, 2 h. of incubation, 5 % PAGE in 1×TBE buffer. Gel was visualized for FITC fluorescence.

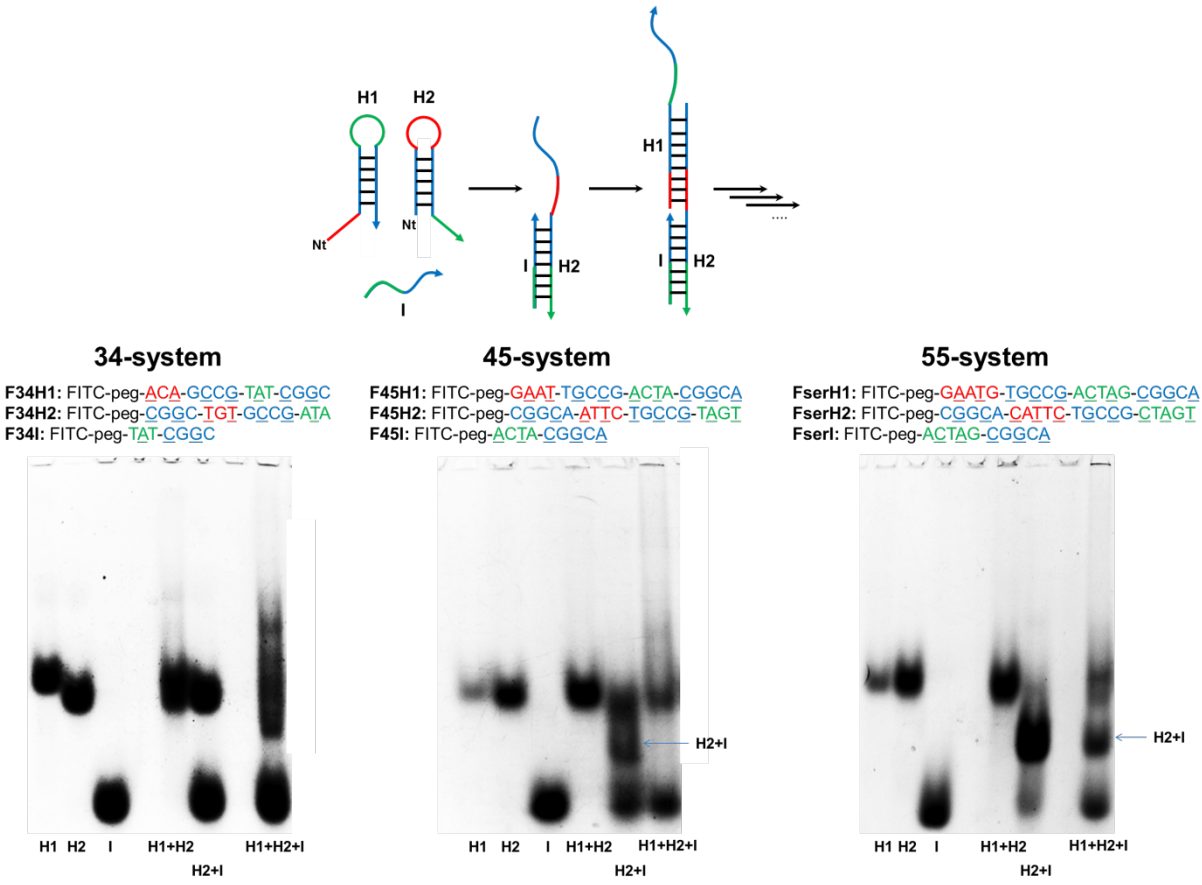
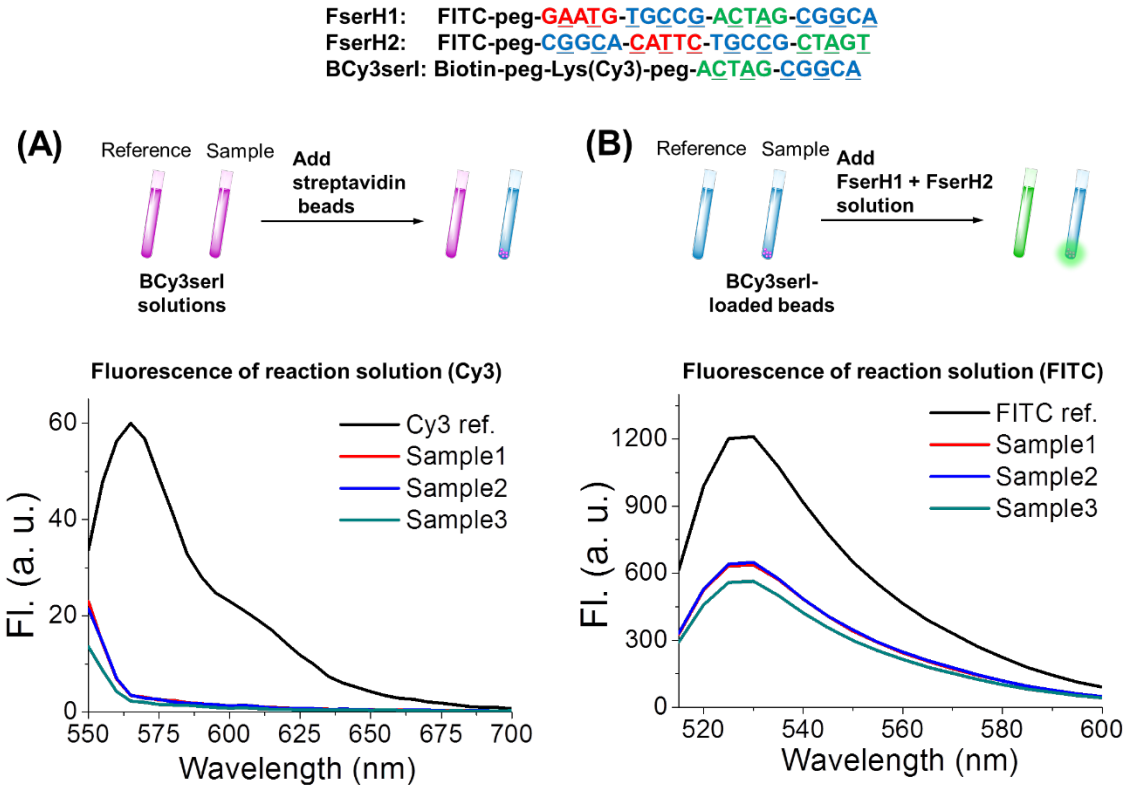


Figure S2 Fluorescence-based quantification method for the estimation of HCR elongation yield. 5,5-system using γ-serine-modified PNAs was used for this experiment. (A) Quantification of loaded initiator sequence based on fluorescence measurement of **BCy3serI** in solution; 30 pmol of **BCy3serI** was used; streptavidin beads loading was found to be >95%. (B) Quantification of hairpins participating in HCR based on fluorescence measurements of the solution after reaction. 150 pmol of each **FserH1** and **FserH2** (total 300 pmol of hairpin / 150 µL) was added to pre-loaded beads. Fluorescence of the resulting solution was measured after 2 h. of incubation. The result showed that 5.2 eq. of hairpins are consumed for HCR. Reaction conditions: pH 7.5 0.1×PBS, 0.02 % tween-20.



Consumed FITC hairpins (pmol)	Loaded BCy3serI (pmol)	Ratio		
141.5	28.5	5.0	Average	5.2
141.1	28.3	5.0		
161.5	29.0	5.6		

Figure S3 Size analysis of PNA complexes using SDS-PAGE.

Complex of **Cy3pegI+pegH2** was found to have higher mobility than **Cy3pegH2** itself. This result indicates that H2 hairpin itself exists as monomeric state, the hairpin, not as dimeric state. Conditions for reaction: pH 7.5 0.5×PBS, 0.02 % tween-20, 500 nM of each component, 15 μL, r.t., 2 h. of incubation.

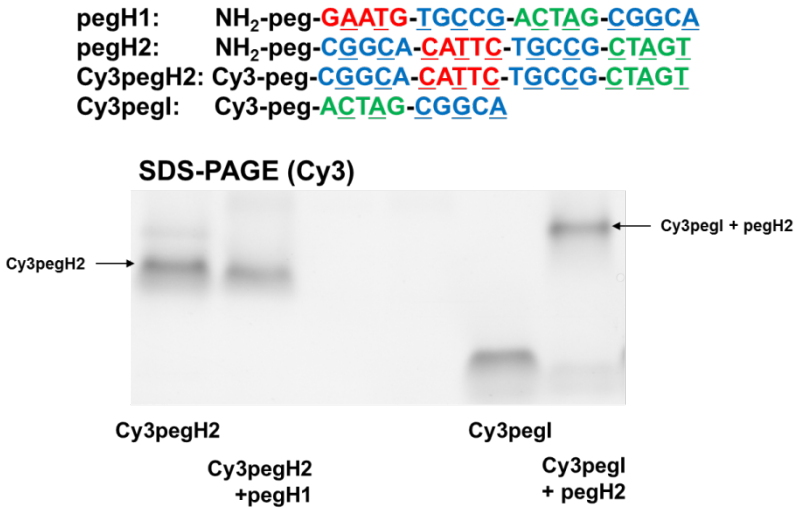


Figure S4 On-bead fluorescent microscopy assay.

Reaction conditions: pH 7 1×SSC buffer, 0.02 % tween-20; (A) 2 pmol of **BpegI**, 32.5 pmol of each hairpin (0.22 μM), total 150 μL, 4 h of reaction; (B) 1 pmol of **BpegI**, 65 pmol of each hairpin (0.43 μM), total 150 μL, 24 h of reaction. Even for long incubation time (24 h), HCR reaction afforded 12-fold of elongation yield.

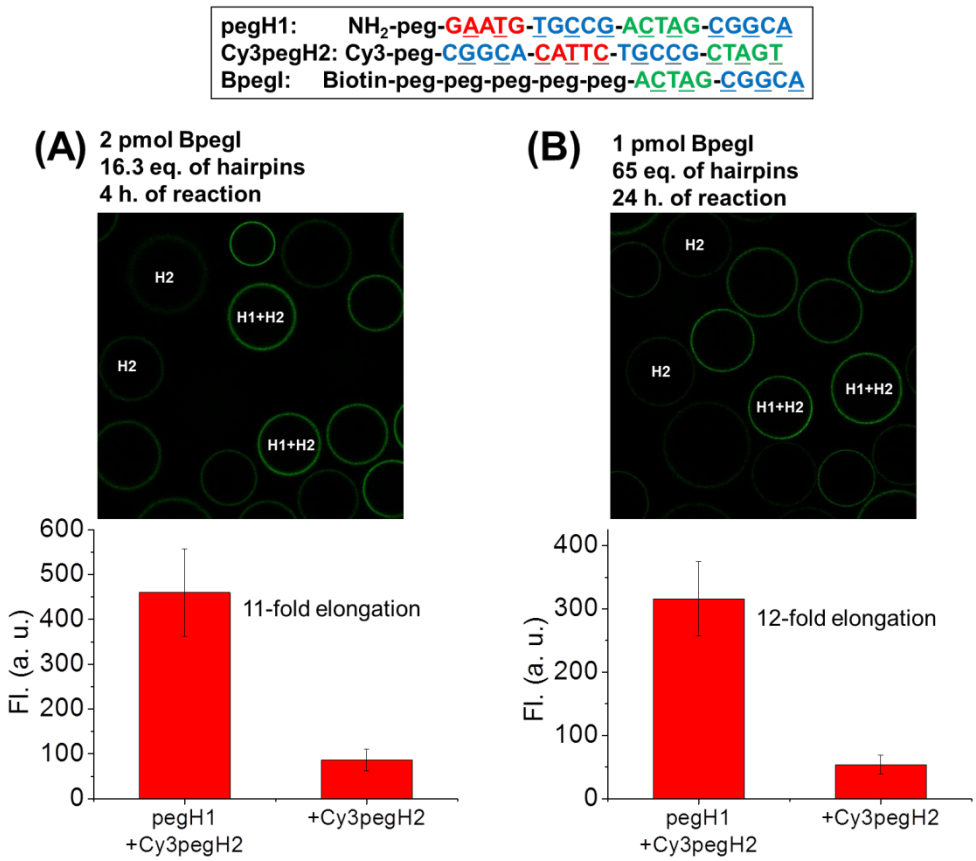


Figure S5 Size estimation of HCR product based on size exclusion chromatography (SEC).
(A) HPLC profile of reference 20-mer (**Ref1**+**Ref2**), 10-mer (**Ref3**+**Ref4**), 6-mer (**Ref5**+**Ref6**) peg-modified PNA duplexes. (B) Injection profiles of H1, H2, I, and their complexes. The major peak of HCR reaction (**pegH1**+**pegH2**+ 0.1 eq. of **pegI**) was found at 7.5 min. (C) Standard curve obtained from reference PNA duplexes in (A). The size of major peak of HCR product was estimated as 57 kDa based on the standard curve.

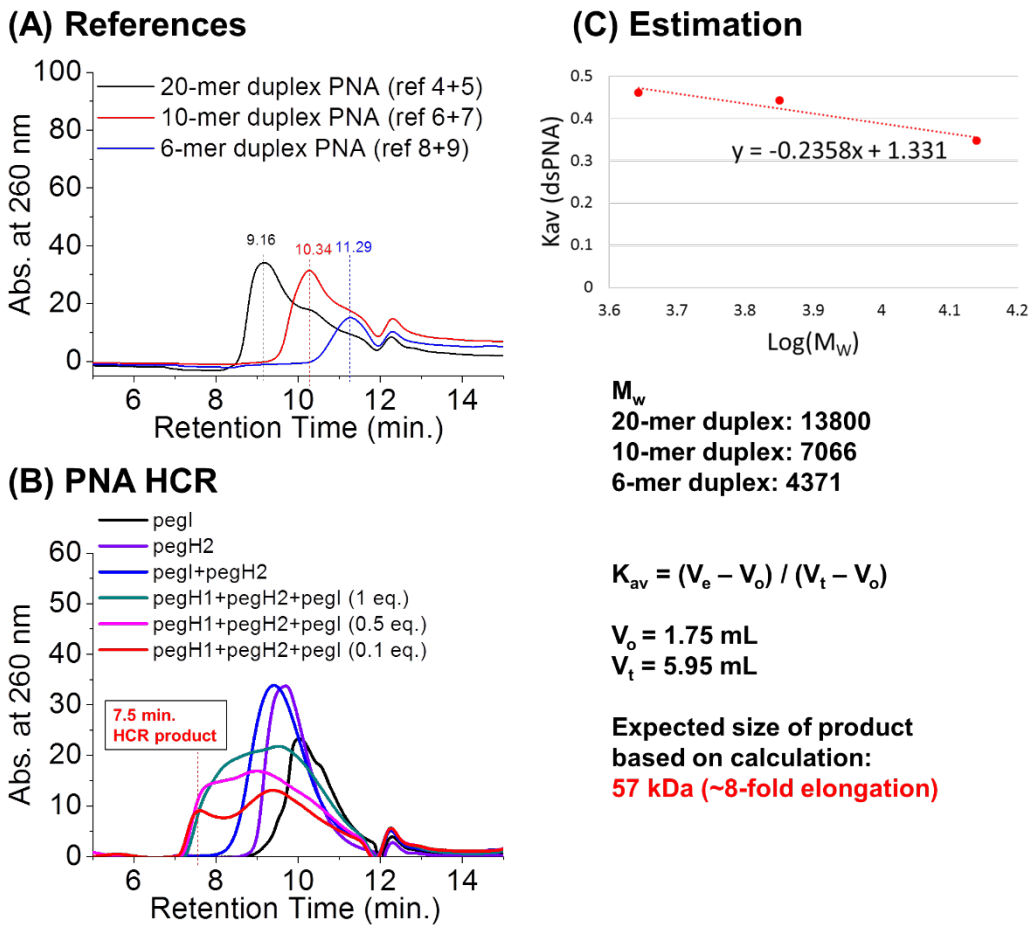
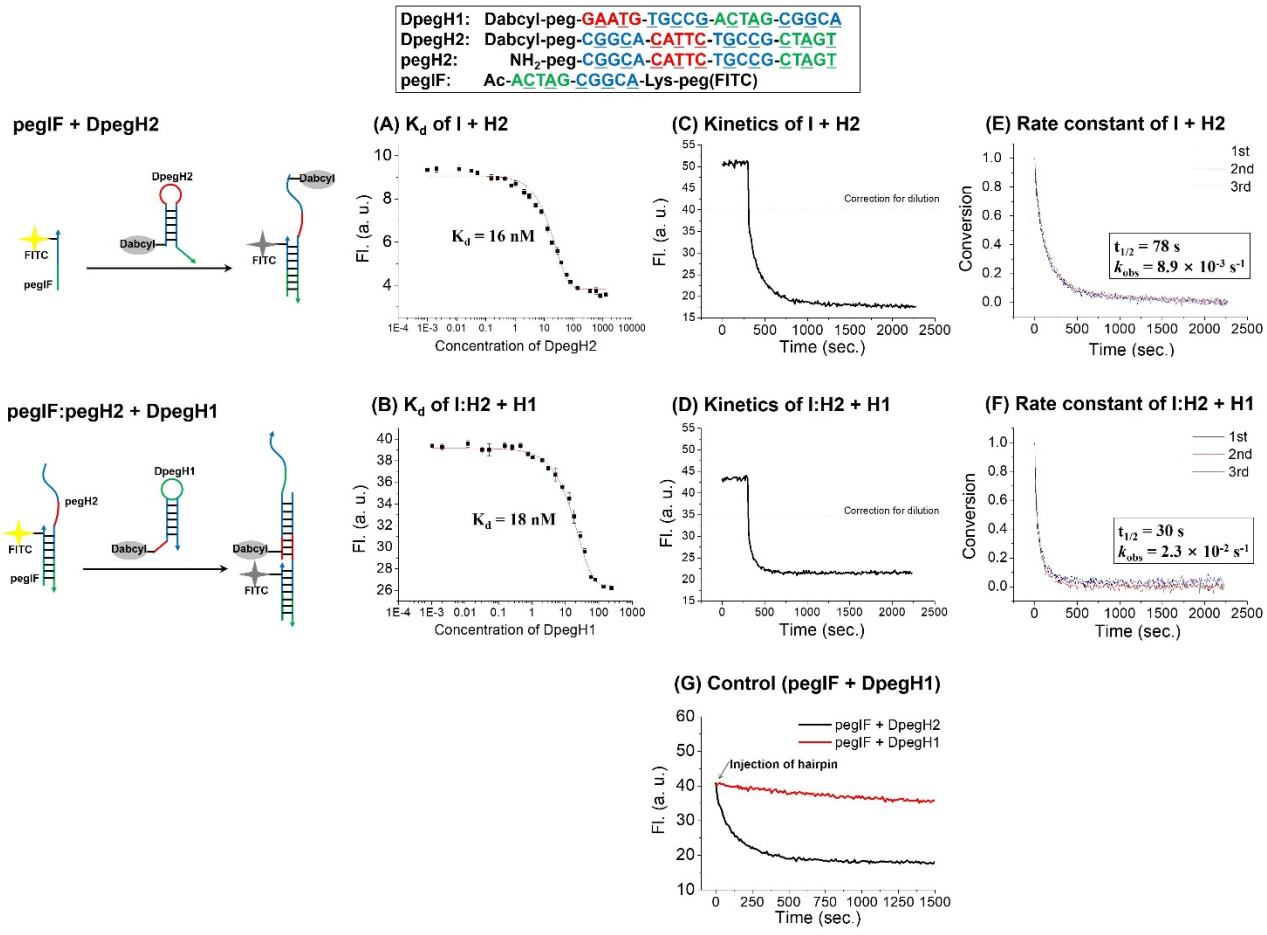
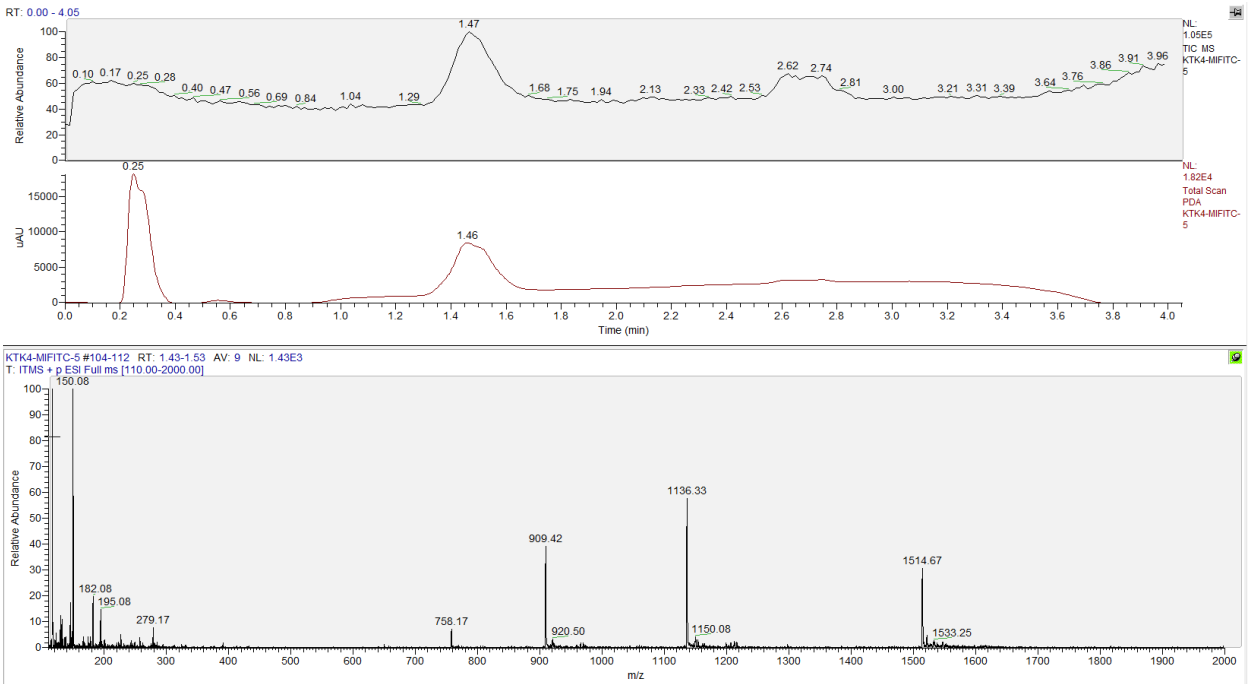


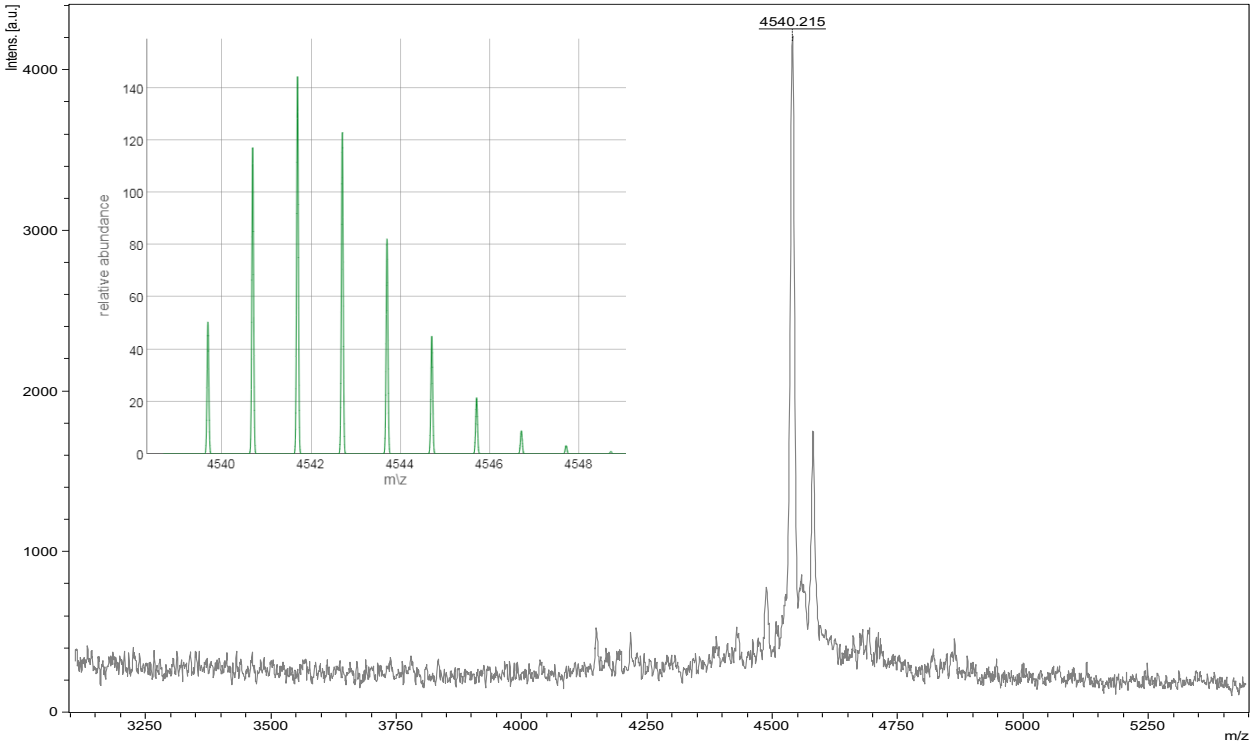
Figure S6 Kinetic data of each HCR step (**pegIF + DpegH2** and **pegIF:pegH2 + DpegH1**).
K_d of I + H2 (A) and I:H2 + H1 (B). Raw kinetic data of I + H2 (C) and I:H2 + H1 (D). (E and F) Half-life and reaction rate constant (Pseudo-first-order kinetics) calculated from processed data of (C) and (D). Conditions: pH 7 1×SSC buffer, 0.02 % tween-20, 2 nM of **pegIF** for (A), 10 nM of **pegIF** + 10 nM of **pegH2** for (B), 10 nM of **pegIF** + 200 nM of **DpegH2** for (C and E), 10 nM of **pegIF** + 20 nM of **pegH2** + 200 nM of **DpegH1** for (D and F). (G) Control reaction using **pegIF** + **DpegH1**. **pegIF** + **DpegH1** showed no fluorescence quenching while fluorescence was rapidly quenched for **pegIF** + **DpegH2.** Conditions: 10 nM of **pegIF** + 200 nM of **DpegH1**.



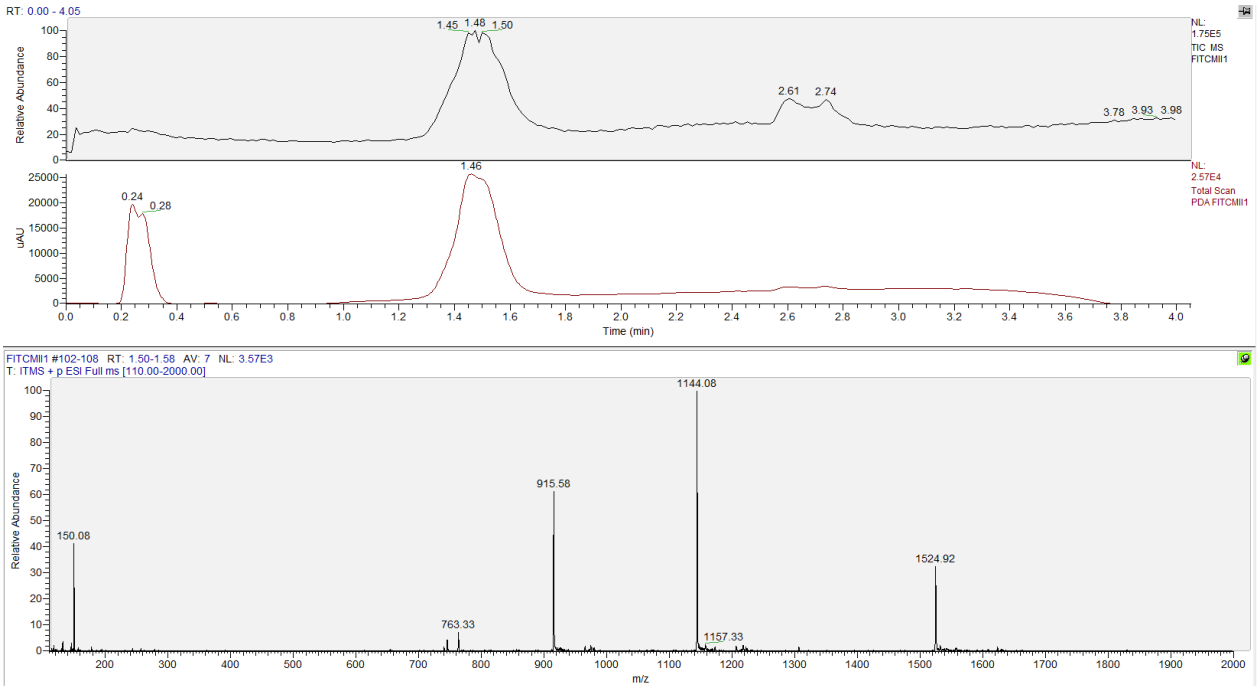
3. Mass spectra of the synthesized DNAs and PNAs

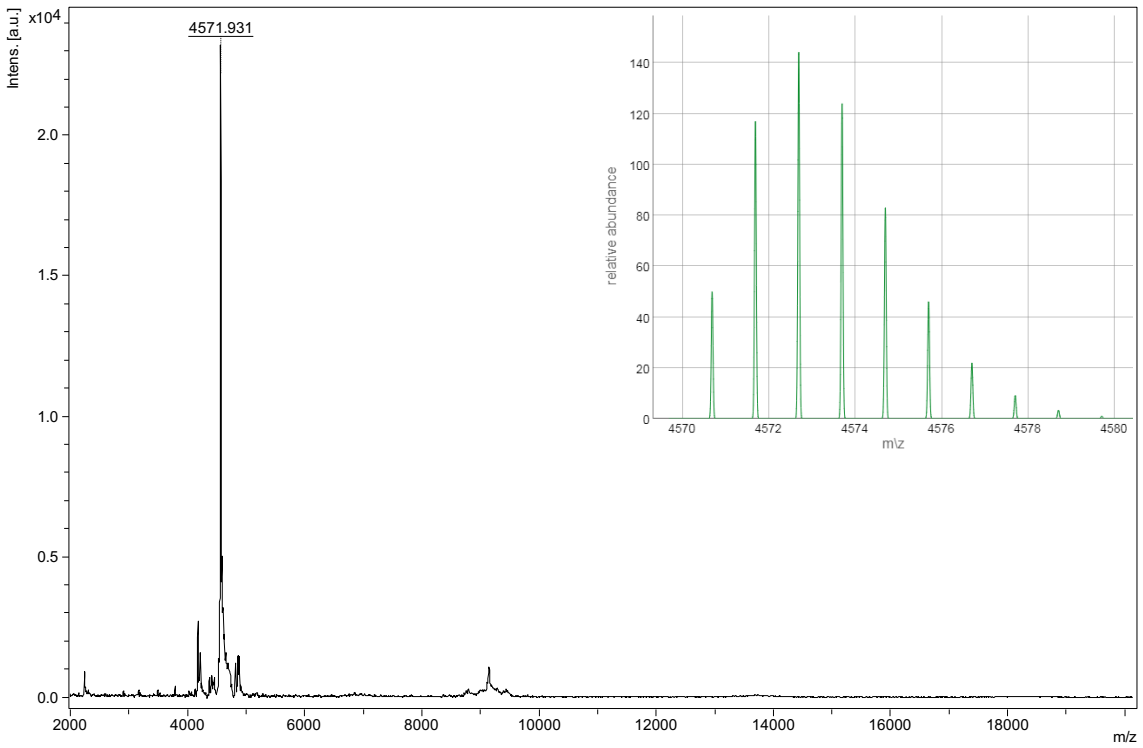
F34H1 (FITC-peg-ACA-GCCG-TAT-CGGC); Expected Mass for $C_{183}H_{223}N_{85}O_{56}S$: 4538.6936; isotopic mass range (green insert). LC-MS (ESI) RT= 1.47 min. m/z: 1514.67 $[M+3H]^{3+}$, 1136.33 $[M+4H]^{4+}$, 909.42 $[M+5H]^{5+}$, 758.17 $[M+6H]^{6+}$, MALDI-TOF m/z found: 4540.21.



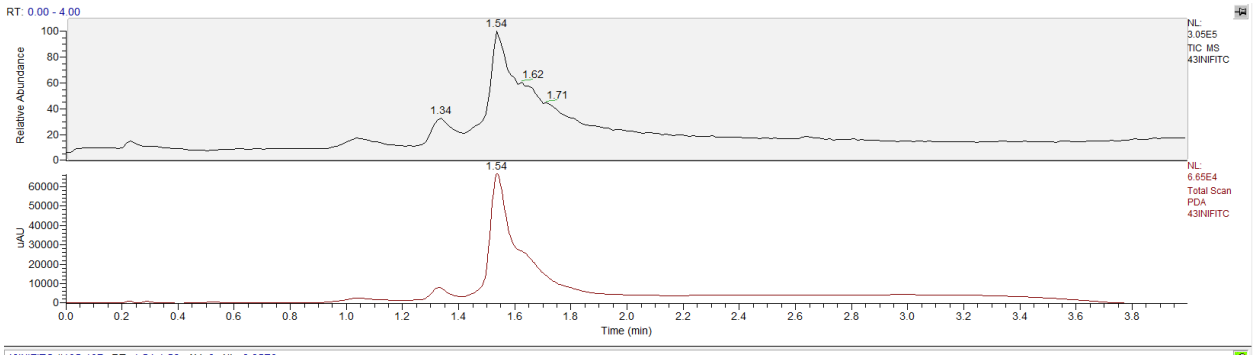


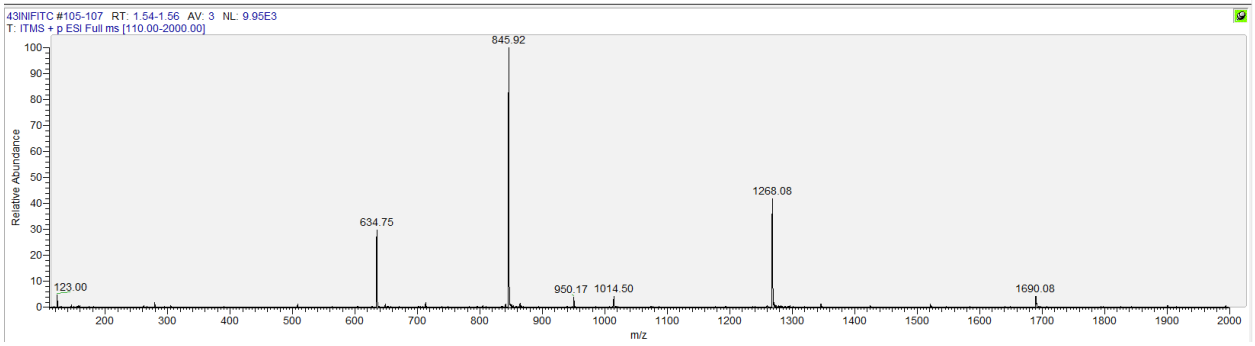
F34H2 (FITC-peg-CGGC-TGT-GCCG-ATA); Expected Mass for $C_{184}H_{224}N_{84}O_{58}S$: 4569.6881, isotopic mass range (green insert). LC-MS (ESI) RT= 1.48 min. m/z: 1524.92 $[M+3H]^{3+}$, 1144.08 $[M+4H]^{4+}$, 915.58 $[M+5H]^{5+}$, 763.33 $[M+6H]^{6+}$, MALDI-TOF m/z found: 4751.931 $[M+H]^{+}$.

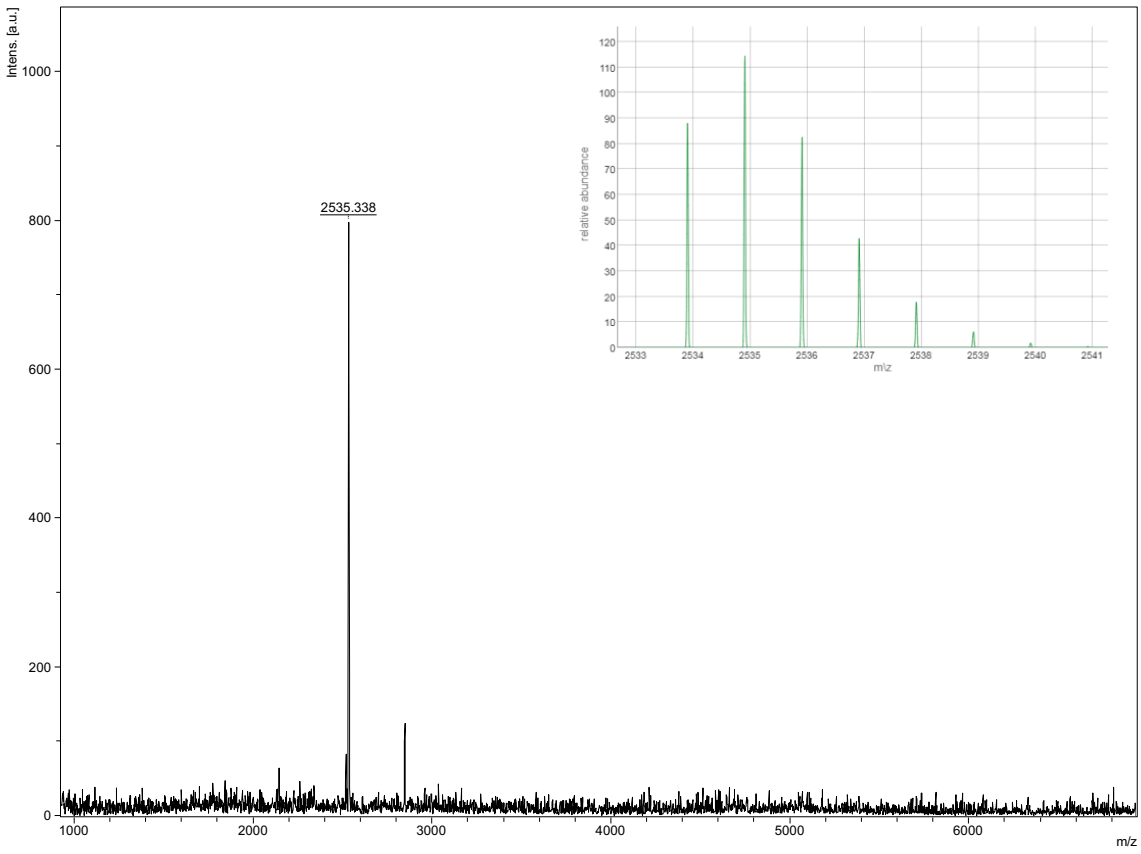




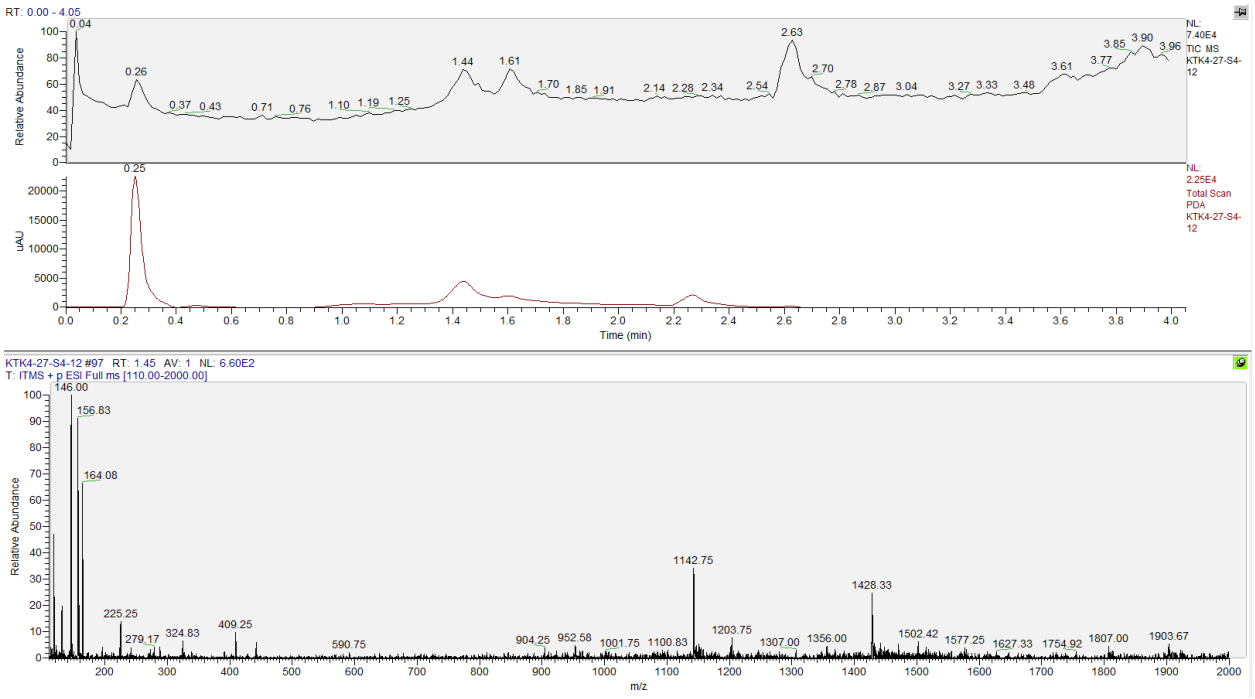
F34I (FITC-peg-TAT-CGGC); Expected Mass for $C_{105}H_{124}N_{42}O_{33}S$: 2532.9037; isotopic mass range (green insert). LC-MS (ESI) RT= 1.54 min. m/z: 1690.08 $[2M+3H]^{3+}$, 1268.08 $[M+2H]^{2+}$, 845.92 $[M+3H]^{3+}$, 634.75 $[M+4H]^{4+}$, MALDI-TOF m/z found: 2535.338 $[M+H]^{+}$

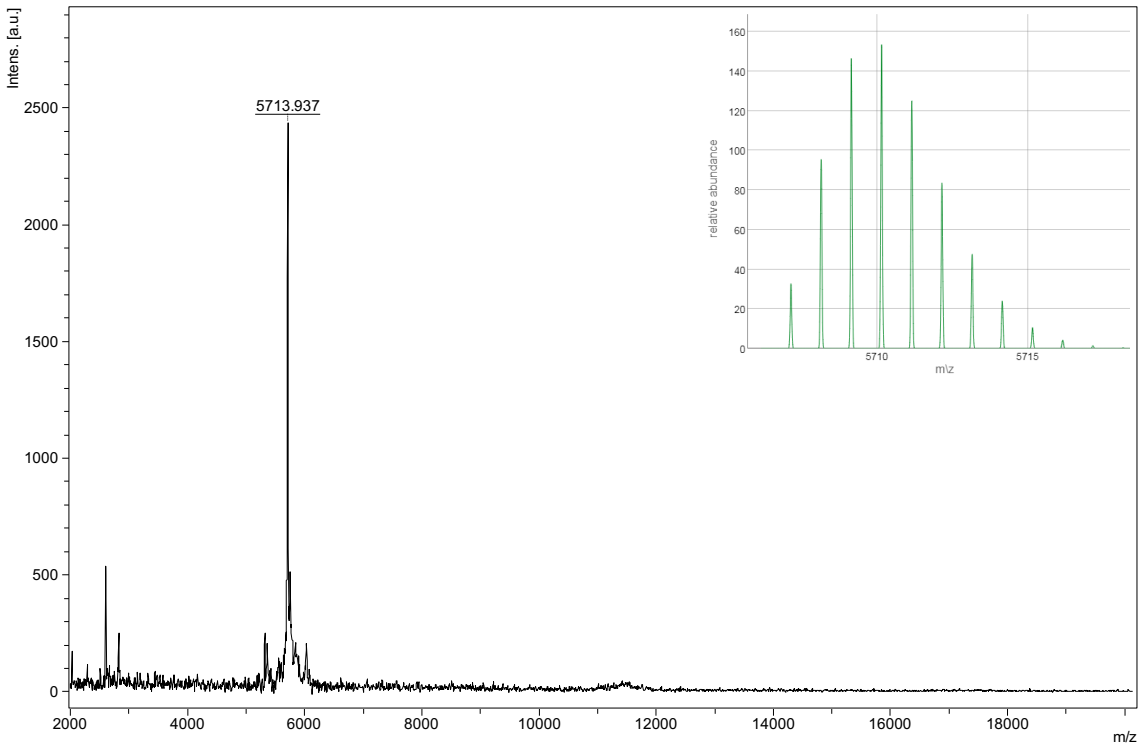




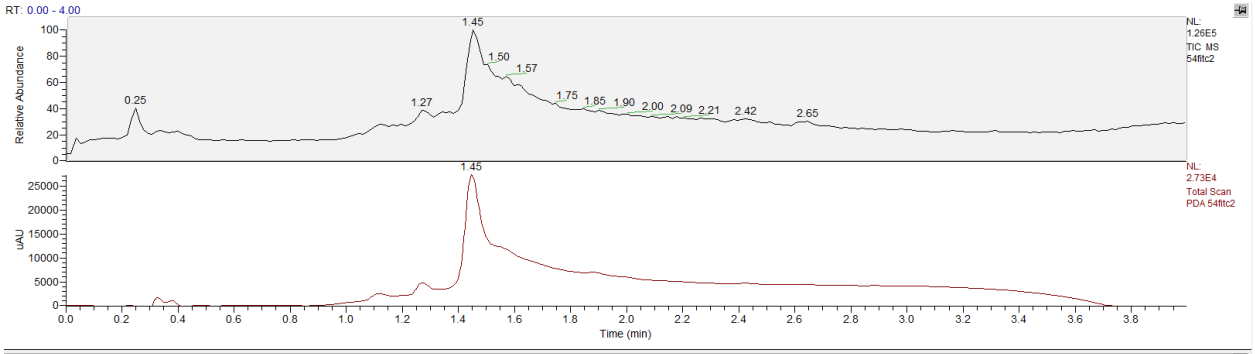


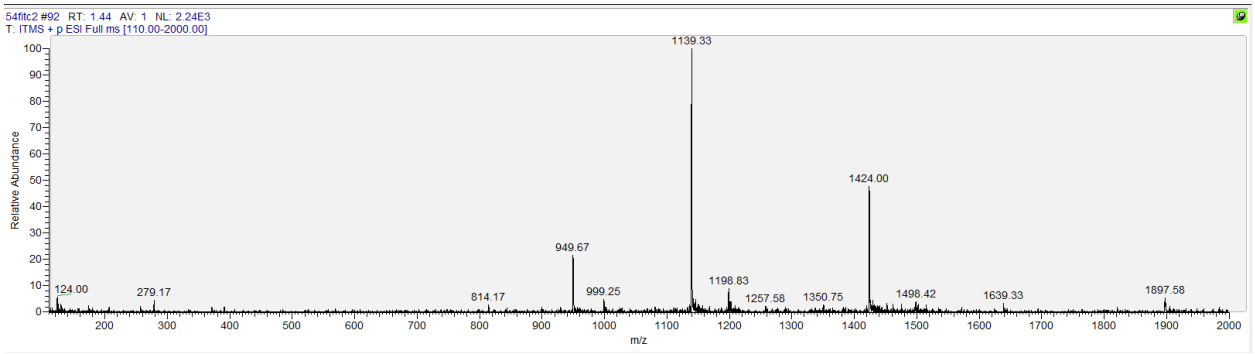
F45H1 (FITC-peg-GAAT-TGCCG-ACTA-CGGCA); Expected Mass for $C_{229}H_{280}N_{110}O_{69}S$: 5706.1503; isotopic mass range (green insert). LC-MS (ESI) RT= 1.44 min. m/z: 1428.33 $[M+4H]^{4+}$, 1142.75 $[M+5H]^{5+}$, MALDI-TOF m/z found: 5713.937 $[M+H]^{+}$

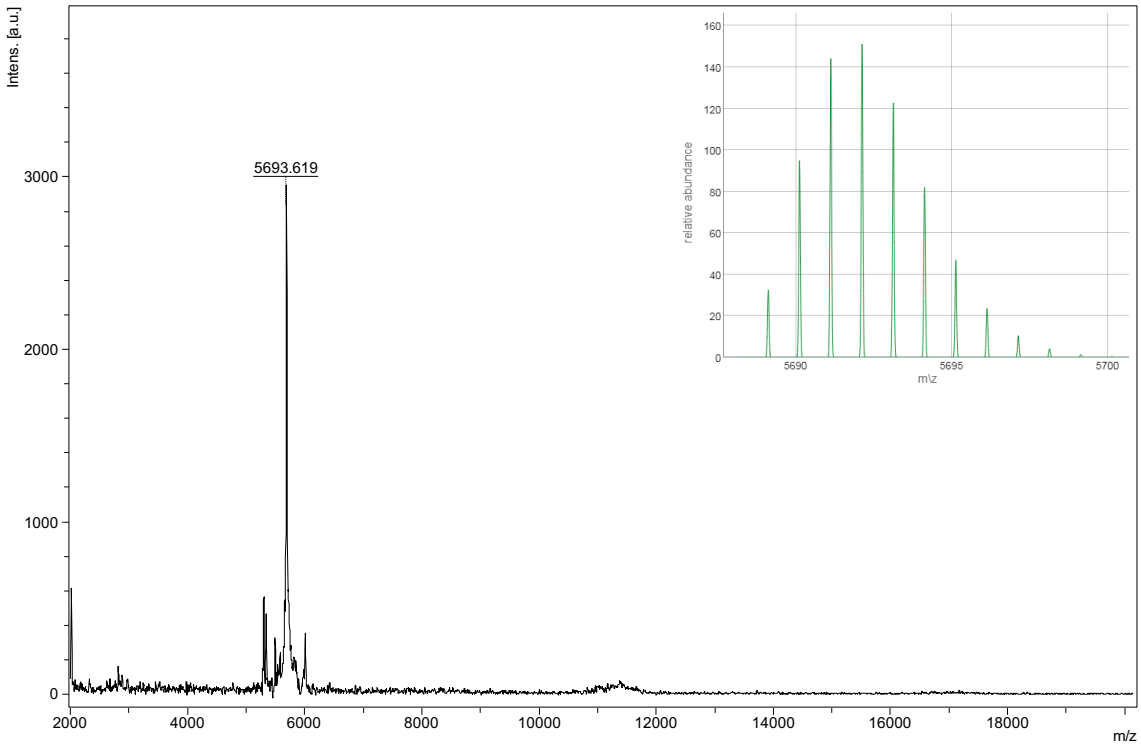




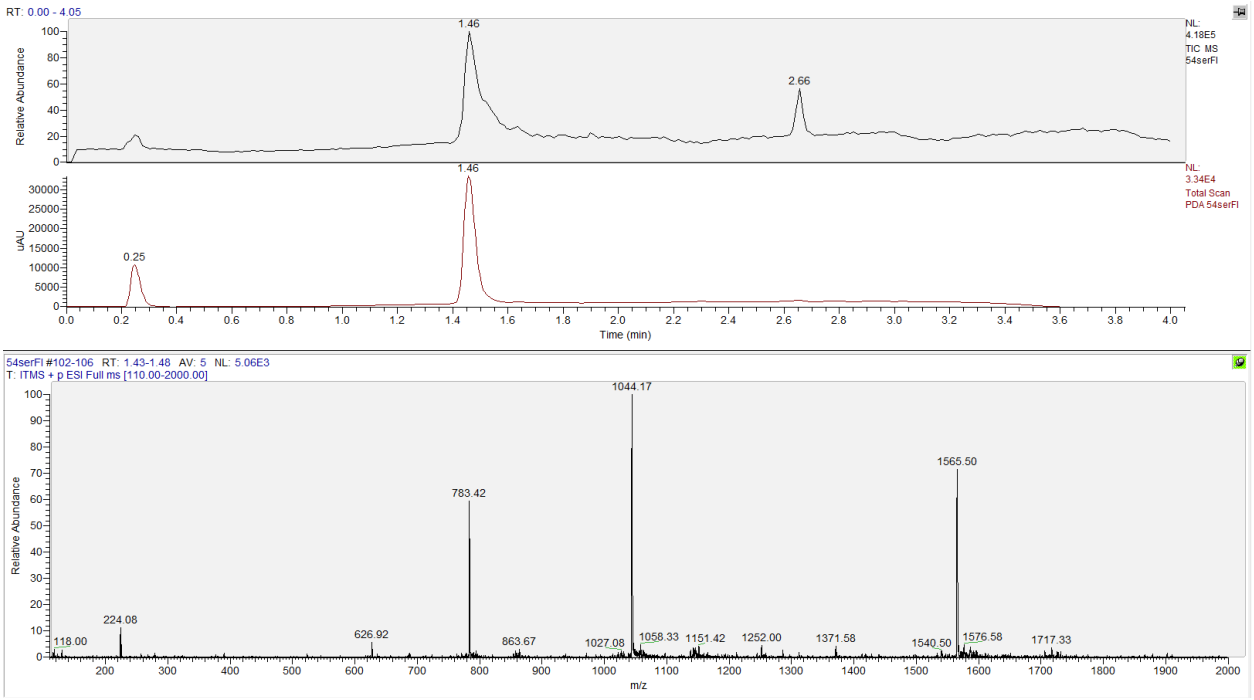
F45H2 (FITC-peg-CGGCA-ATTC-TGCCG-TAGT); Expected Mass for $C_{229}H_{282}N_{104}O_{73}S$: 5688.1272; isotopic mass range (green insert). LC-MS (ESI) RT= 1.45 min. m/z: 1897.58 $[M+3H]^{3+}$, 1424.00 $[M+4H]^{4+}$, 1139.33 $[M+5H]^{5+}$, 949.67 $[M+6H]^{6+}$, MALDI-TOF m/z found: 5693.619 $[M+H]^{+}$

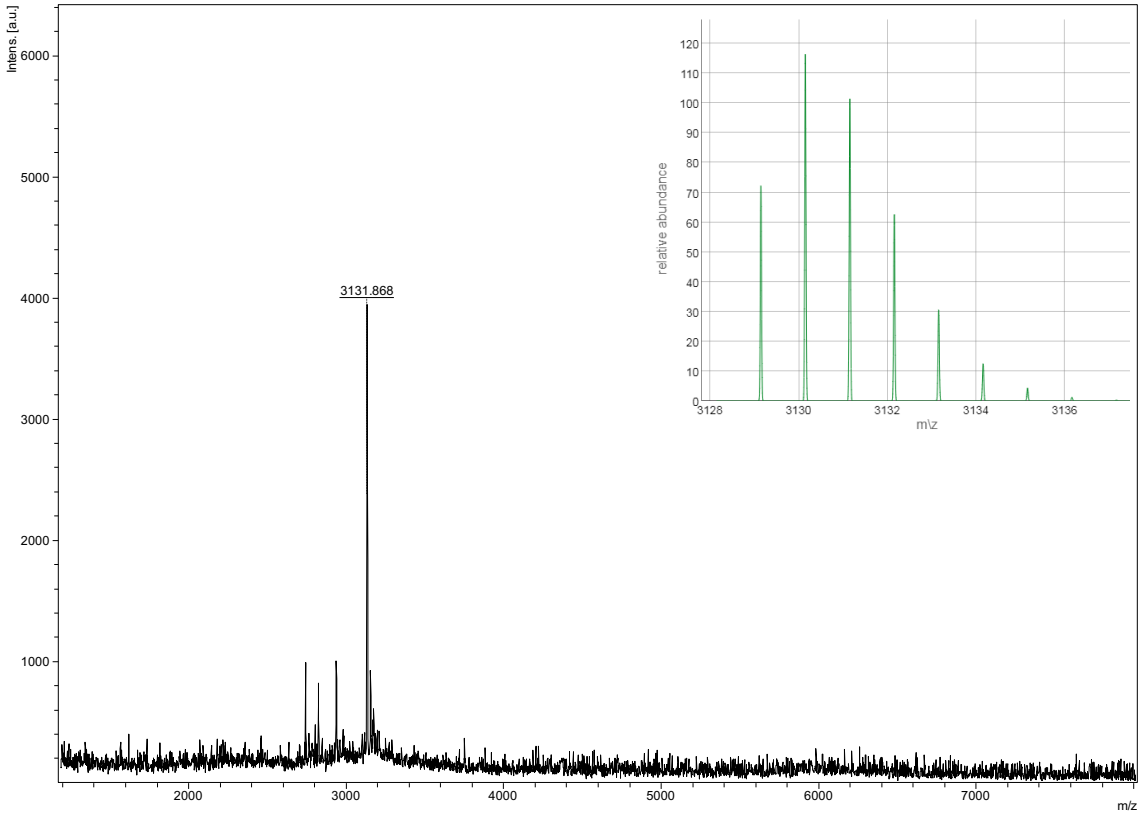




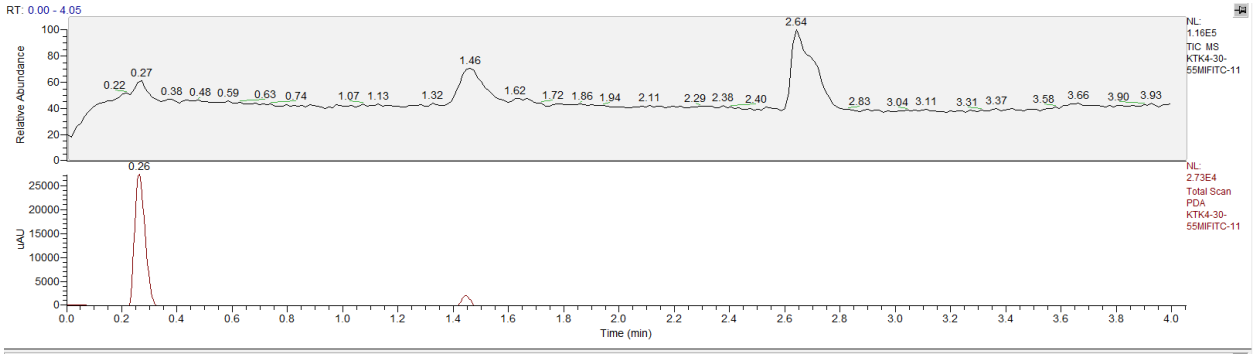


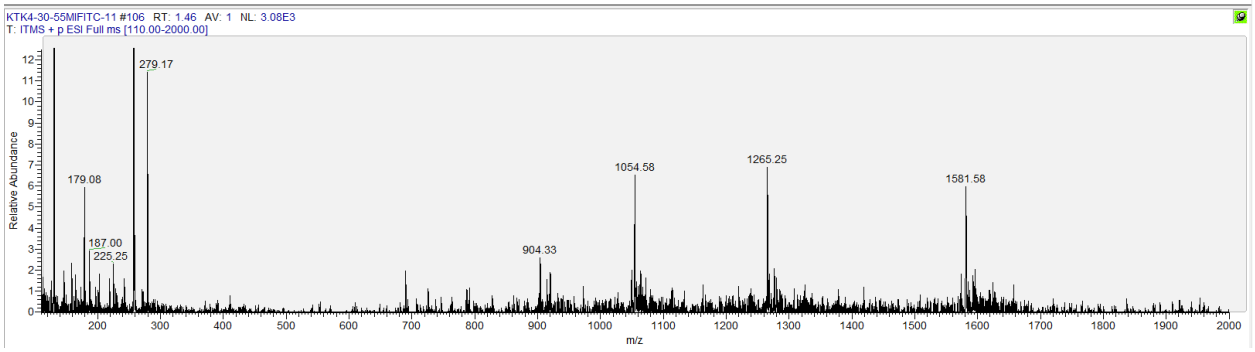
F45I (FITC-peg-ACTA-CGGCA); Expected Mass for $C_{128}H_{153}N_{57}O_{38}S$: 3128.1513; isotopic mass range (green insert). LC-MS (ESI) RT= 1.45 min. m/z: 1565.50 $[M+2H]^{2+}$, 1044.17 $[M+3H]^{3+}$, 783.42 $[M+4H]^{4+}$, 626.92 $[M+5H]^{5+}$, MALDI-TOF m/z found: 3131.868 $[M+H]^{+}$

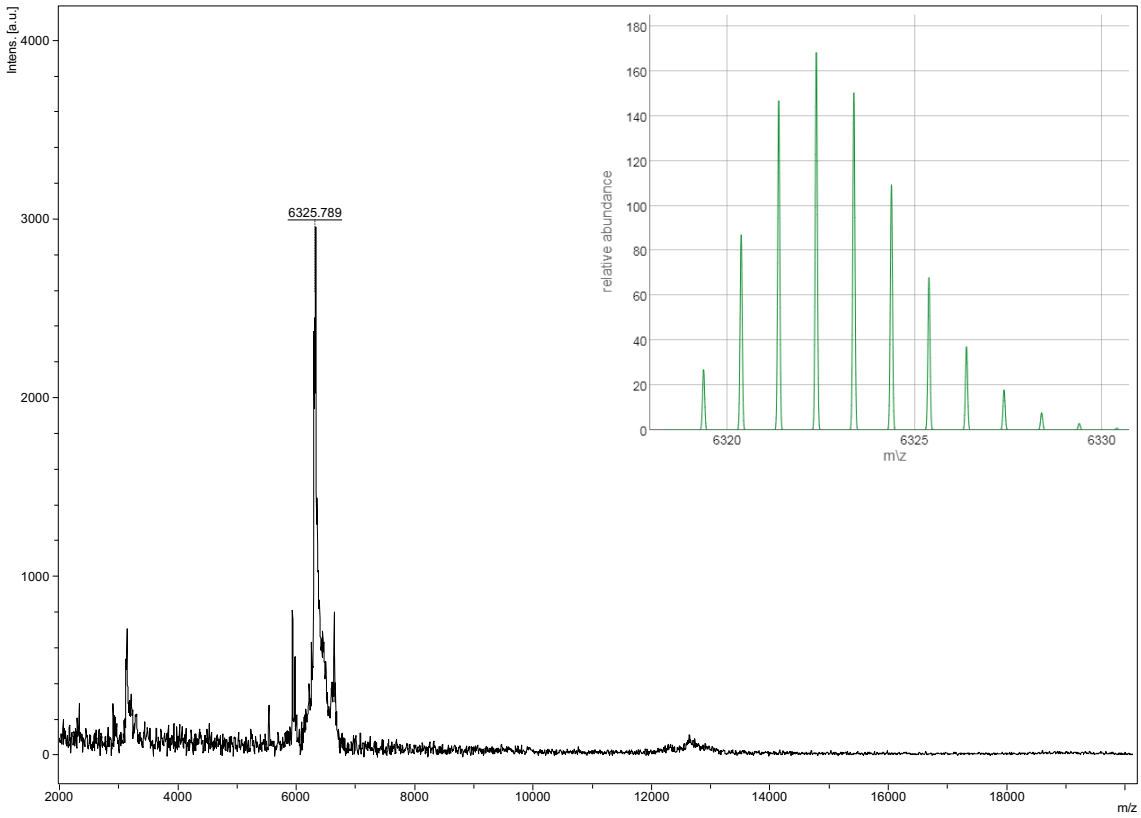




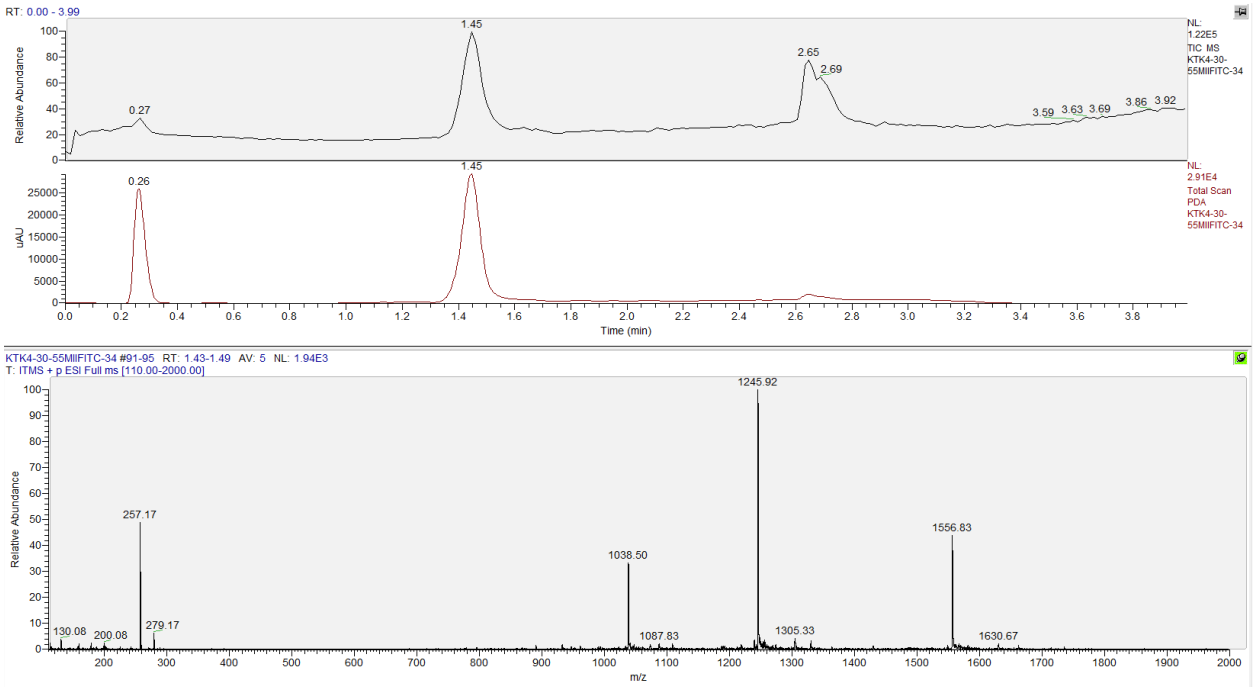
FserH1 (FITC-peg-GAATG-TGCCG-ACTAG-CGGCA); Expected Mass for $C_{252}H_{308}N_{124}O_{76}S$: 6318.3769; isotopic mass range (green insert). LC-MS (ESI) RT= 1.46 min. m/z: 1581.58 $[M+4H]^{4+}$, 1265.25 $[M+5H]^{5+}$, 1054.58 $[M+6H]^{6+}$, 904.33 $[M+7H]^{7+}$, MALDI-TOF m/z found: 6325.789 $[M+H]^{+}$

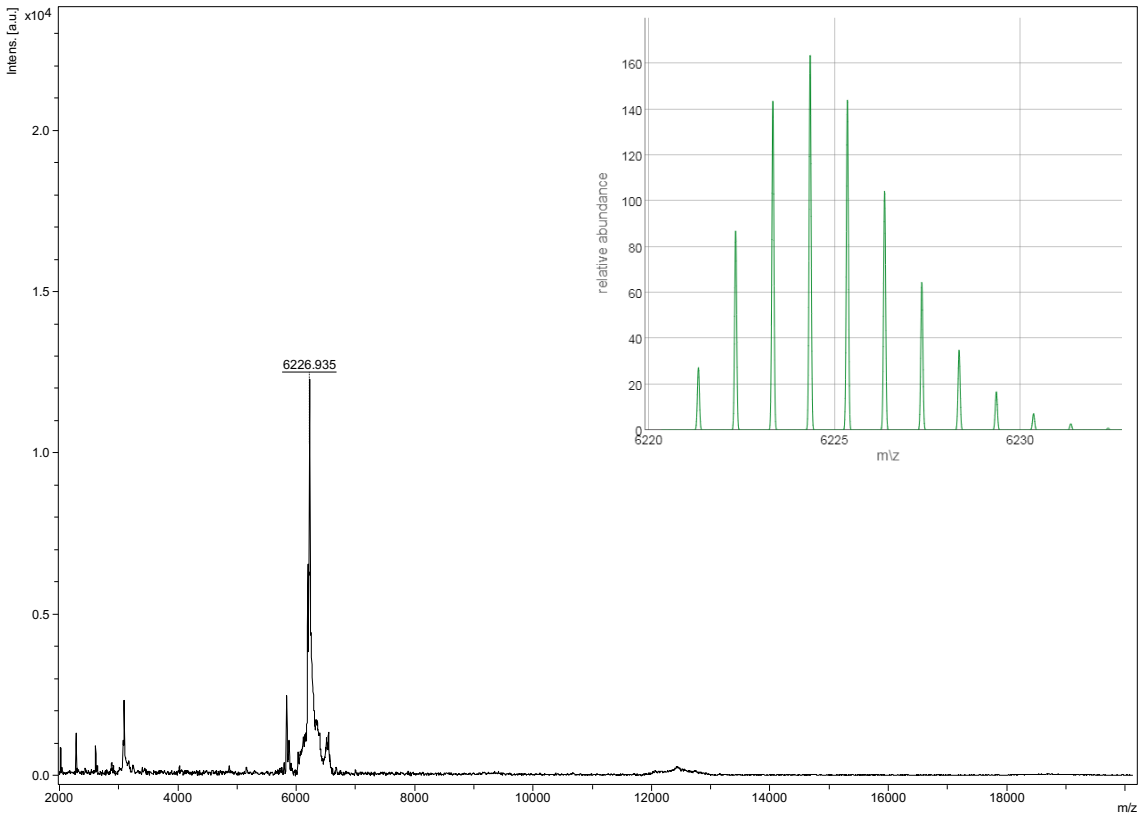




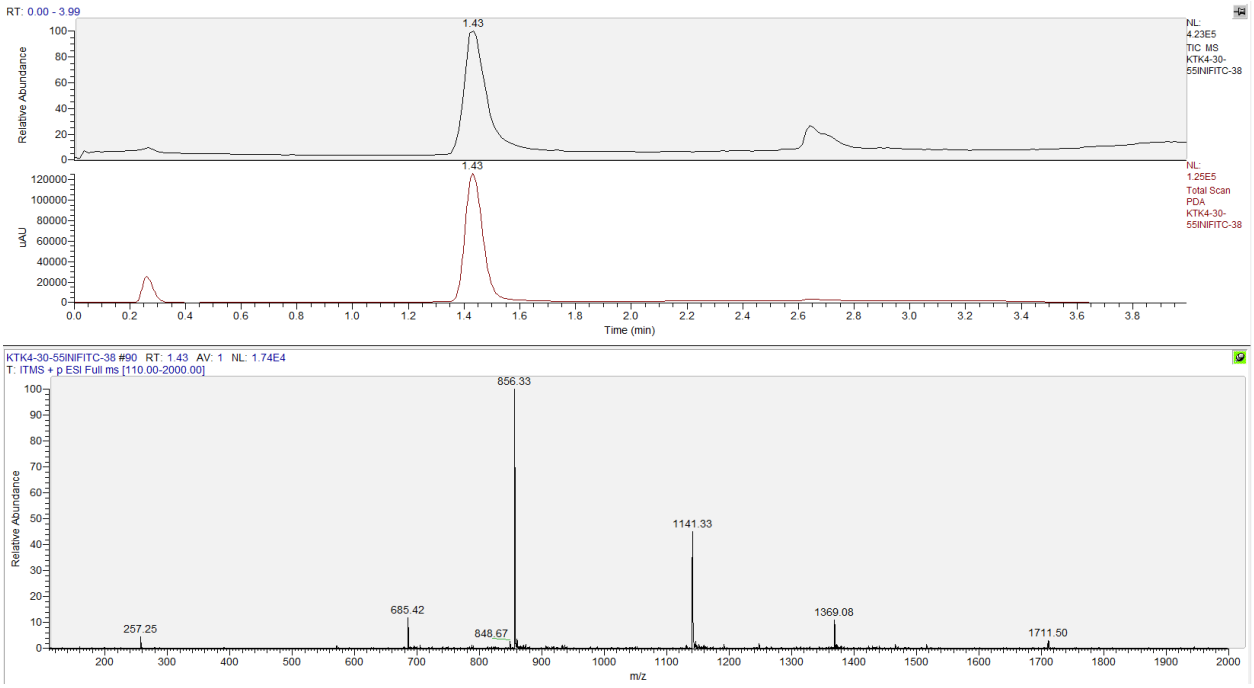


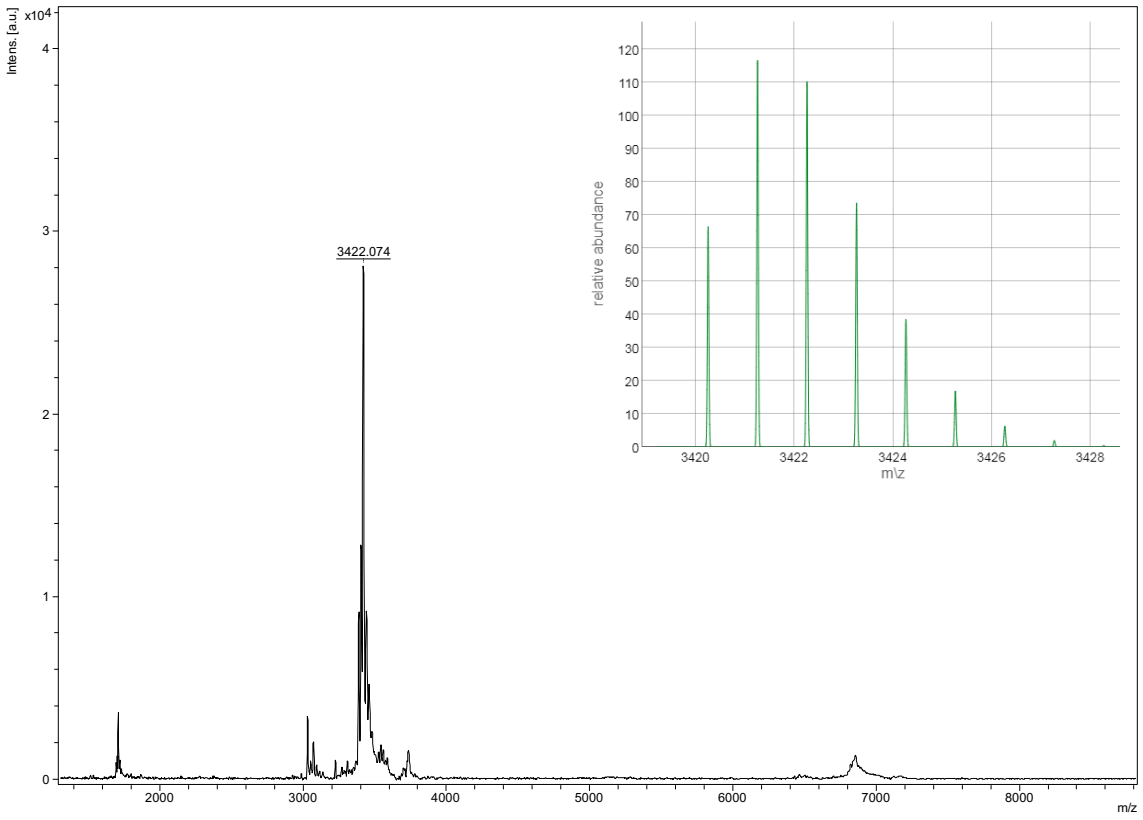
FserH2 (FITC-peg-CGGCA-CATTC-TGCCG-CTAGT); Expected Mass for $C_{250}H_{310}N_{114}O_{80}S$: 6220.3414; isotopic mass range (green insert). LC-MS (ESI) RT= 1.45 min. m/z: 1556.83 $[M+4H]^{4+}$, 1245.92 $[M+5H]^{5+}$, 1038.50 $[M+6H]^{6+}$, MALDI-TOF m/z found: 6226.935 $[M+H]^{+}$



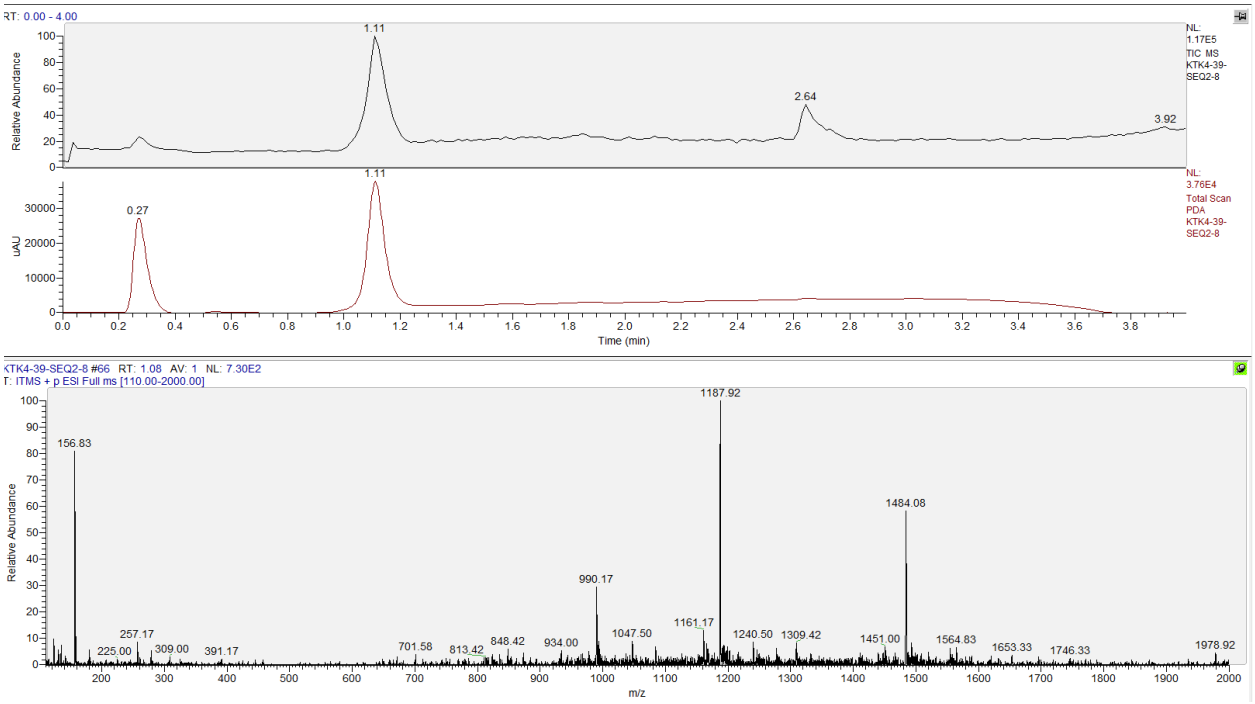


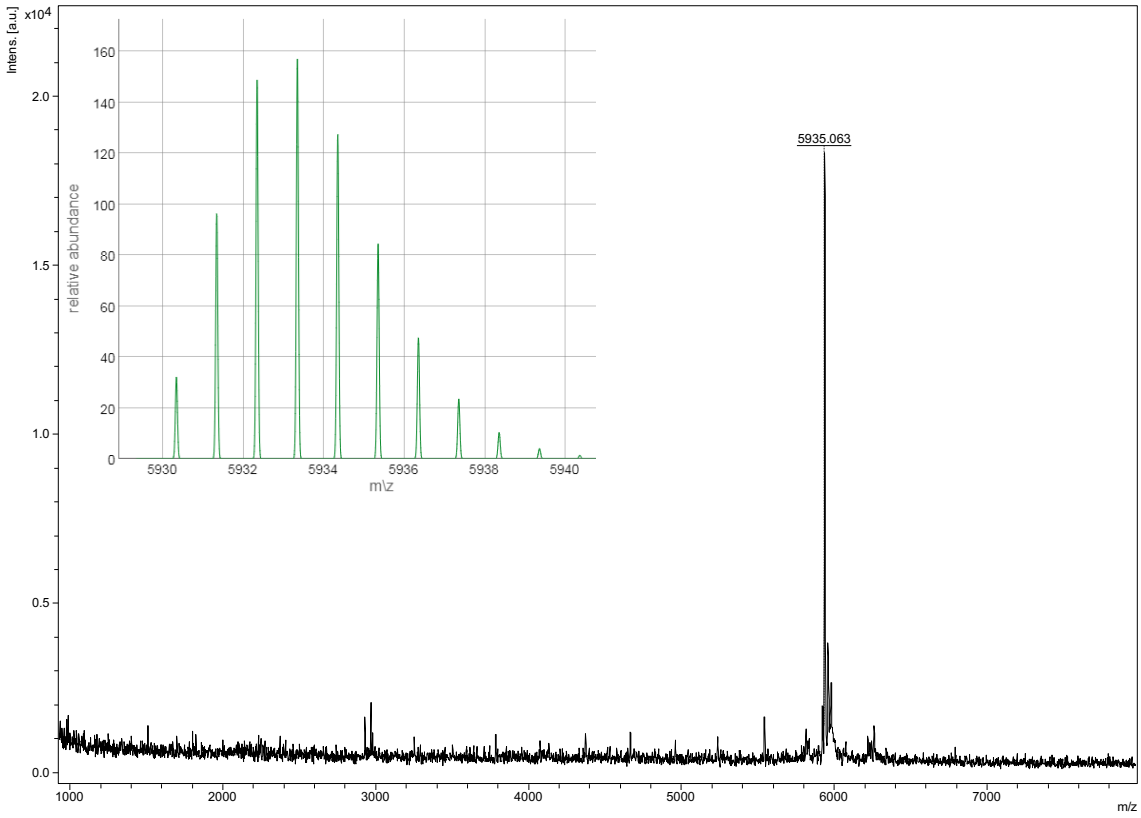
FserI (FITC-peg-ACTAG-CGGCA); Expected Mass for $C_{139}H_{166}N_{64}O_{41}S$: 3419.2593; isotopic mass range (green insert). LC-MS (ESI) RT= 1.43 min. m/z: 1711.50 $[M+2H]^{2+}$, 1141.33 $[M+3H]^{3+}$, 856.33 $[M+4H]^{4+}$, 685.42 $[M+5H]^{5+}$, MALDI-TOF m/z found: 3422.074 $[M+H]^{+}$



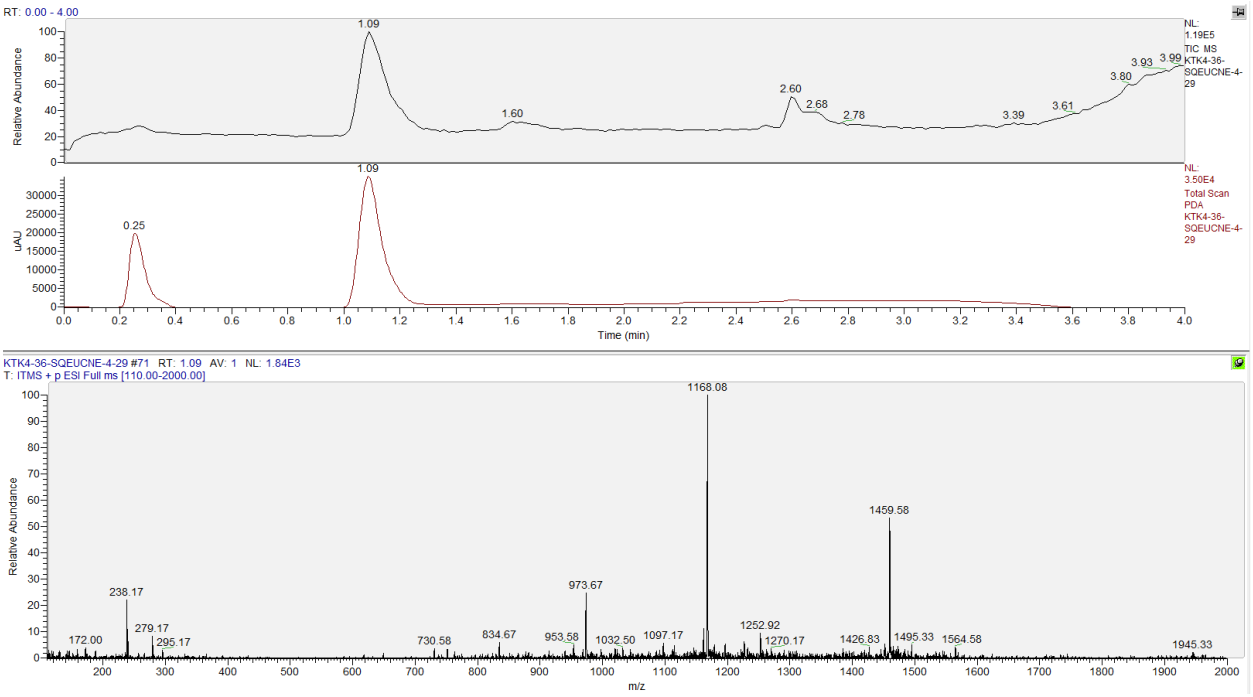


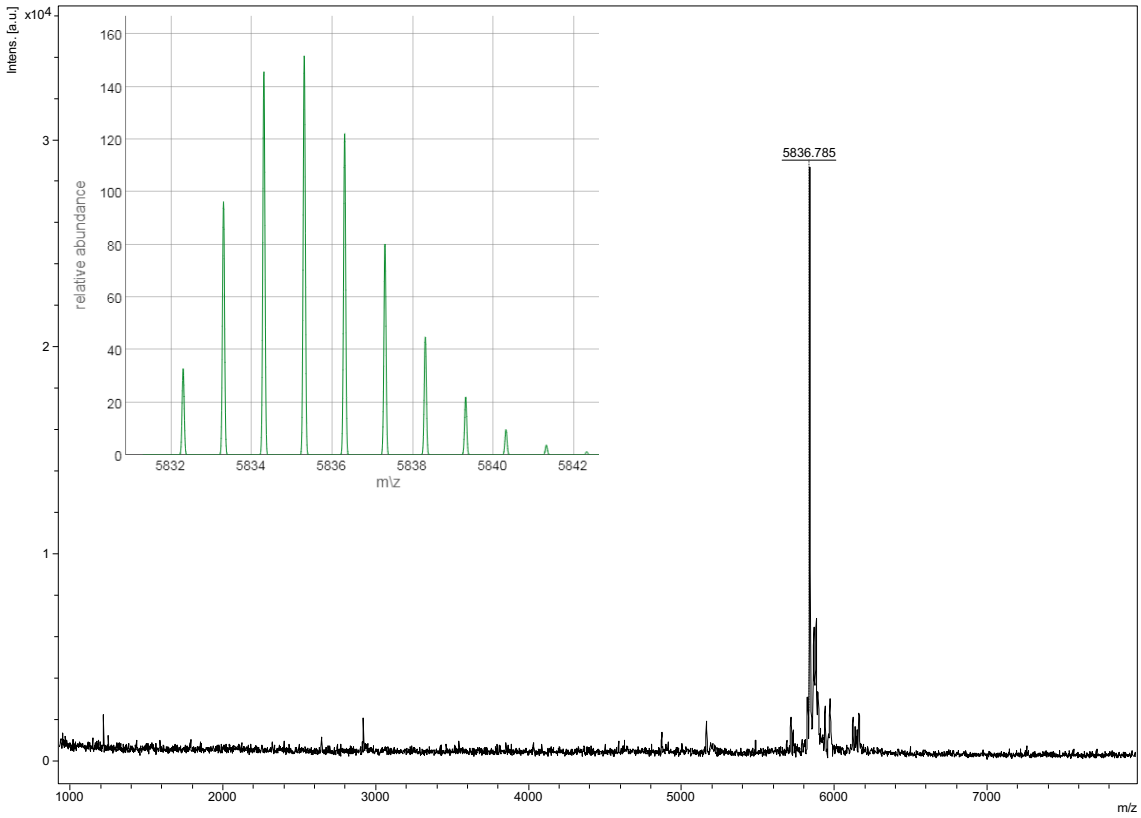
serH1 (NH_2-peg-G<u>A</u>A<u>T</u>G-<u>T</u>G<u>C</u>C<u>G</u>-A<u>C</u>T<u>A</u>G-<u>C</u>G<u>G</u>C<u>A</u>); Expected Mass for $C_{231}H_{297}N_{123}O_{71}$: 5929.3411; isotopic mass range (green insert). LC-MS (ESI) RT= 1.11 min. m/z: 1484.08 $[M+4H]^{4+}$, 1187.92 $[M+5H]^{5+}$, 990.17 $[M+6H]^{6+}$, MALDI-TOF m/z found: 5935.063 $[M+H]^{+}$



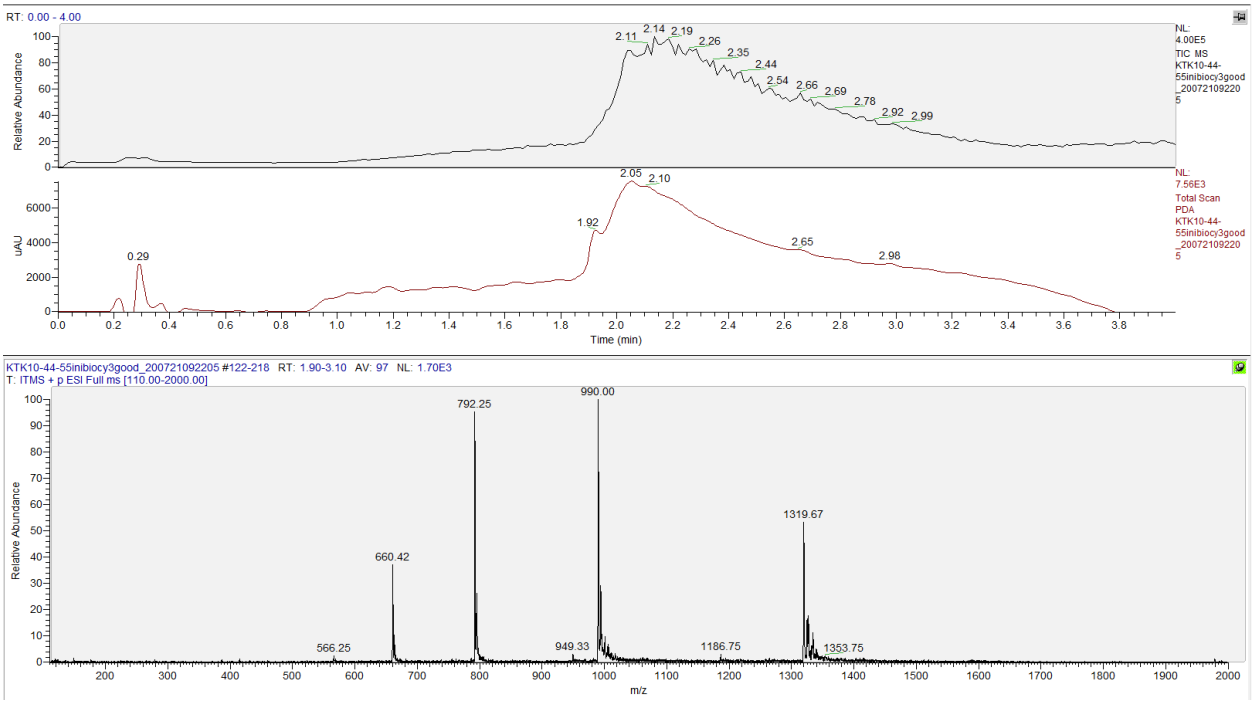


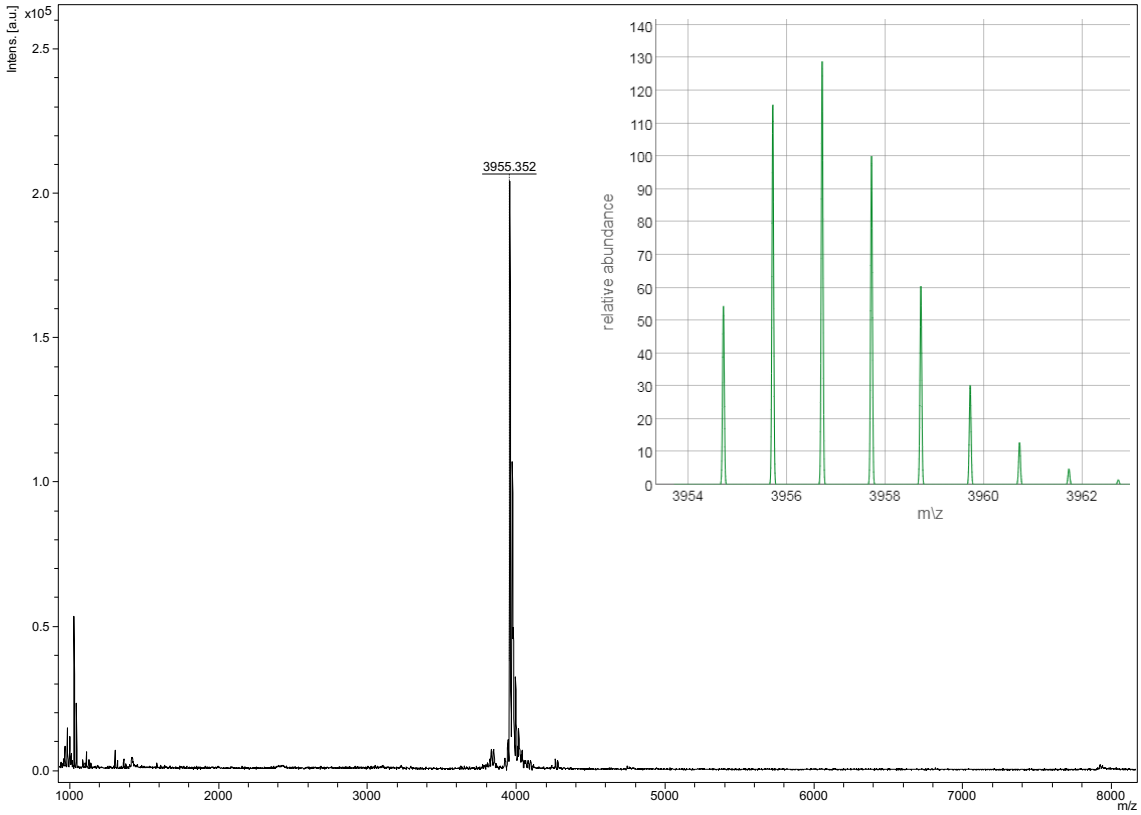
serH2 (NH_2-peg-CGGCA-CATTC-TGCCG-CTAGT); Expected Mass for $C_{229}H_{299}N_{113}O_{75}$: 5831.3056; isotopic mass range (green insert). LC-MS (ESI) RT= 1.09 min. m/z: 1459.58 $[M+4H]^{4+}$, 1168.08 $[M+5H]^{5+}$, 973.67 $[M+6H]^{6+}$, MALDI-TOF m/z found: 5836.785 $[M+H]^{+}$



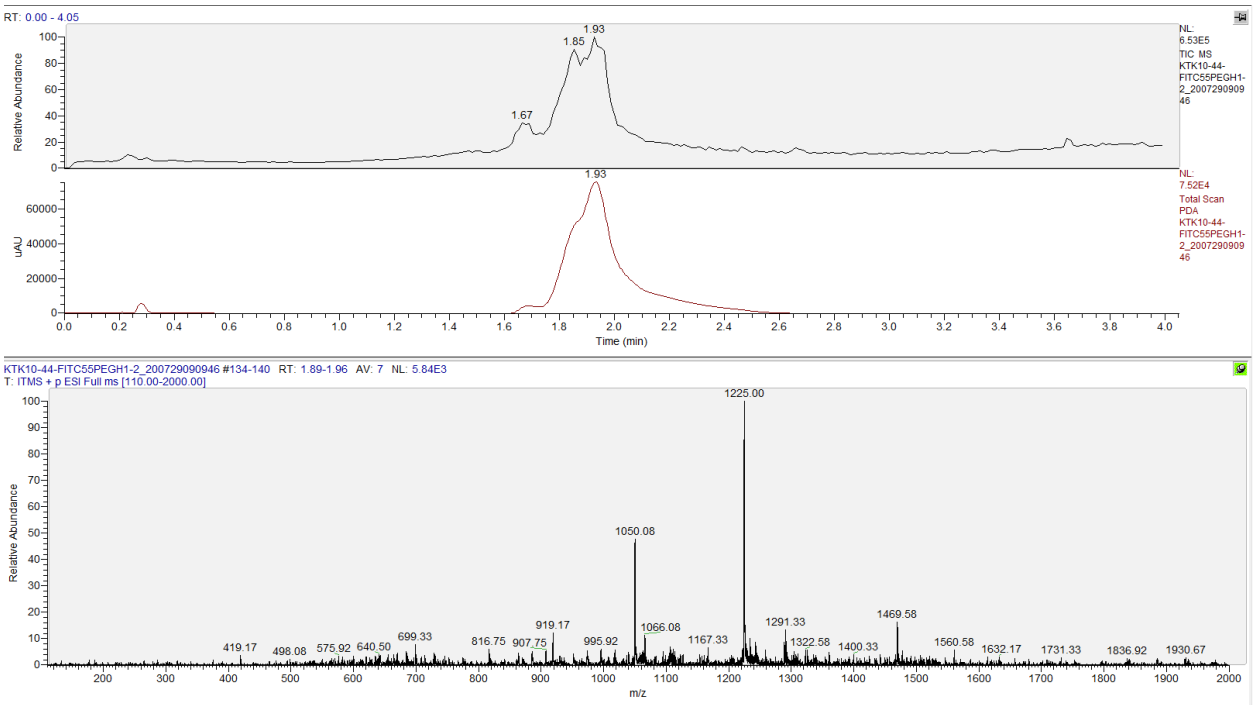


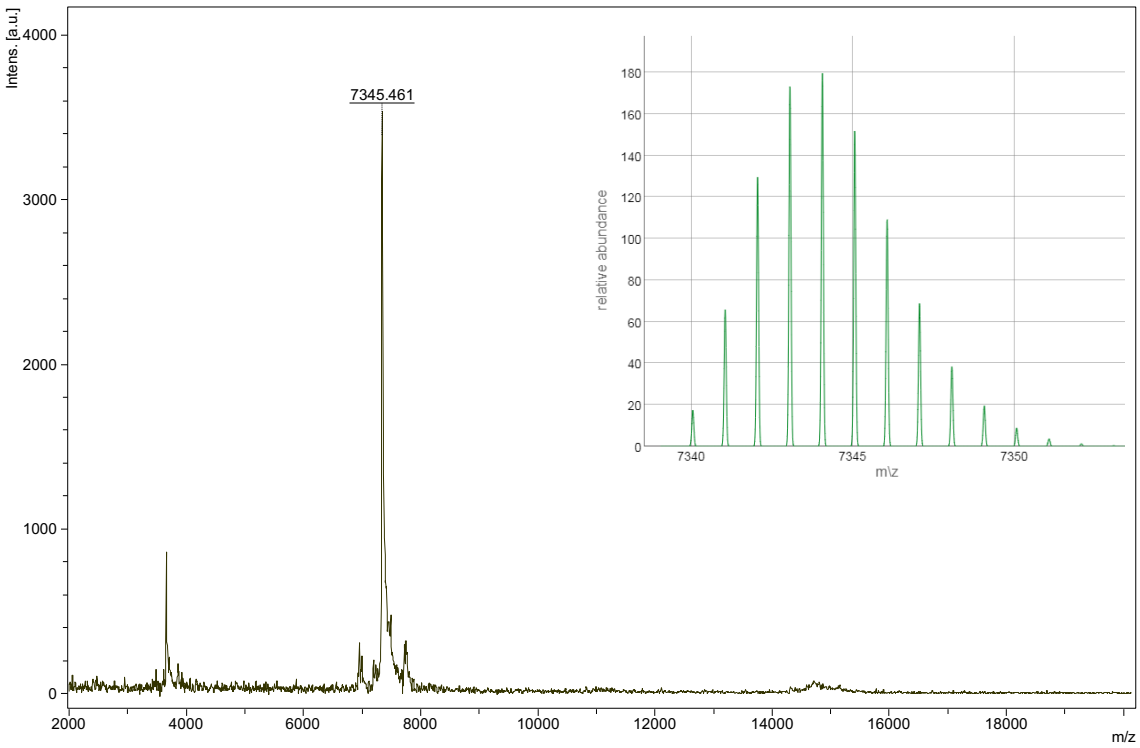
Cy3serI (biotin-peg-Lys(cy3)-peg-ACTAG-CGGCA); Expected Mass for $C_{169}H_{225}N_{70}O_{43}S^{+}$: 3954.7287; isotopic mass range (green insert). LC-MS (ESI) RT= 2.11 min. m/z: 1319.67 $[M+2H]^{3+}$, 990.00 $[M+3H]^{4+}$, 792.25 $[M+4H]^{5+}$, 660.42 $[M+5H]^{6+}$, MALDI-TOF m/z found: 3955.352 $[M]^{+}$



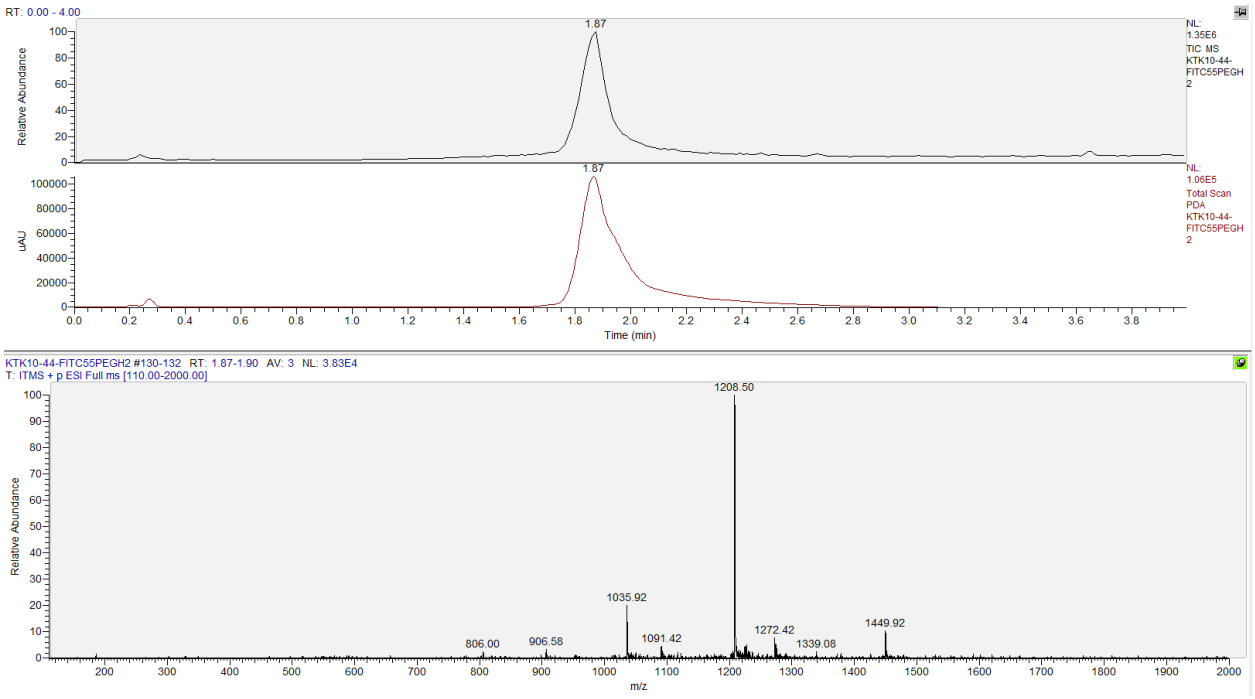


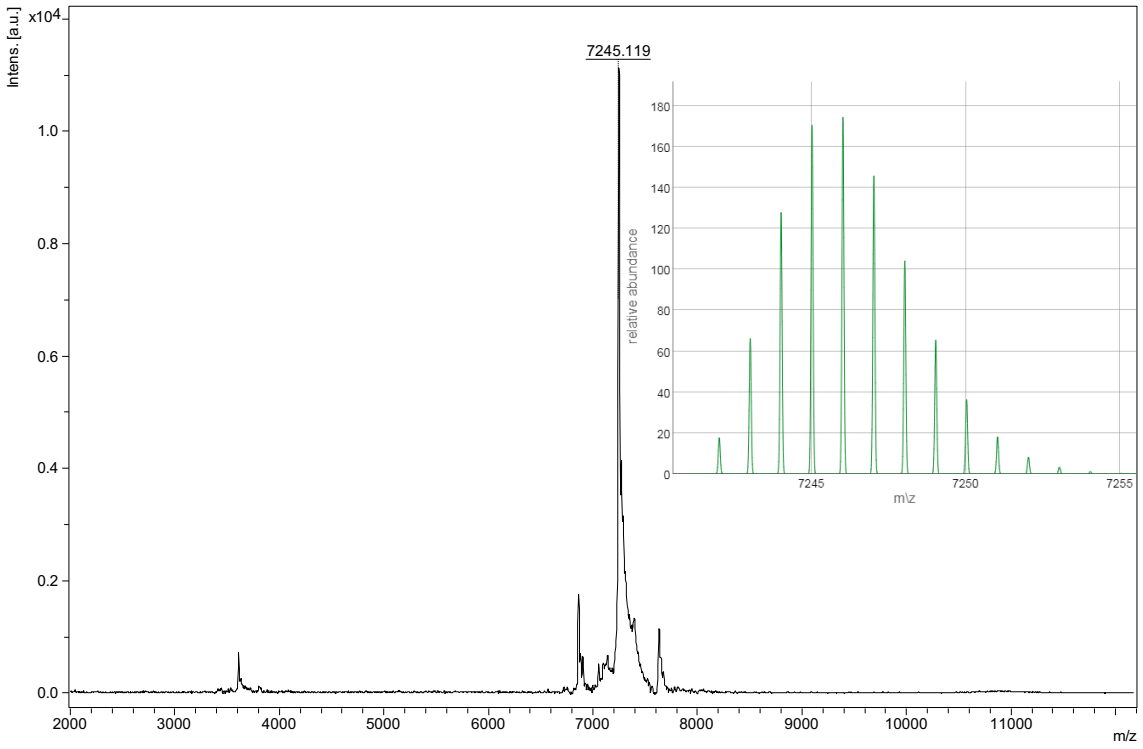
FpegH1 (FITC-peg-GAATG-TGCCG-ACTAG-CGGCA); Expected Mass for $C_{302}H_{408}N_{124}O_{96}S$: 7339.0577; isotopic mass range (green insert). LC-MS (ESI) RT= 1.93 min. m/z: 1469.58 $[M+5H]^{5+}$, 1225.00 $[M+6H]^{6+}$, 1050.08 $[M+7H]^{7+}$, 919.17 $[M+8H]^{8+}$, MALDI-TOF m/z found: 7345.461 $[M+H]^{+}$



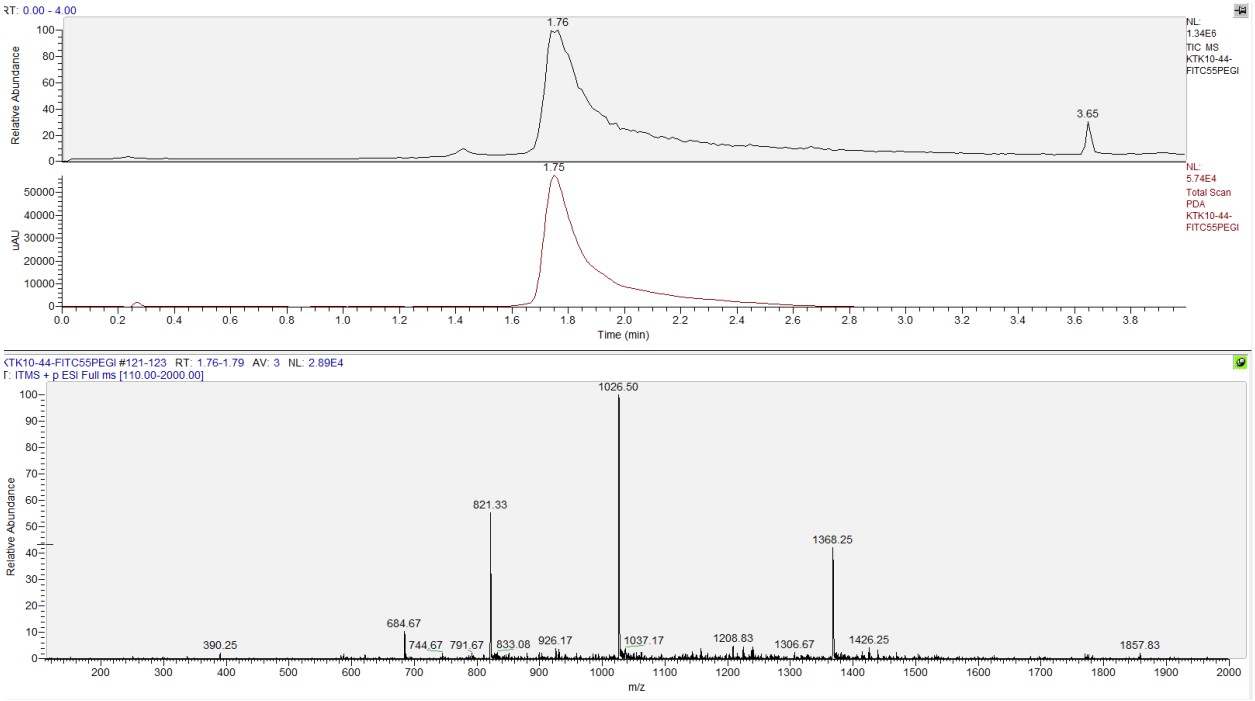


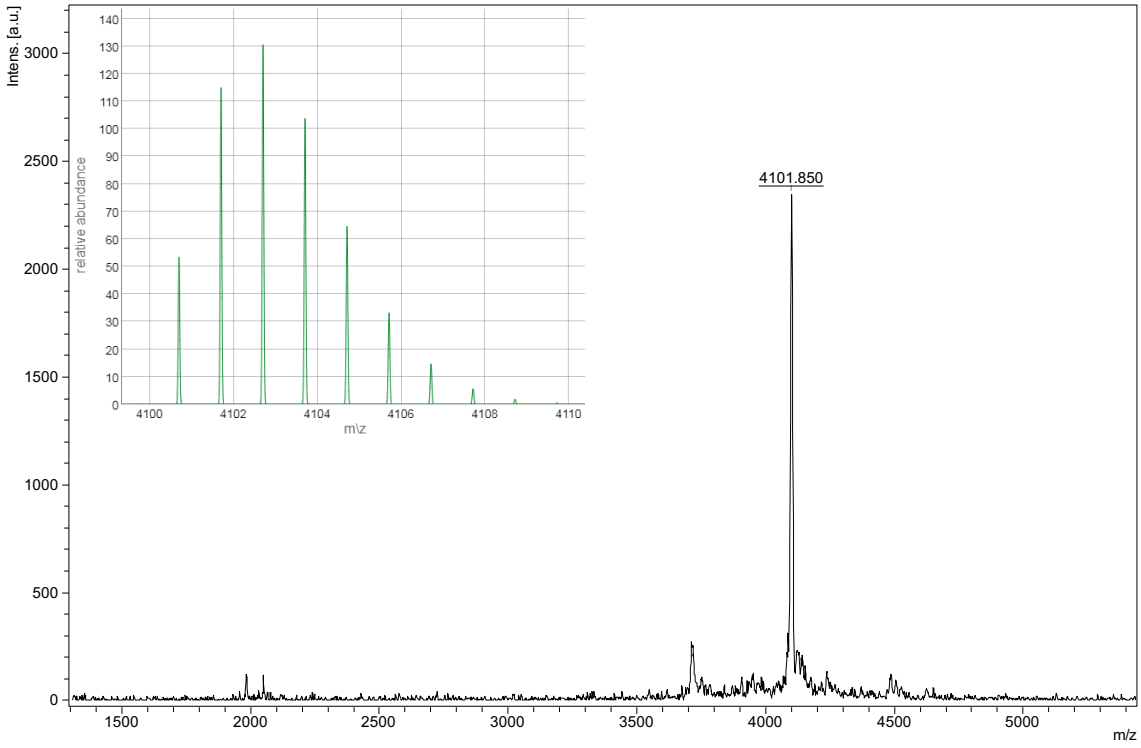
FpegH2 (FITC-peg-CGGCA-CATTC-TGCCG-CTAGT); Expected Mass for $C_{300}H_{410}N_{114}O_{100}S$: 7241.0222; isotopic mass range (green insert). LC-MS (ESI) RT= 1.87 min. m/z: 1449.92 $[M+5H]^{5+}$, 1208.50 $[M+6H]^{6+}$, 1035.92 $[M+7H]^{7+}$, 906.58 $[M+8H]^{8+}$, MALDI-TOF m/z found: 7245.119 $[M+H]^{+}$



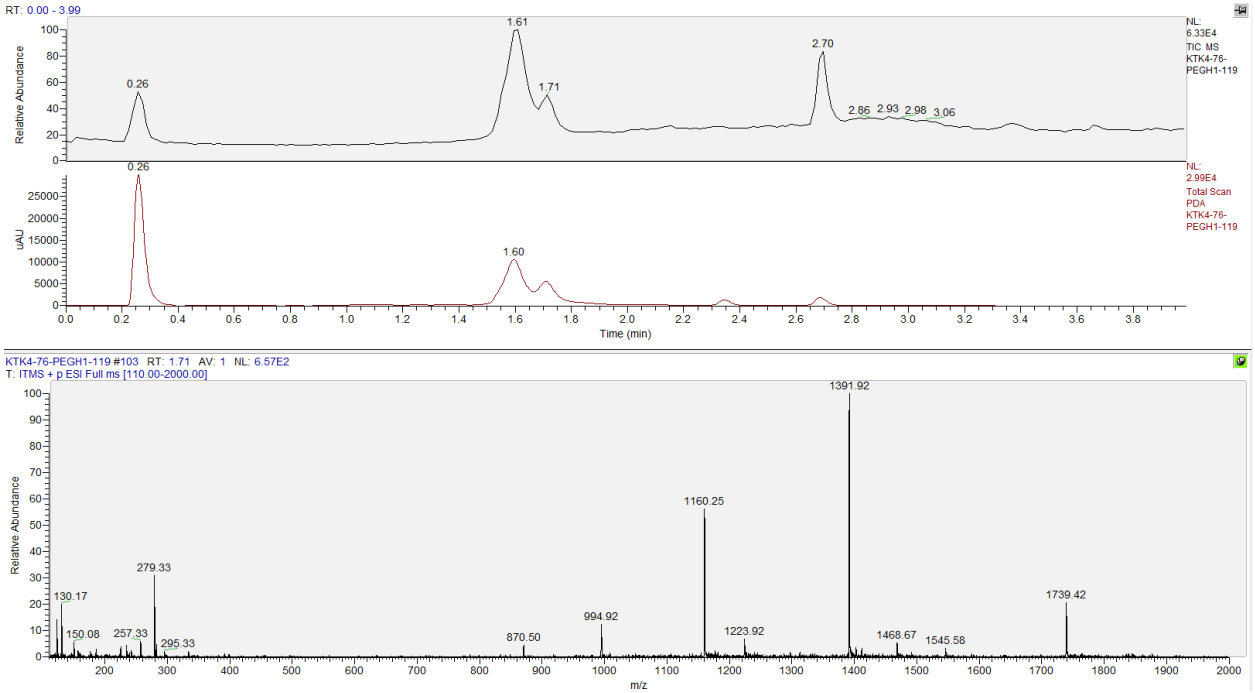


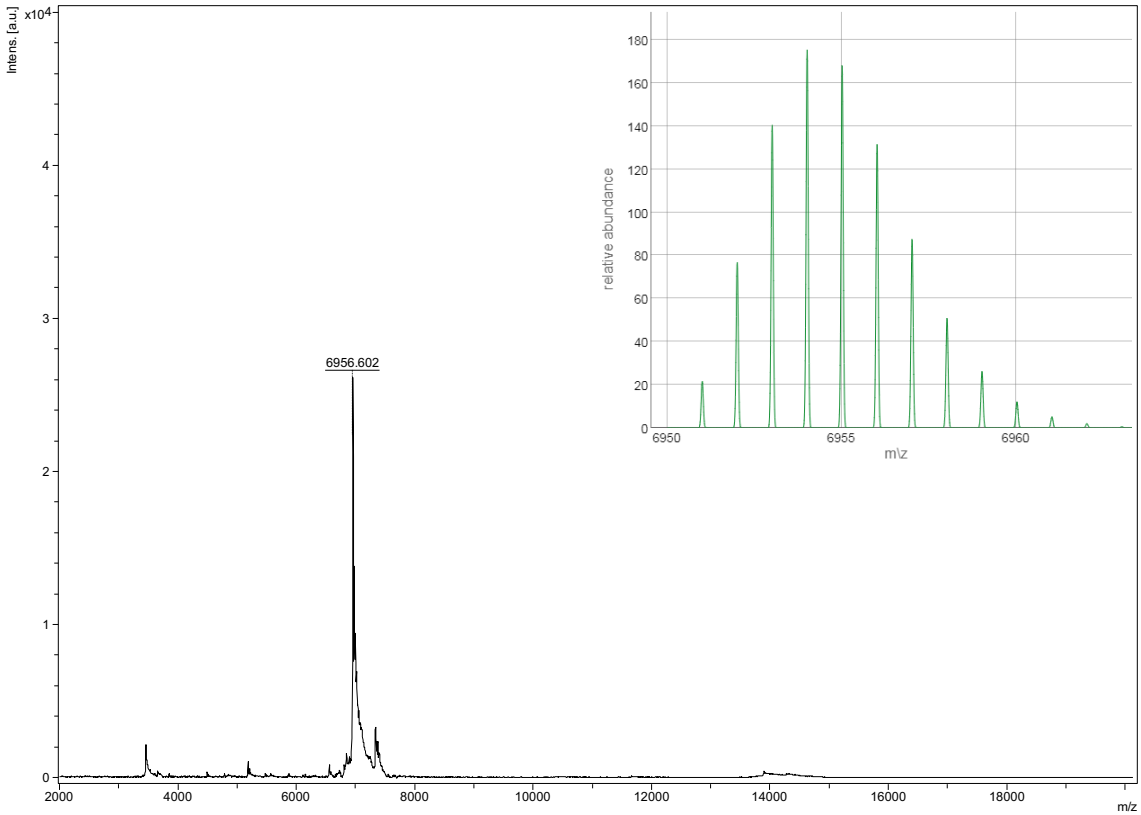
FpegI (FITC-peg-ACTAG-CGGCA-Lys-Ac); Expected Mass for $C_{172}H_{230}N_{66}O_{53}S$: 4099.7052; isotopic mass range (green insert). LC-MS (ESI) RT= 1.76 min. m/z: 1368.25 $[M+3H]^{3+}$, 1026.50 $[M+4H]^{4+}$, 821.33 $[M+5H]^{5+}$, 684.67 $[M+6H]^{6+}$, MALDI-TOF m/z found: 4101.85 $[M+H]^{+}$



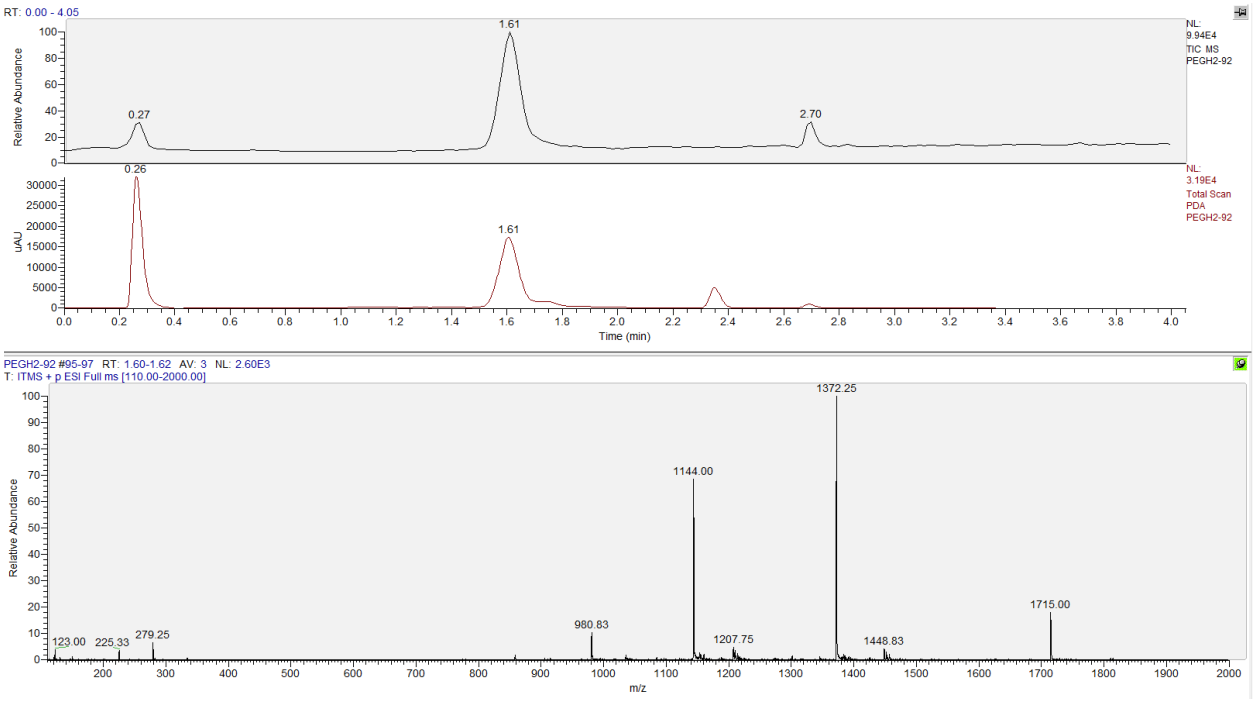


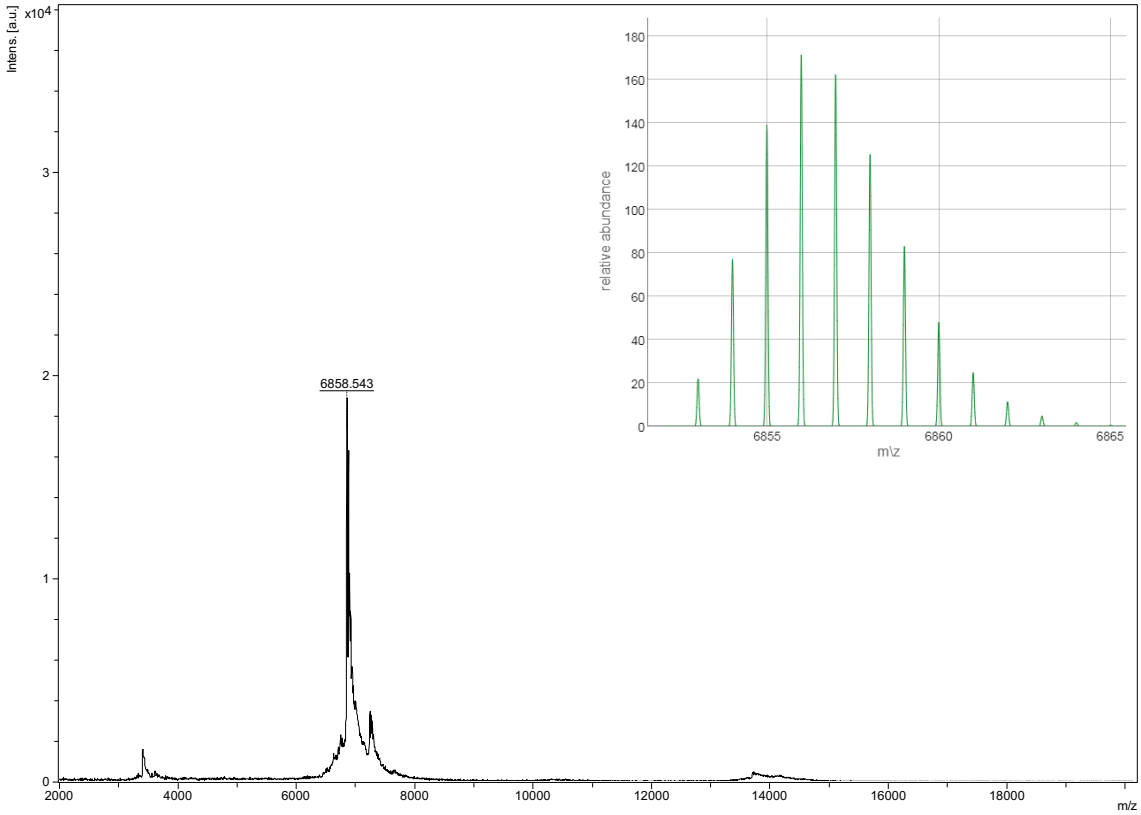
pegH1 (NH_2-peg-GAATG-TGCCG-ACTAG-CGGCA); Expected Mass for $C_{281}H_{397}N_{123}O_{91}$: 6950.0219; isotopic mass range (green insert). LC-MS (ESI) RT= 1.61 min. m/z: 1739.42 $[M+4H]^{4+}$, 1391.92 $[M+5H]^{5+}$, 1160.25 $[M+6H]^{6+}$, 994.92 $[M+7H]^{7+}$, MALDI-TOF m/z found: 6956.602 $[M+H]^{+}$



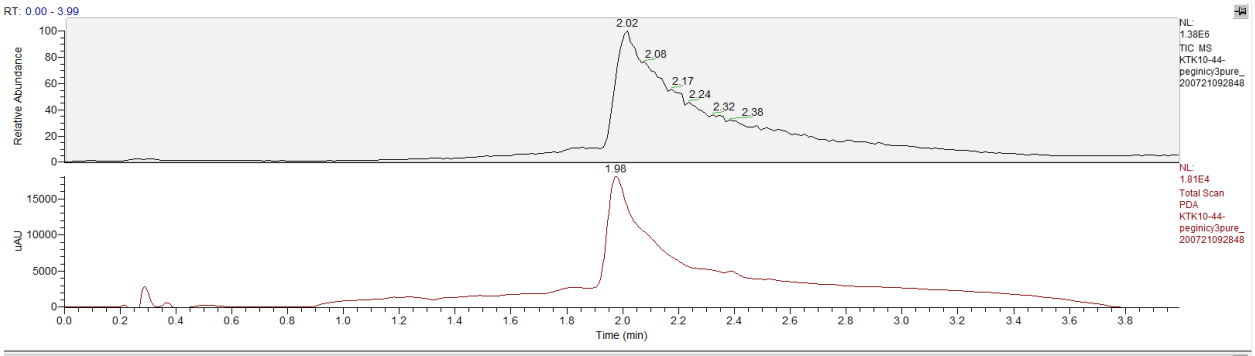


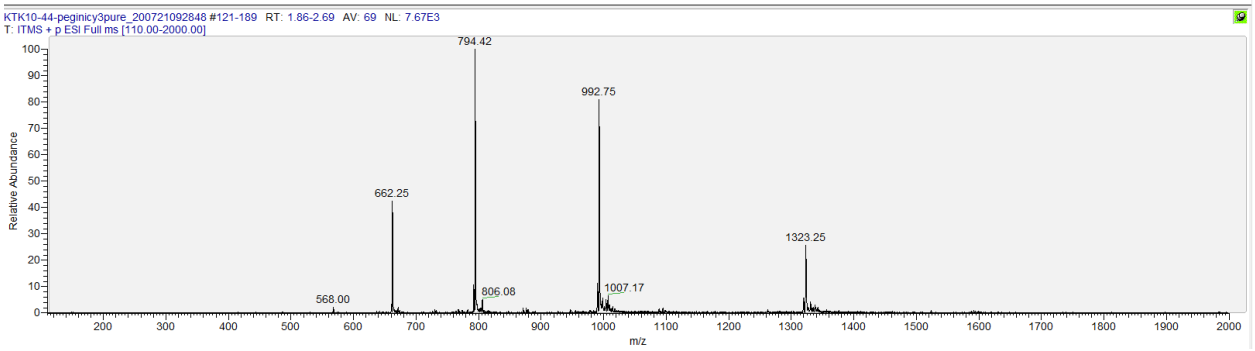
pegH2 (NH_2-peg-C<u>G</u><u>G</u><u>C</u>A-<u>C</u><u>A</u><u>T</u><u>T</u><u>C</u>-T<u>G</u><u>C</u><u>C</u>G-<u>C</u><u>T</u><u>A</u><u>G</u><u>T</u>); Expected Mass for $C_{279}H_{399}N_{113}O_{95}$: 6851.9864; isotopic mass range (green insert). LC-MS (ESI) RT= 1.61 min. m/z: 1715.00 $[M+4H]^{4+}$, 1372.25 $[M+5H]^{5+}$, 1144.00 $[M+6H]^{6+}$, 980.83 $[M+7H]^{7+}$, MALDI-TOF m/z found: 6858.543 $[M+H]^{+}$

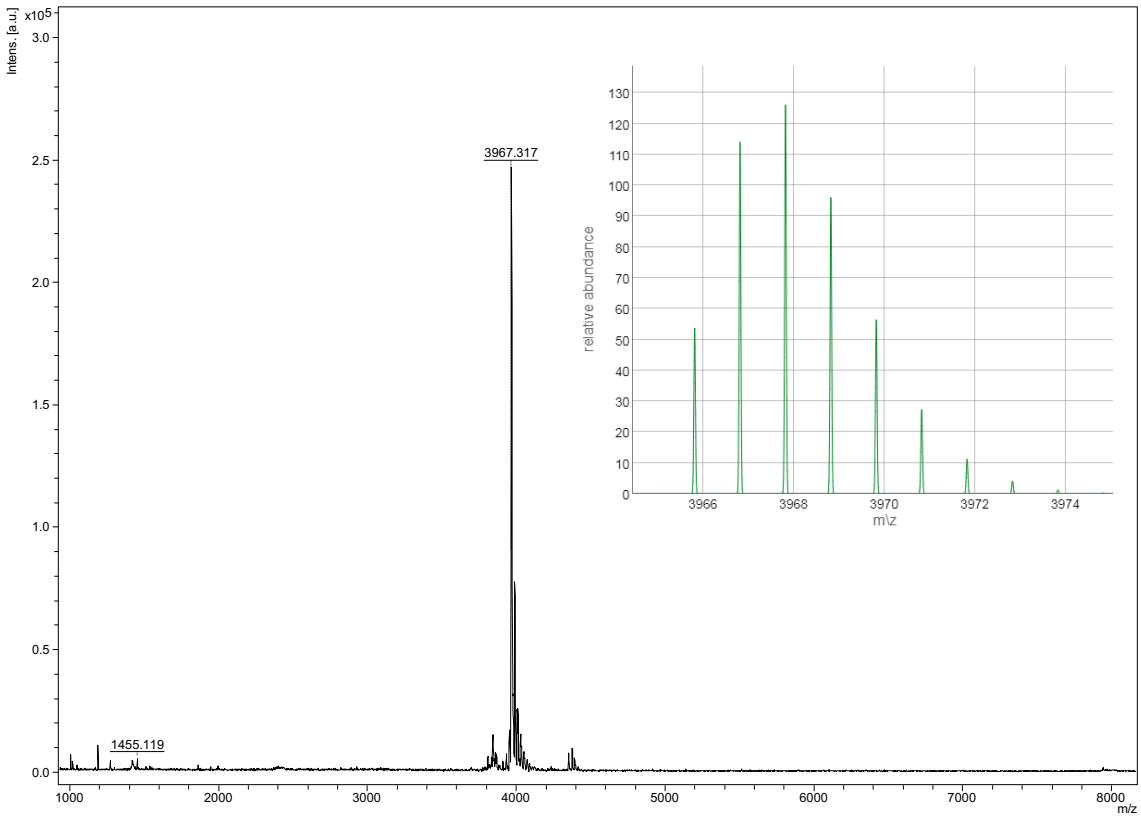




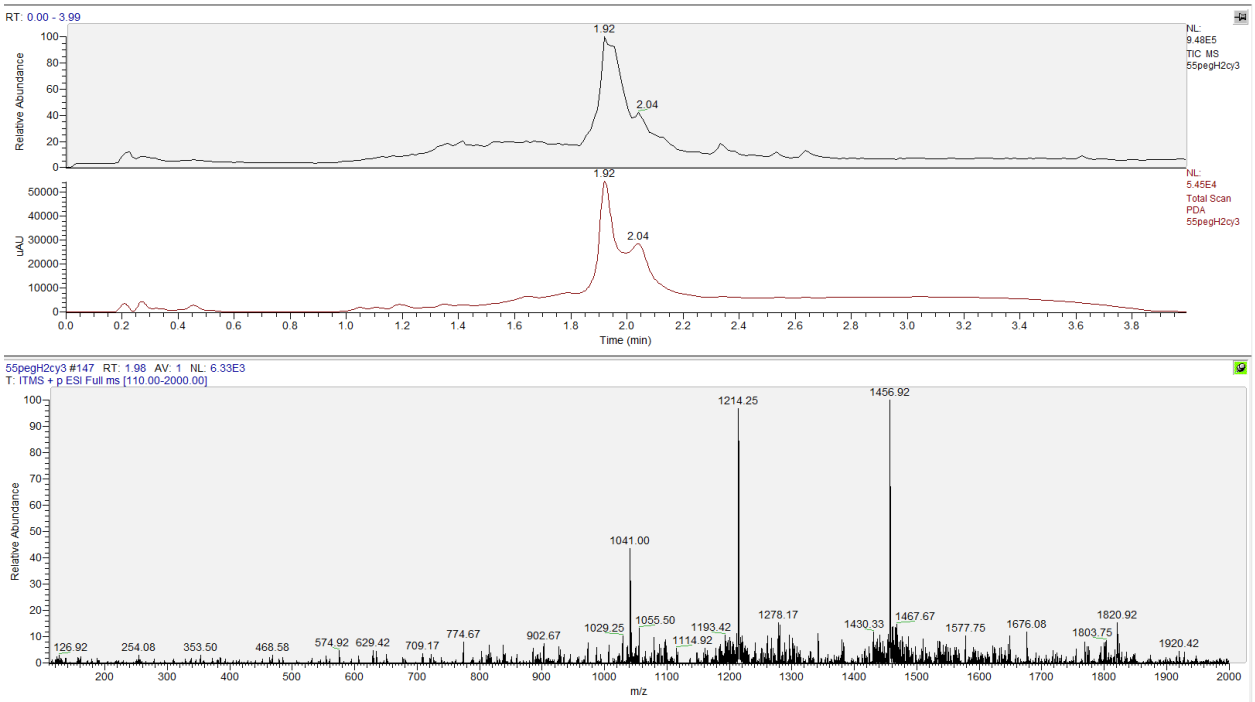
Cy3pegI (cy3-peg-A<u>C</u>T<u>A</u>G-<u>C</u>G<u>G</u>C<u>A</u>); Expected Mass for $C_{172}H_{238}N_{65}O_{47}^{+}$: 3965.8232; isotopic mass range (green insert). LC-MS (ESI) RT= 2.02 min. m/z: 1323.25 $[M+2H]^{3+}$, 992.75 $[M+3H]^{4+}$, 794.42 $[M+4H]^{5+}$, 662.25 $[M+5H]^{6+}$, MALDI-TOF m/z found: 3967.317 $[M]^{+}$

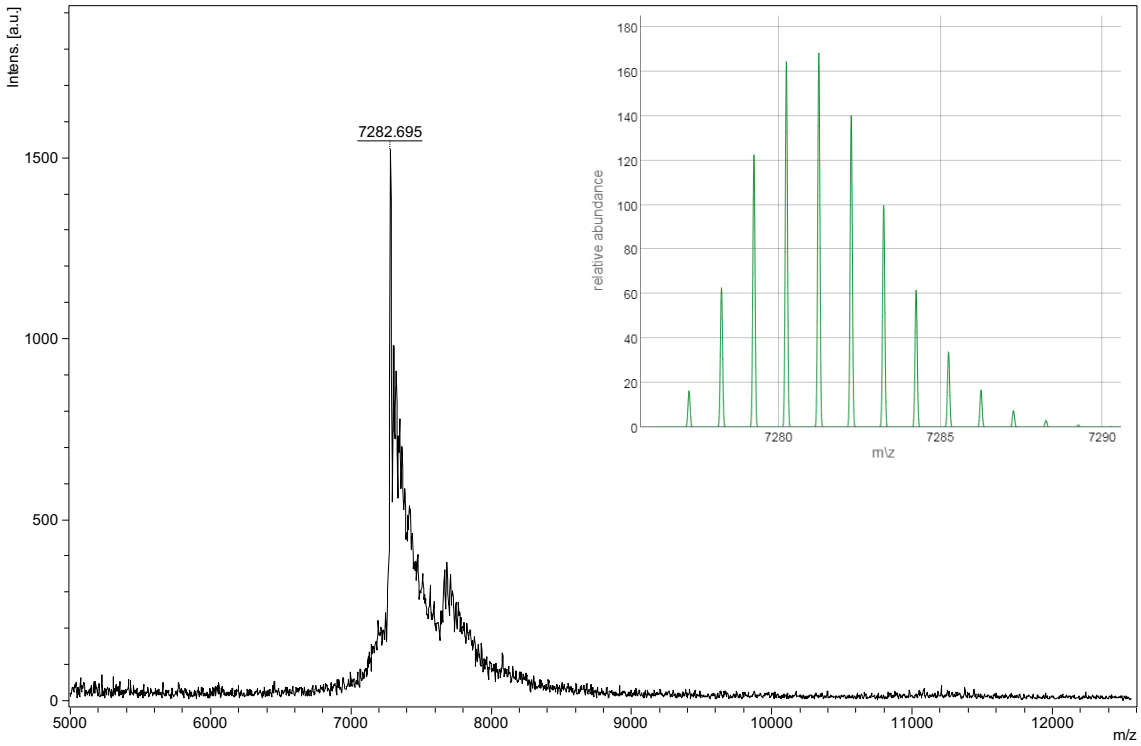




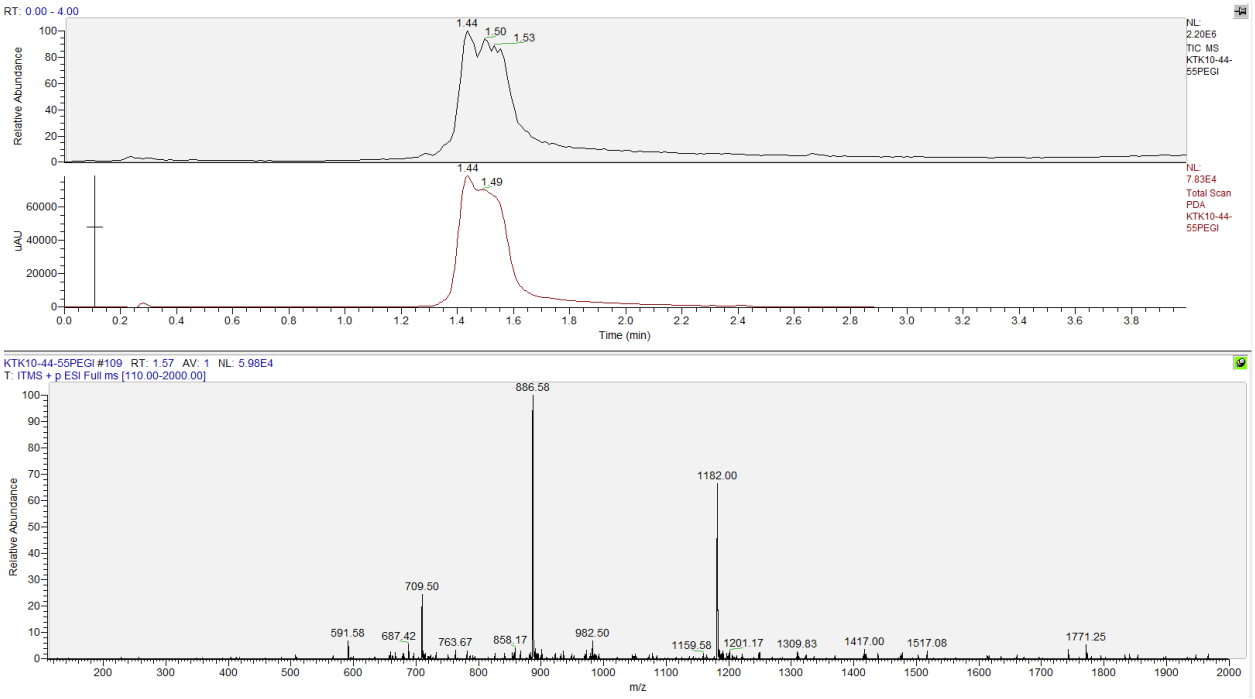


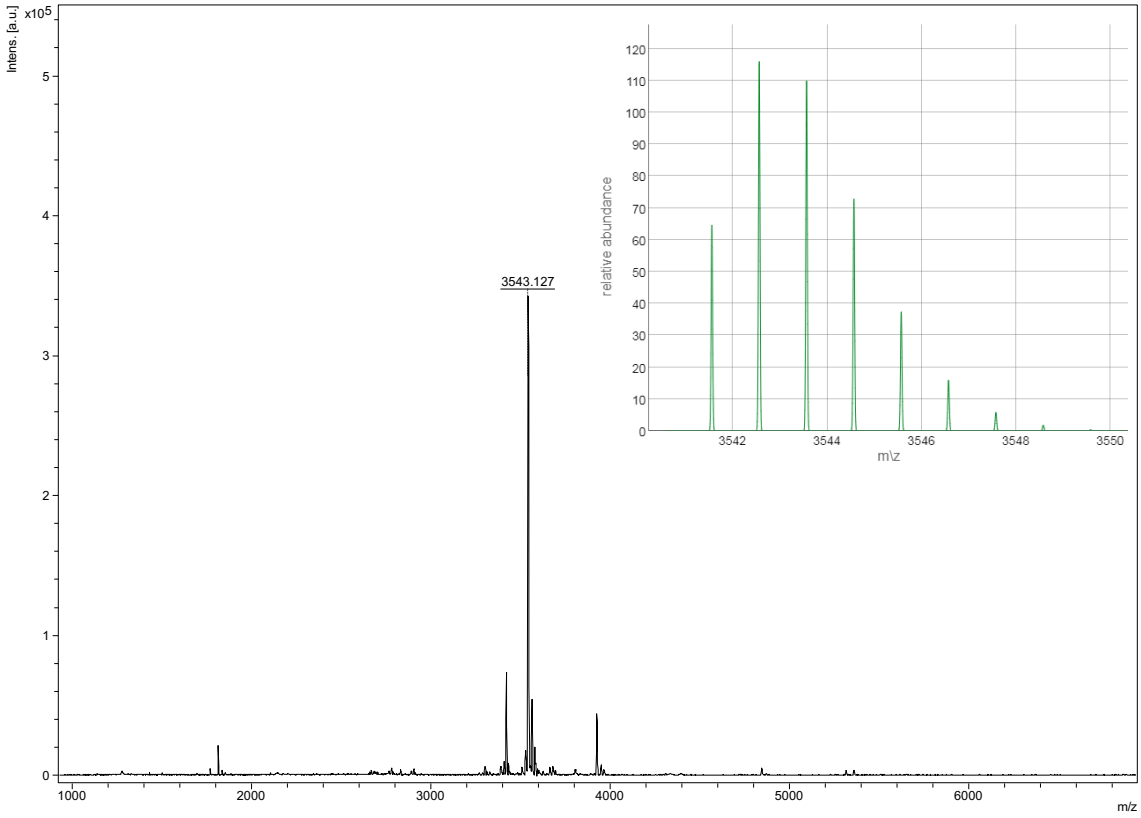
Cy3pegH2 (cy3-peg-CGGCA-CATTC-TGCCG-CTAGT); Expected Mass for $C_{308}H_{432}N_{115}O_{96}^{+}$: 7277.2457; isotopic mass range (green insert). LC-MS (ESI) RT= 1.92 min. m/z: 1456.92 $[M+4H]^{5+}$, 1214.25 $[M+5H]^{6+}$, 1041.00 $[M+6H]^{7+}$, MALDI-TOF m/z found: 7282.695 $[M]^{+}$



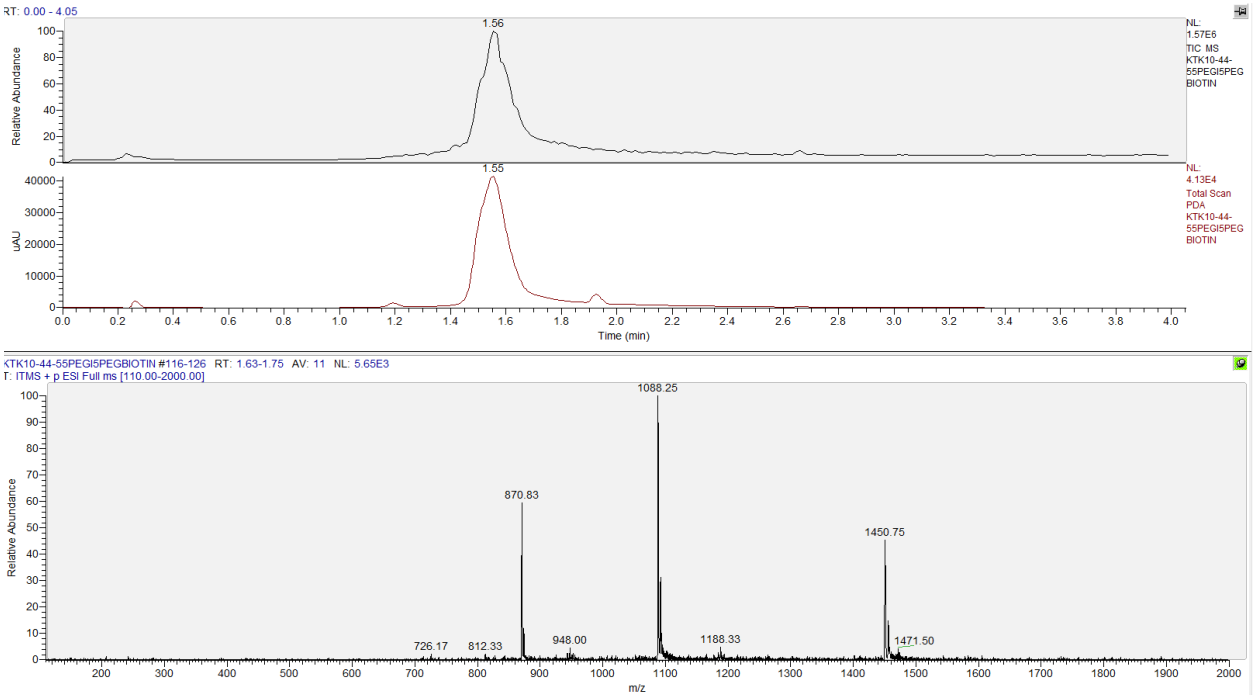


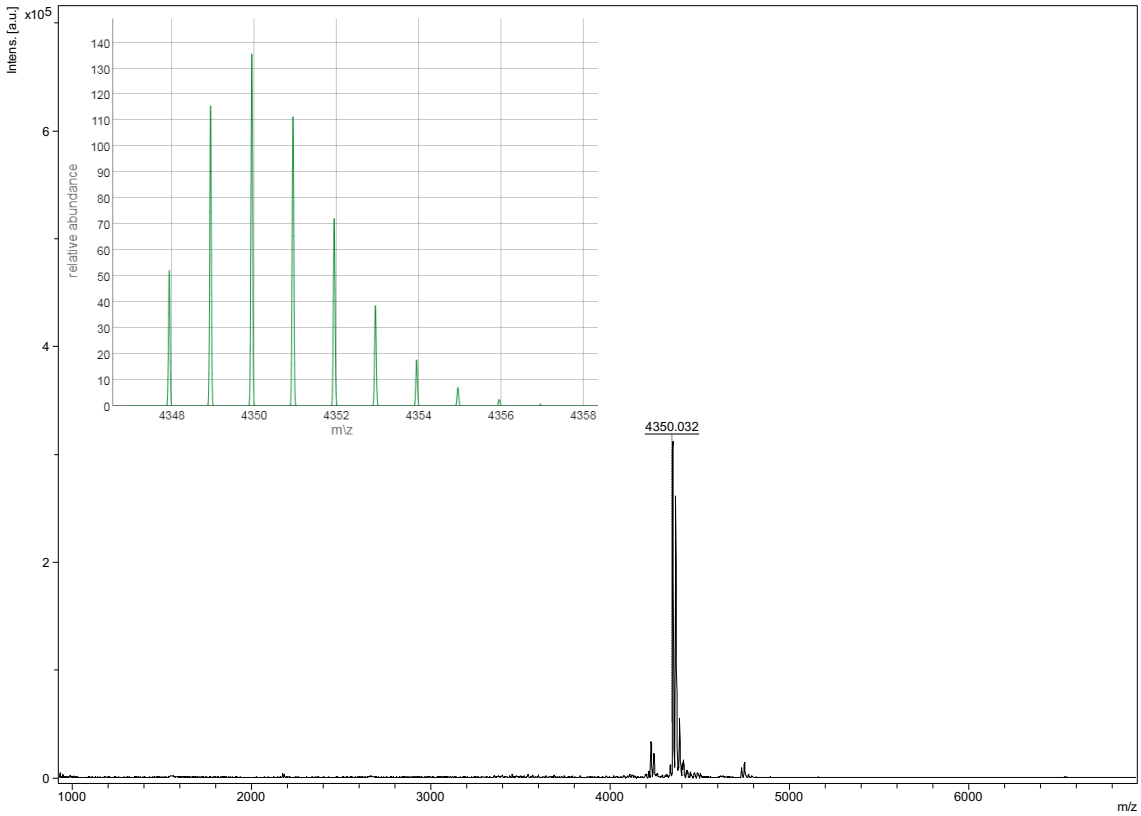
pegI (NH_2-peg-A<u>C</u>T<u>A</u>G-<u>C</u>G<u>G</u>C<u>A</u>); Expected Mass for $C_{143}H_{205}N_{63}O_{46}$: 3540.5639; isotopic mass range (green insert). LC-MS (ESI) RT= 1.76 min. m/z: 1182.00 $[M+3H]^{3+}$, 886.58 $[M+4H]^{4+}$, 709.50 $[M+5H]^{5+}$, MALDI-TOF m/z found: 3543.127 $[M+H]^{+}$



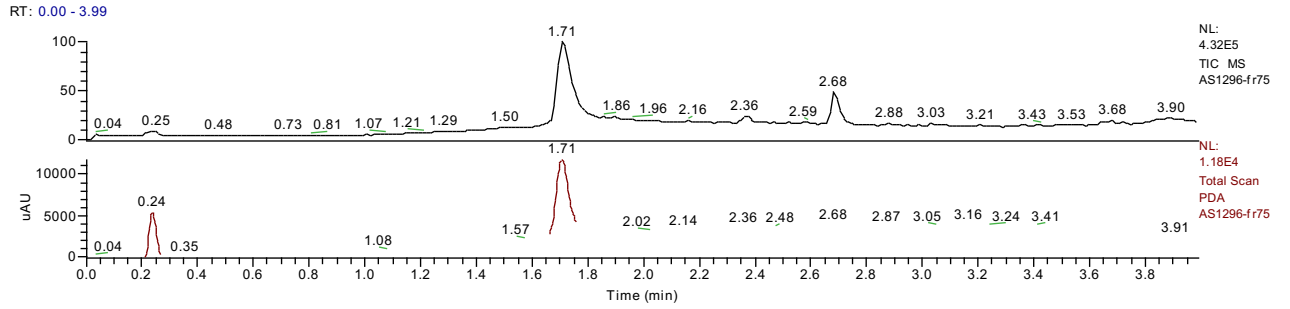


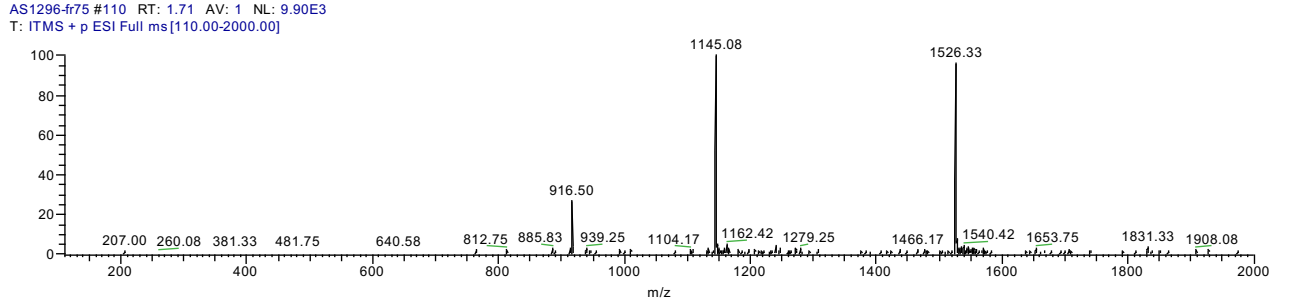
BpegI (Biotin-peg-peg-peg-peg-peg-ACTAG-CGGCA); Expected Mass for $C_{177}H_{263}N_{69}O_{60}S$: 4346.9370; isotopic mass range (green insert). LC-MS (ESI) RT= 1.56 min. m/z: 1450.75 $[M+3H]^{3+}$, 1088.25 $[M+4H]^{4+}$, 870.33 $[M+5H]^{5+}$, MALDI-TOF m/z found: 4350.032 $[M+H]^{+}$

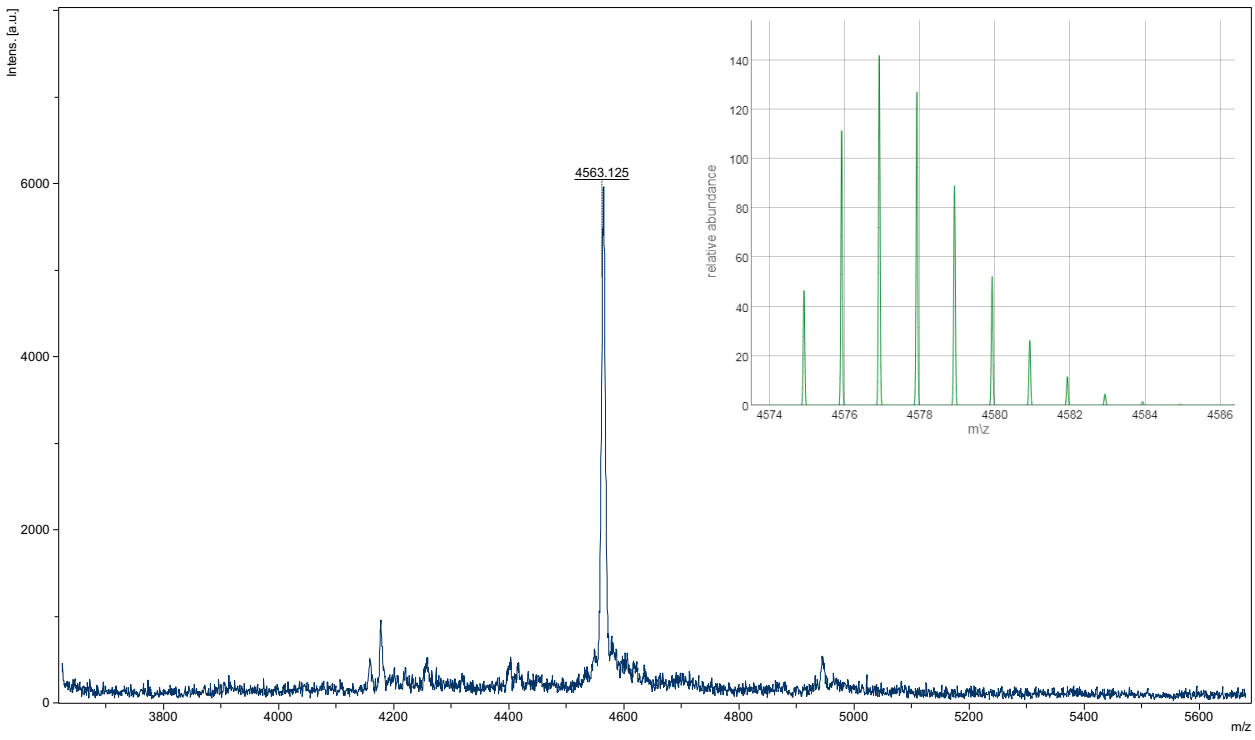




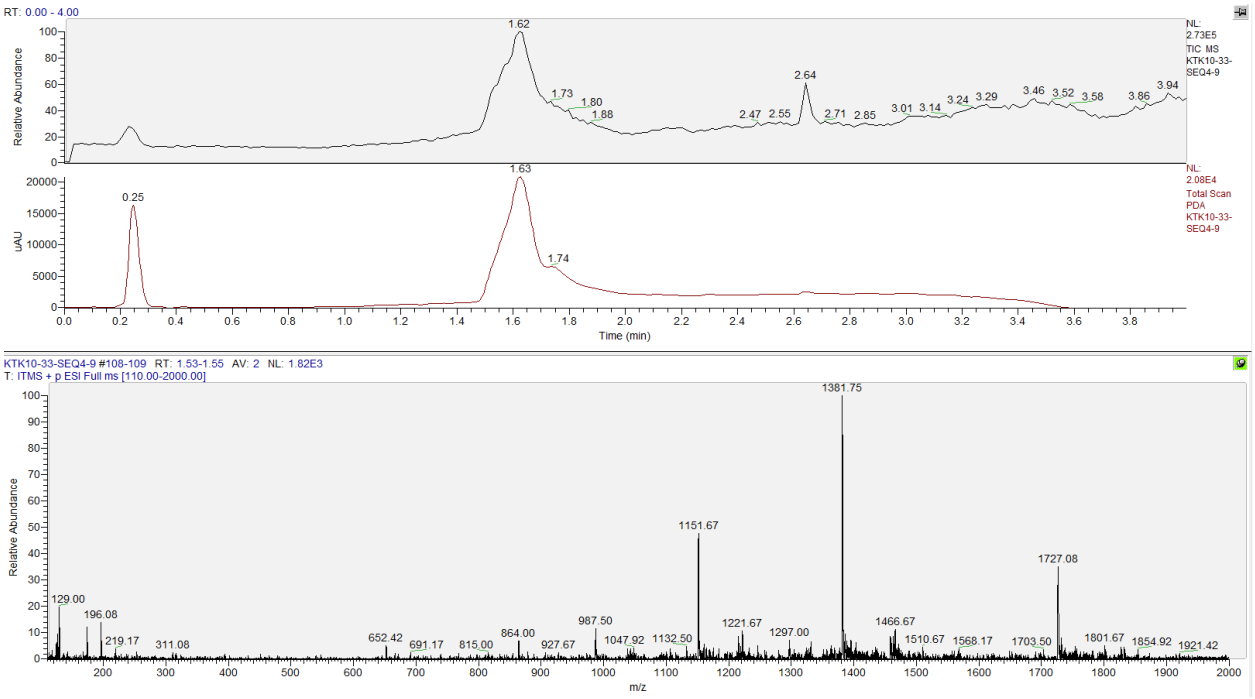
FBpegI (Biotin-peg-peg-Lys(FITC)-peg-ACTAG-CGGCA); Expected Mass for $C_{192}H_{264}N_{70}O_{60}S_2$: 4557.9300; isotopic mass range (green insert). LC-MS (ESI) RT= 1.71 min. m/z: 1526.33 $[M+4H]^{3+}$,1145.08 $[M+4H]^{4+}$, 916.50 $[M+5H]^{5+}$, MALDI-TOF m/z found: 4563 $[M+H]^{+}$

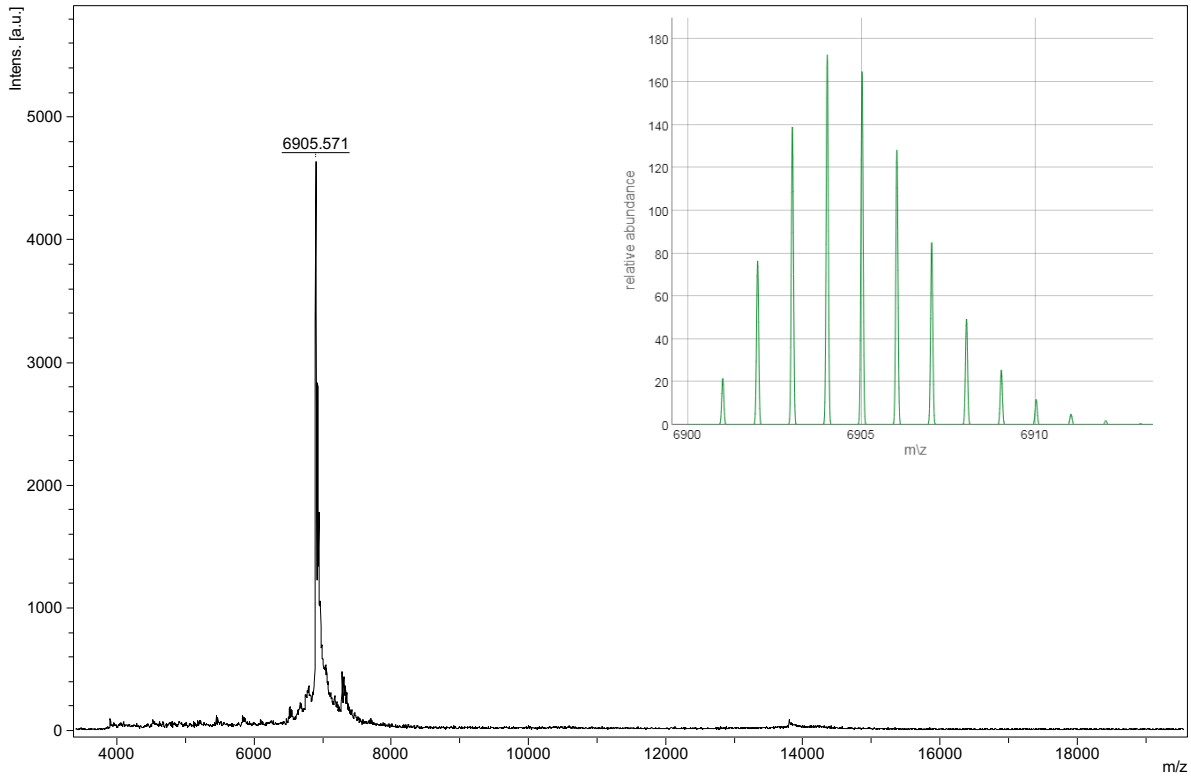




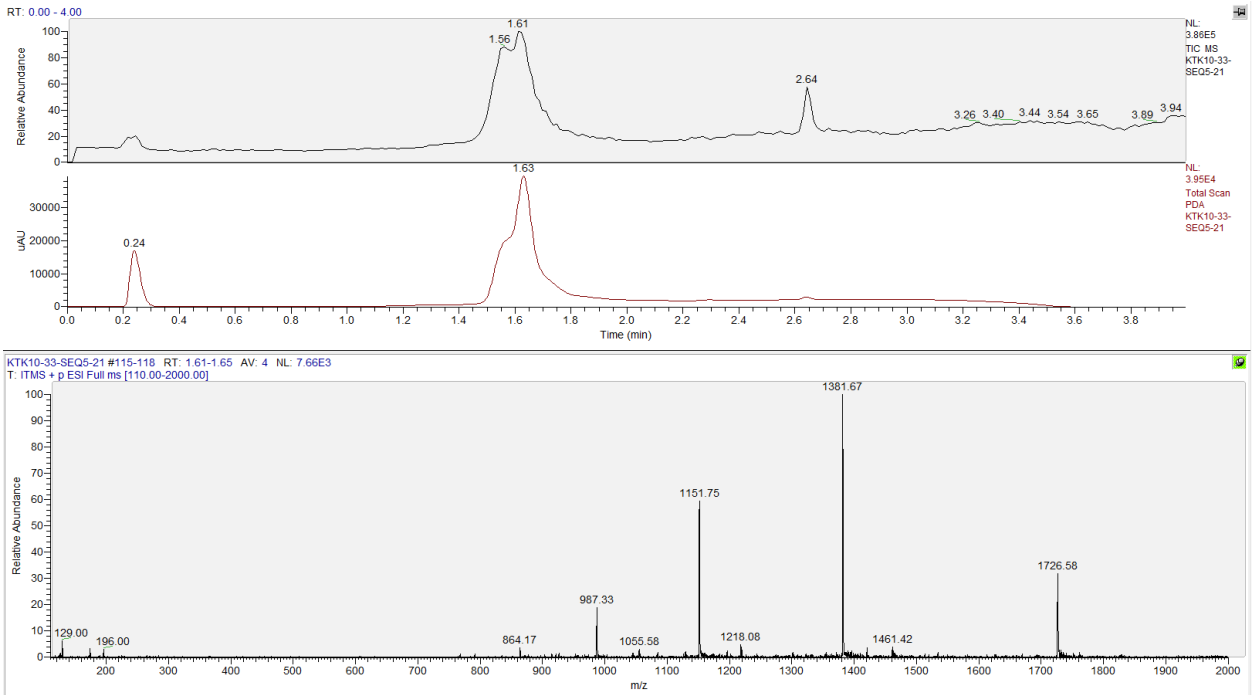


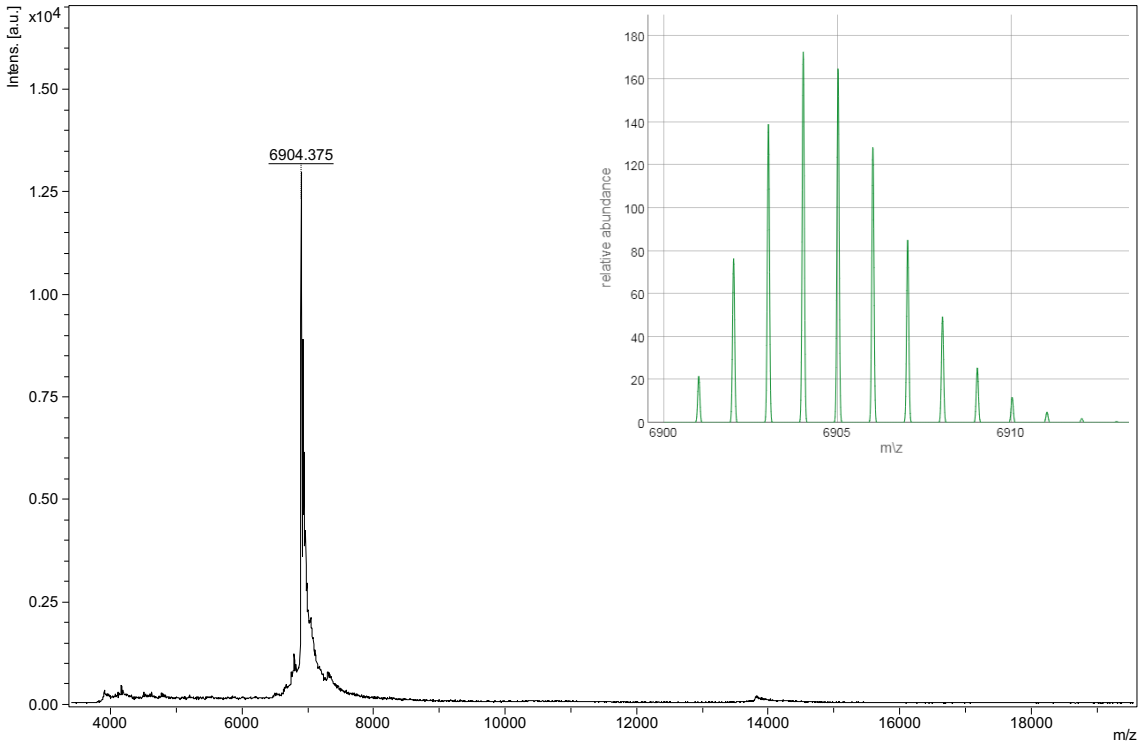
Ref1 (NH_2-peg-AGTCTCAGTGTCAACGTACG); Expected Mass for $C_{281}H_{399}N_{117}O_{93}$: 6900.0098; isotopic mass range (green insert). LC-MS (ESI) RT= 1.62 min. m/z: 1727.08 $[M+4H]^{4+}$, 1381.75 $[M+5H]^{5+}$, 1151.67 $[M+6H]^{6+}$, 987.50 $[M+7H]^{7+}$, MALDI-TOF m/z found: 6905.571 $[M+H]^{+}$



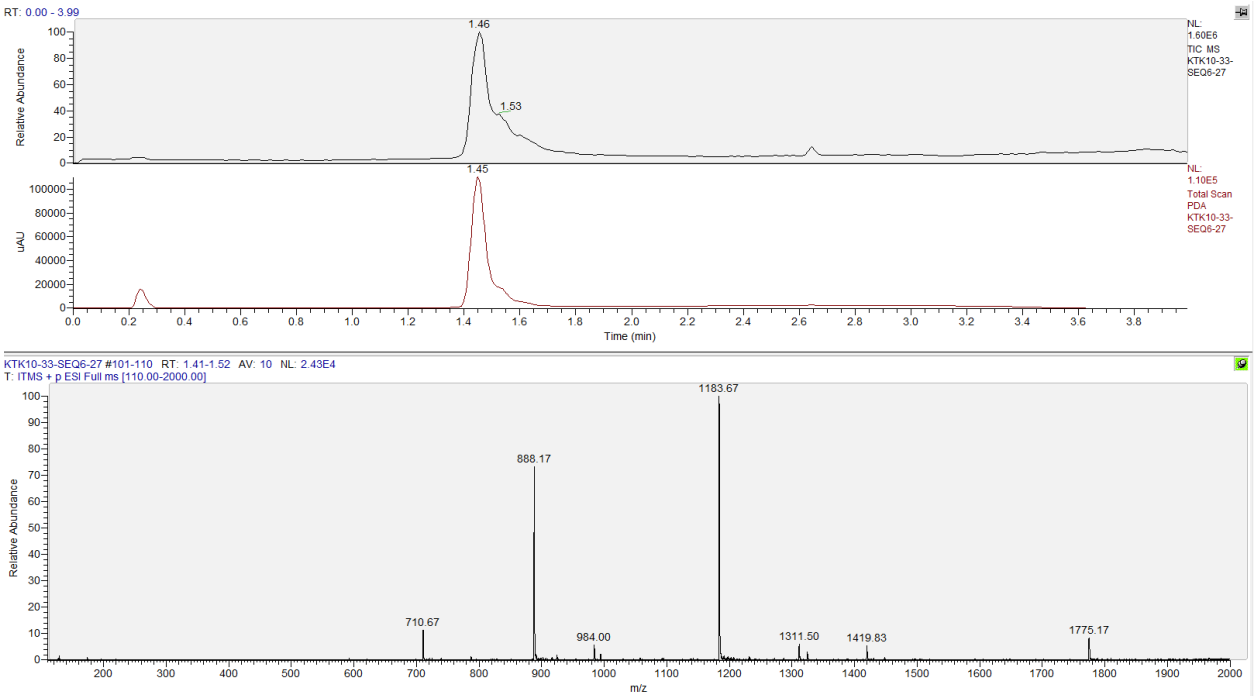


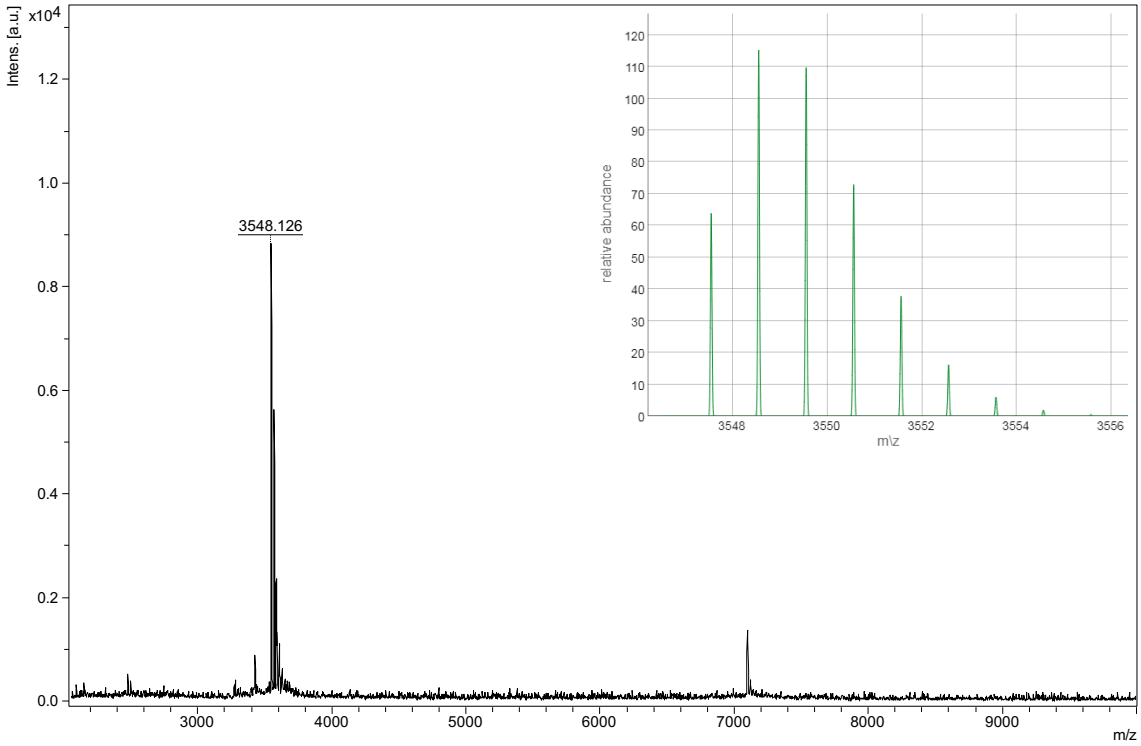
Ref2 (NH_2-peg-CGTACGTTGACACTGAGACT); Expected Mass for $C_{281}H_{399}N_{117}O_{93}$: 6900.0098; isotopic mass range (green insert). LC-MS (ESI) RT= 1.61 min. m/z: 1726.58 $[M+4H]^{4+}$, 1381.67 $[M+5H]^{5+}$, 1151.75 $[M+6H]^{6+}$, 987.33 $[M+7H]^{7+}$, MALDI-TOF m/z found: 6904.375 $[M+H]^+$



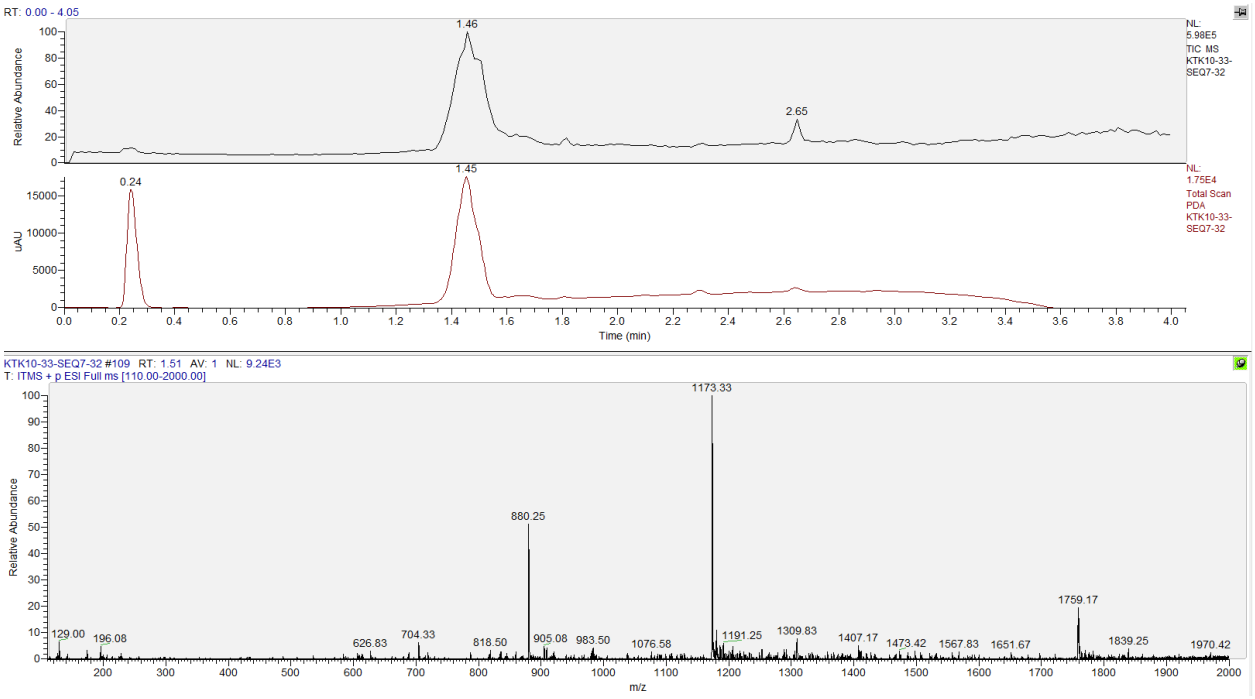


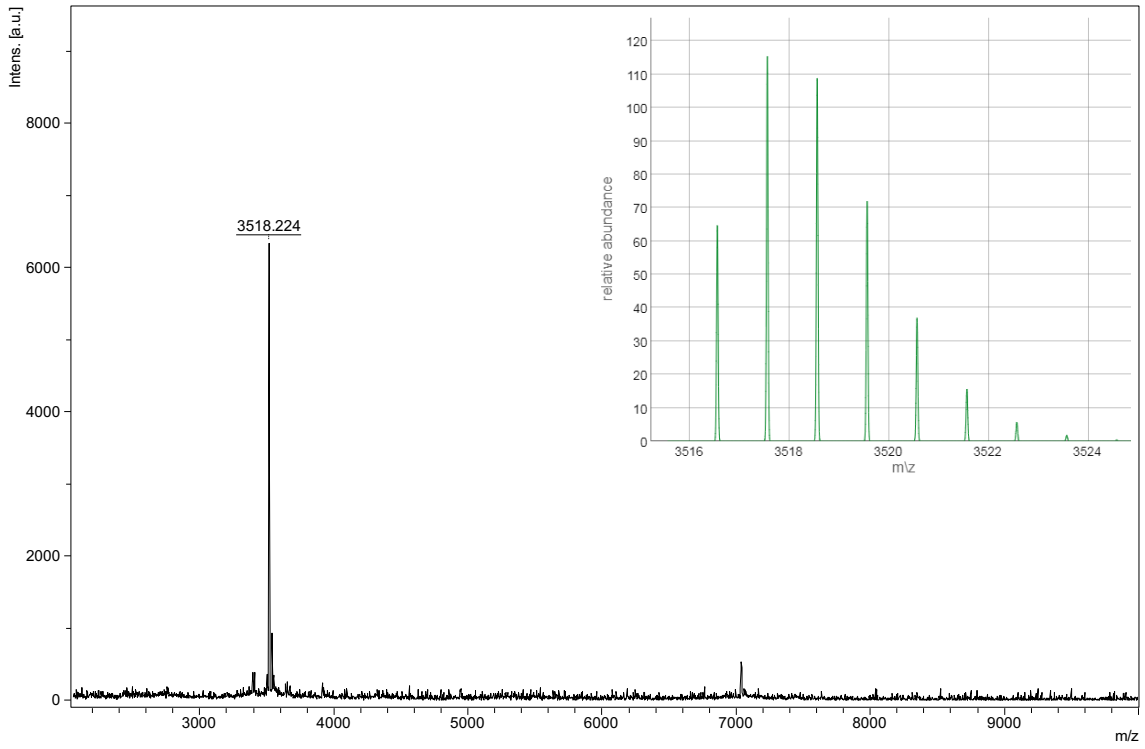
Ref3 (NH_2-peg-AGTCTCAGTG); Expected Mass for $C_{144}H_{207}N_{59}O_{49}$: 3546.5520; isotopic mass range (green insert). LC-MS (ESI) RT= 1.46 min. m/z: 1775.17 $[M+2H]^{2+}$, 1183.67 $[M+3H]^{3+}$, 888.17 $[M+4H]^{4+}$, 710.67 $[M+5H]^{5+}$, MALDI-TOF m/z found: 3548.126 $[M+H]^{+}$



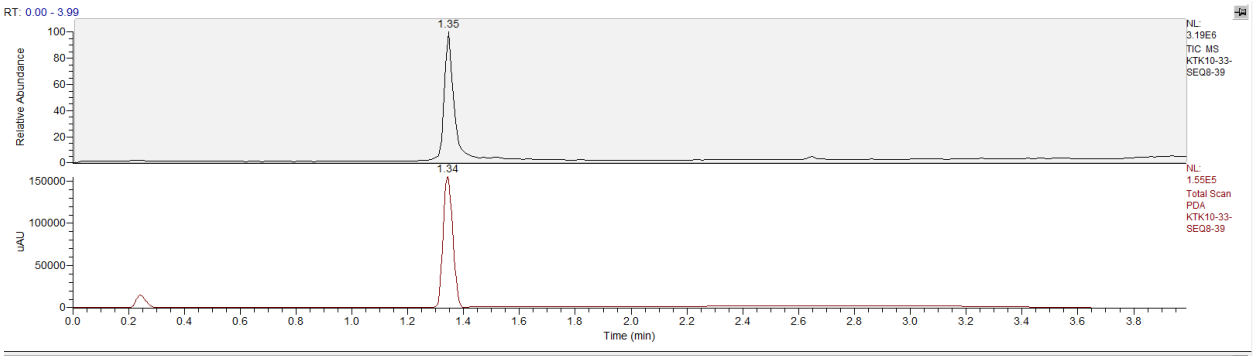


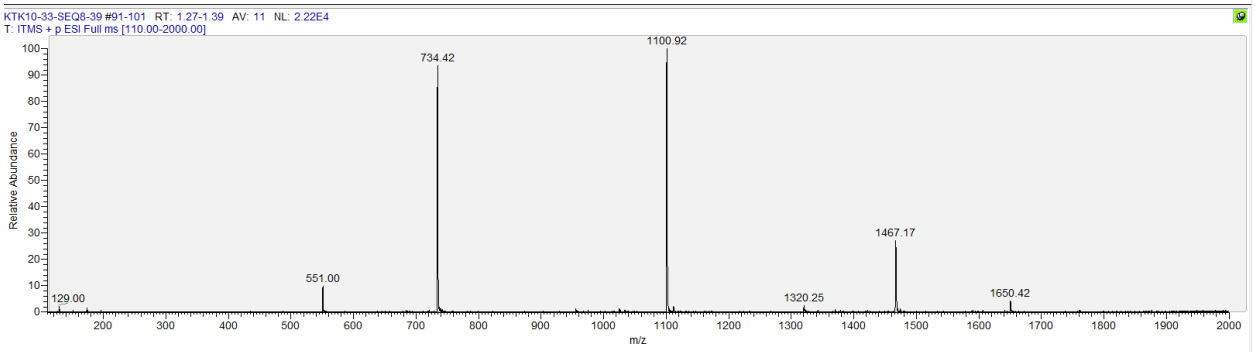
Ref4 (NH_2-peg-C<u>A</u>C<u>T</u>G<u>A</u>G<u>A</u>C<u>T</u>); Expected Mass for $C_{143}H_{206}N_{60}O_{47}$: 3515.5574; isotopic mass range (green insert). LC-MS (ESI) RT= 1.46 min. m/z: 1759.17 $[M+2H]^{2+}$, 1173.33 $[M+3H]^{3+}$, 880.25 $[M+4H]^{4+}$, 704.33 $[M+5H]^{5+}$, MALDI-TOF m/z found: 3518.224 $[M+H]^{+}$

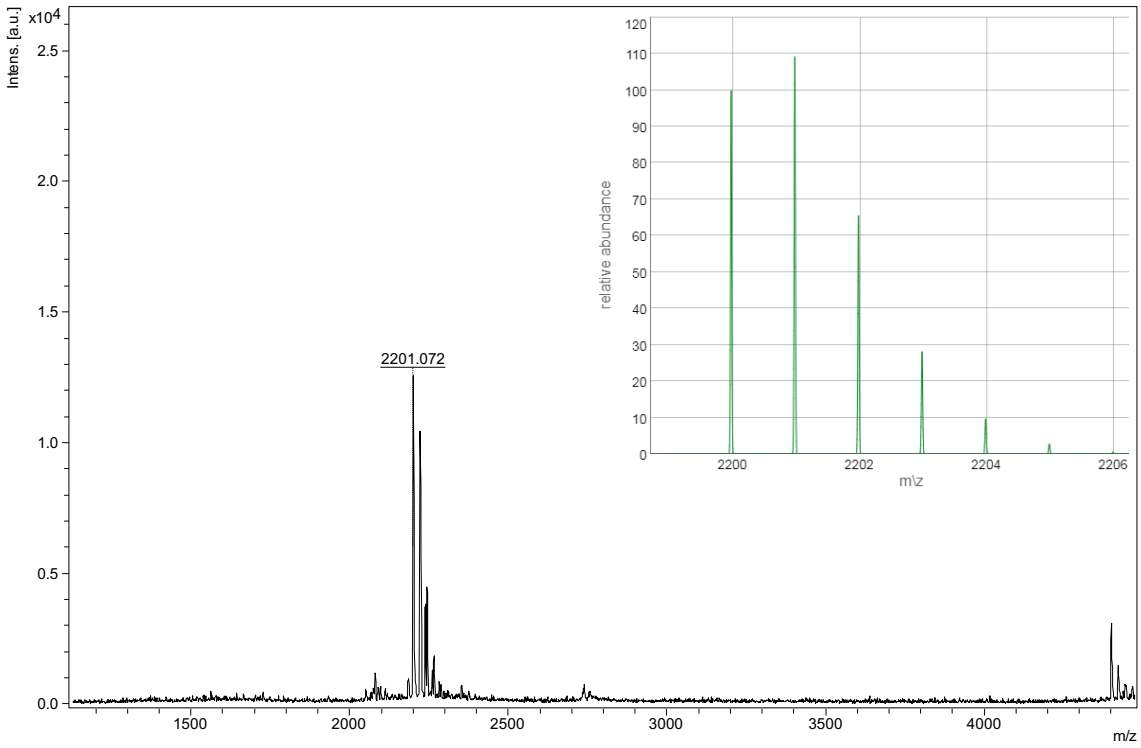




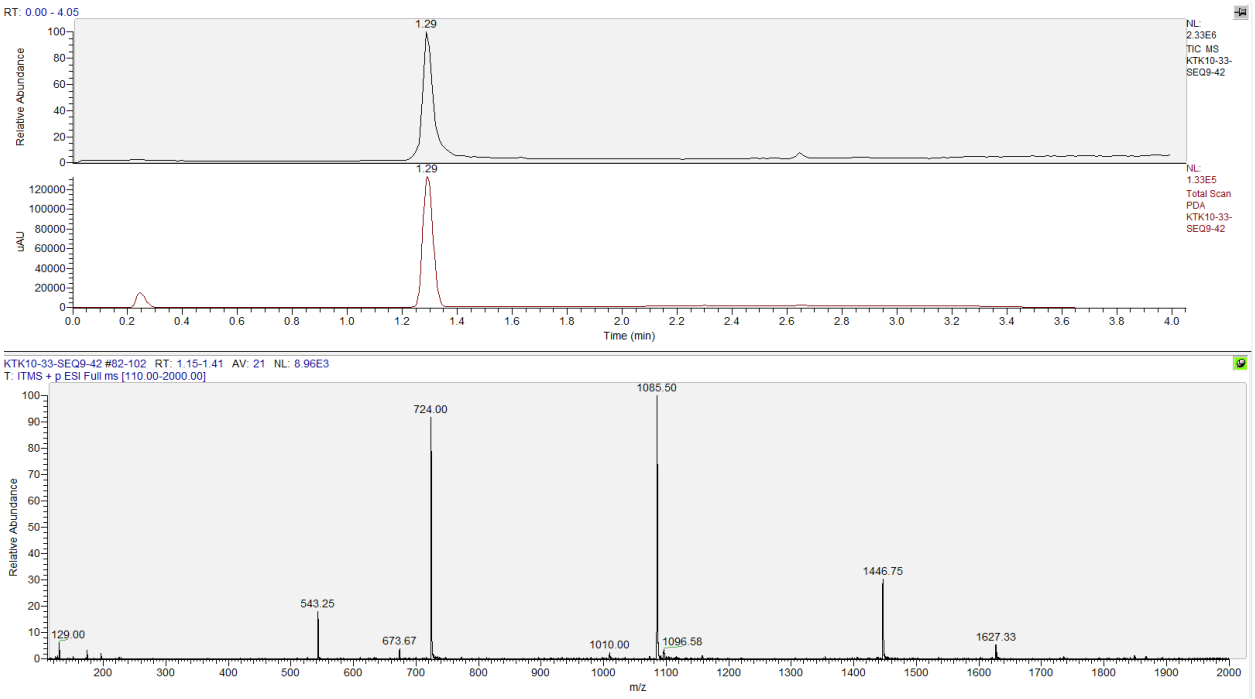
Ref5 (NH_2-peg-G<u>A</u>T<u>G</u>C<u>T</u>); Expected Mass for $C_{89}H_{130}N_{36}O_{31}$: 2198.9703; isotopic mass range (green insert). LC-MS (ESI) RT= 1.35 min. m/z: 1467.17 $[2M+3H]^{3+}$, 1100.92 $[M+2H]^{2+}$, 734.42 $[M+3H]^{3+}$, 551.00 $[M+4H]^{4+}$, MALDI-TOF m/z found: 2201.072 $[M+H]^{+}$

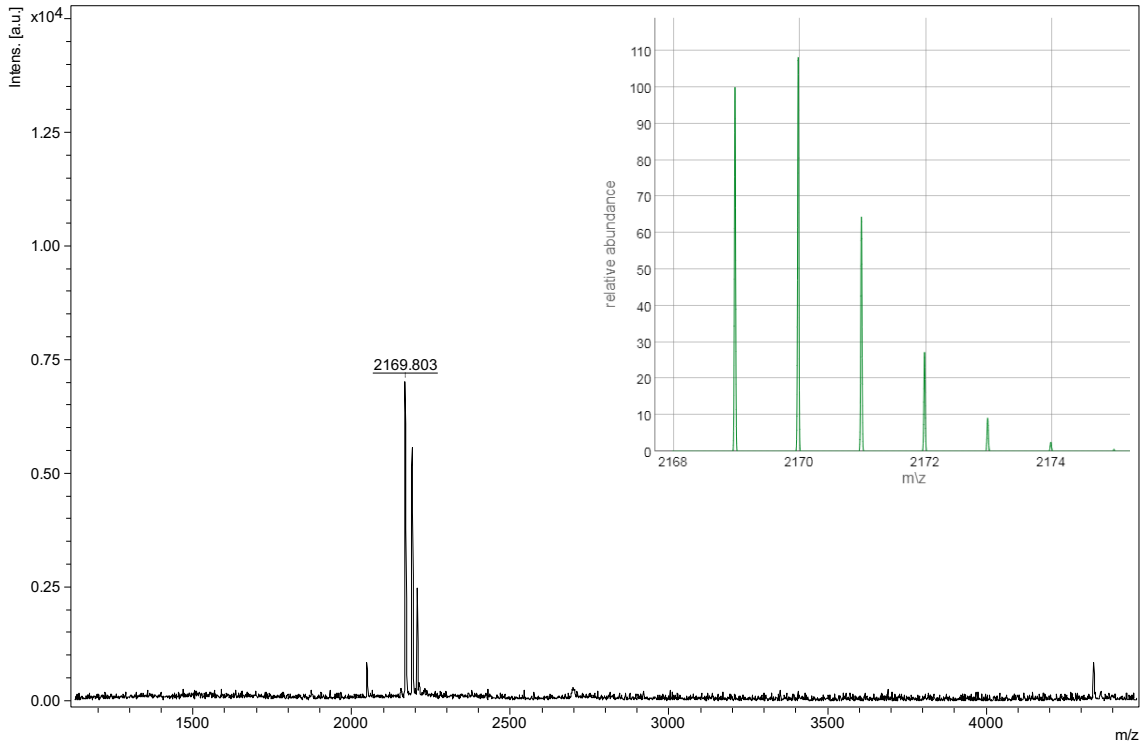




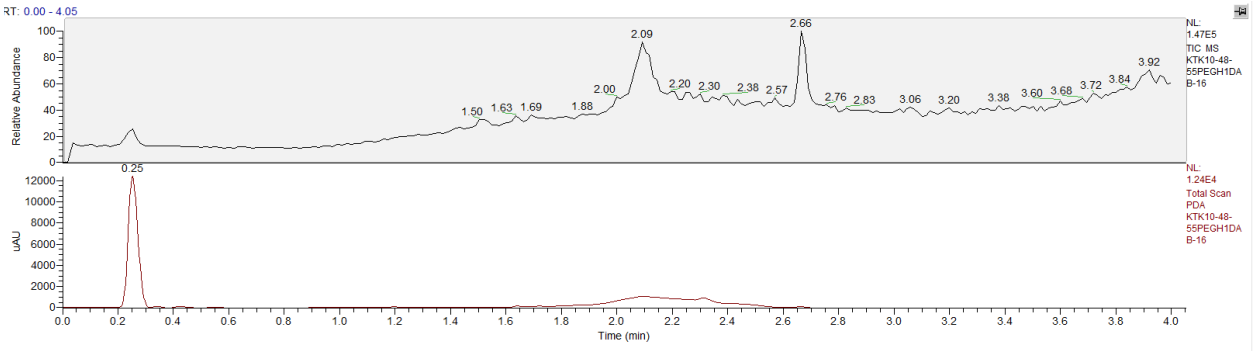


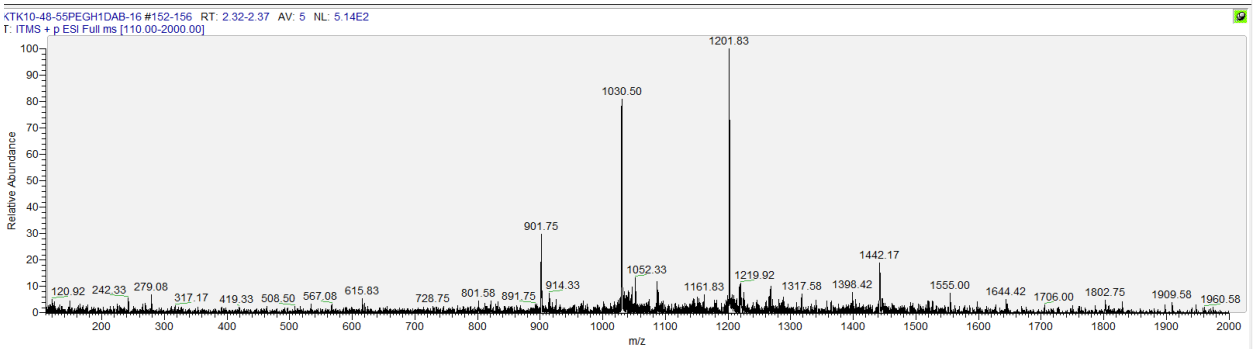
Ref6 (NH_2-peg-AGCATC); Expected Mass for $C_{88}H_{129}N_{37}O_{29}$: 2167.9757; isotopic mass range (green insert). LC-MS (ESI) RT= 1.29 min. m/z: 1446.75 $[2M+3H]^{3+}$, 1085.50 $[M+2H]^{2+}$, 724.00 $[M+3H]^{3+}$, 543.25 $[M+4H]^{4+}$, MALDI-TOF m/z found: 2169.803 $[M+H]^+$

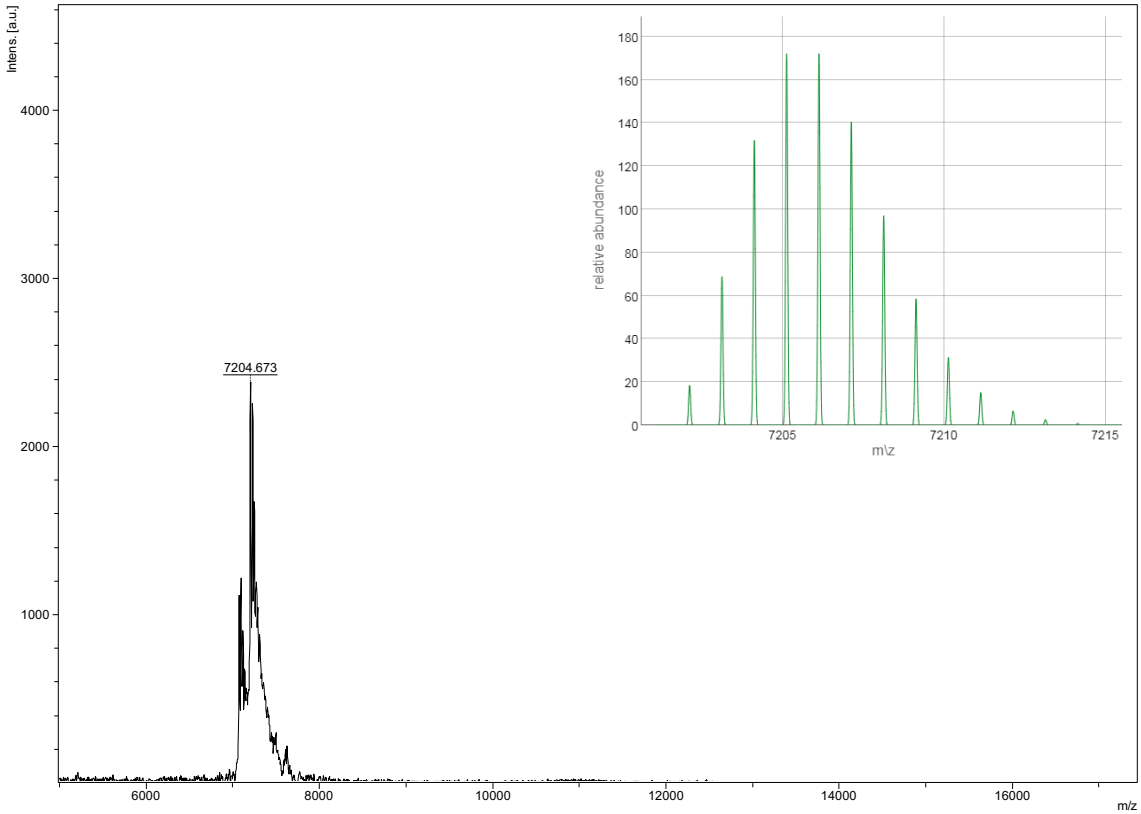




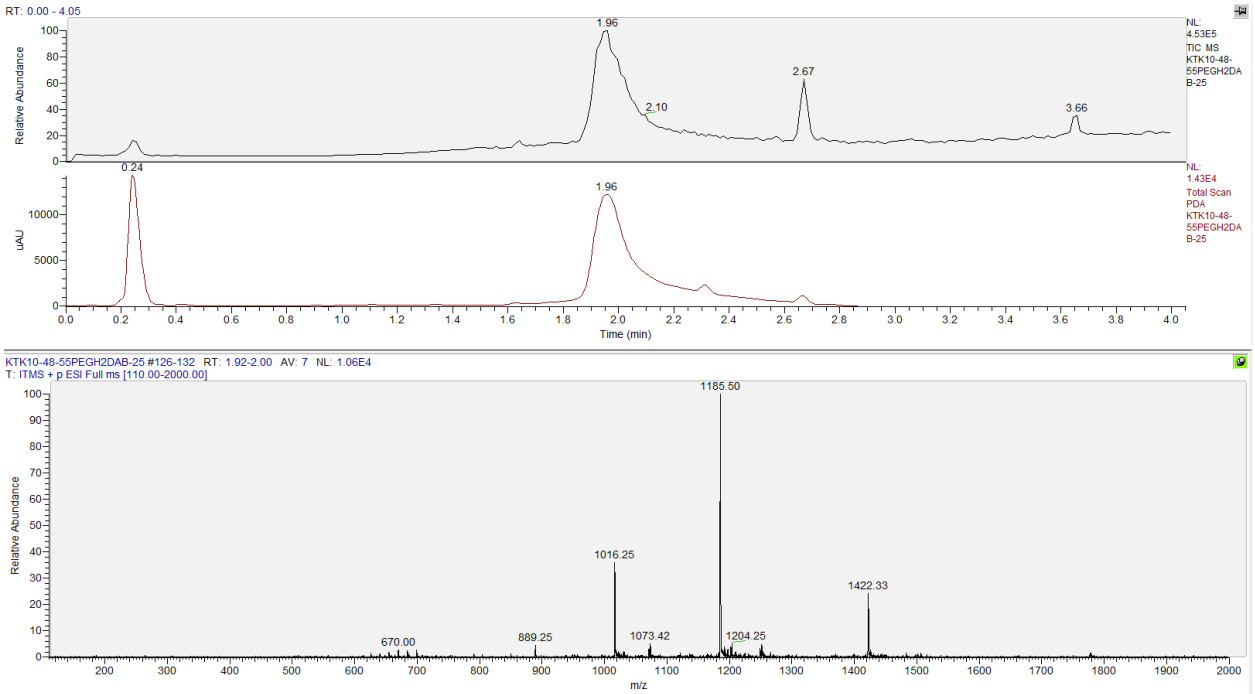
DpegH1 (Dabcyl-peg-GAATG-TGCCG-ACTAG-CGGCA); Expected Mass for $C_{296}H_{410}N_{126}O_{92}$: 7201.1277; isotopic mass range (green insert). LC-MS (ESI) RT= 2.09 min. m/z: 1442.17 $[M+5H]^{5+}$, 1201.83 $[M+6H]^{6+}$, 1030.50 $[M+7H]^{7+}$, 901.75 $[M+8H]^{8+}$, MALDI-TOF m/z found: 7204.673 $[M+H]^{+}$

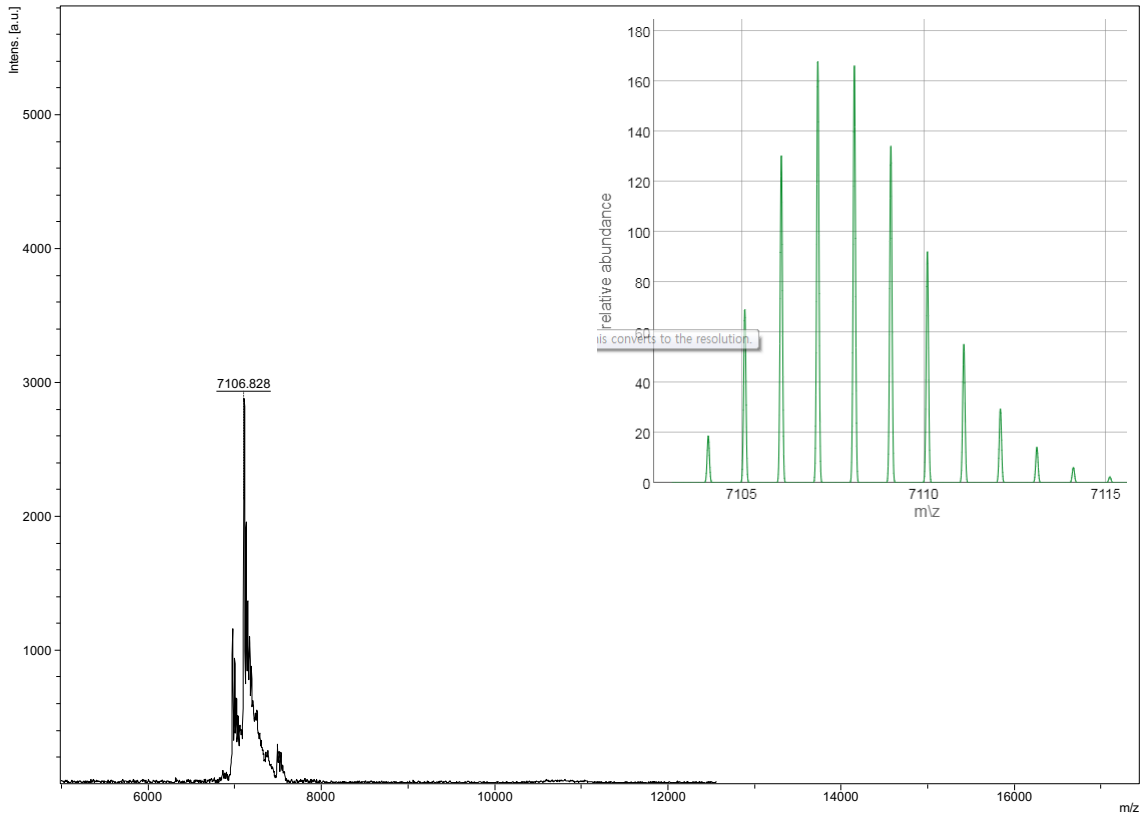




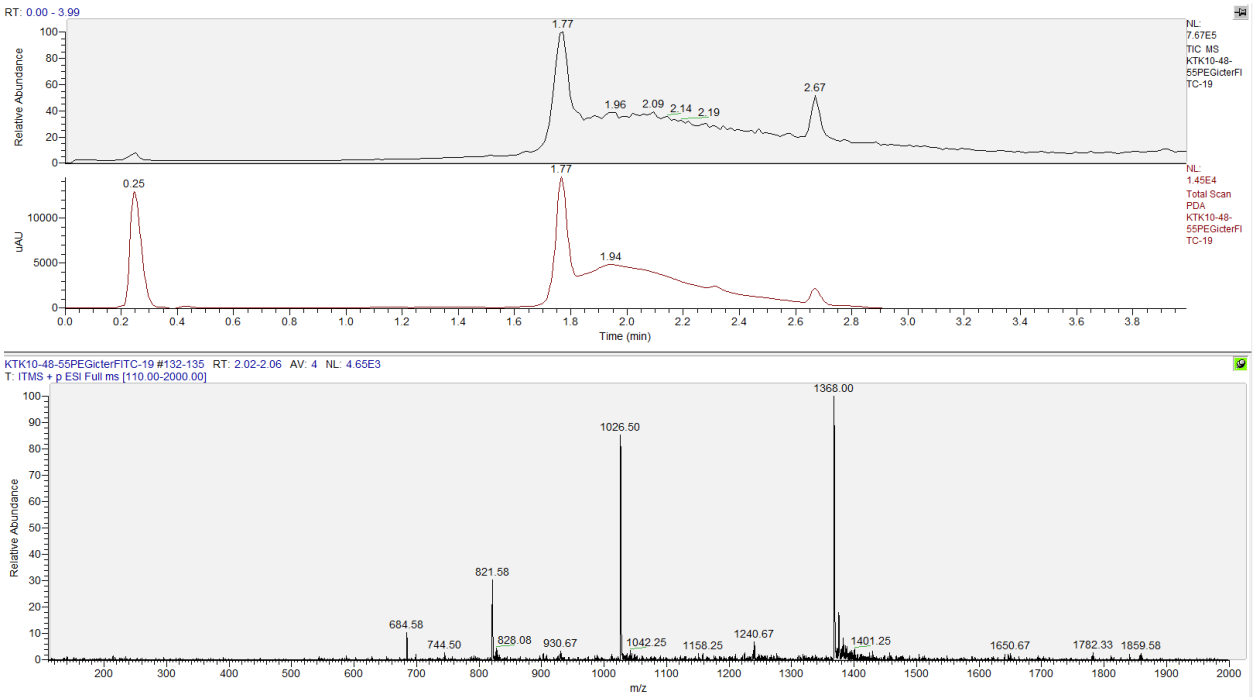


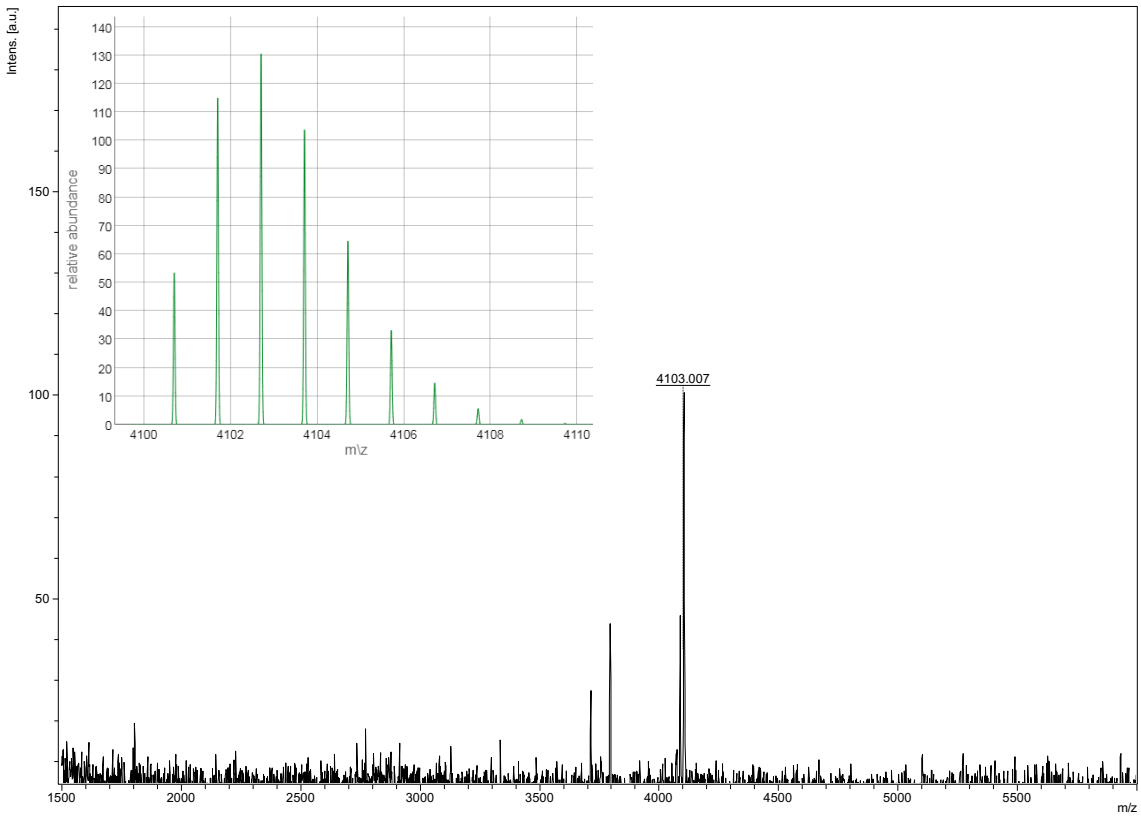
DpegH2 (Dabcyl-peg-CGGCA-CATTC-TGCCG-CTAGT); Expected Mass for $C_{294}H_{412}N_{116}O_{96}$: 7103.0923; isotopic mass range (green insert). LC-MS (ESI) RT= 1.96 min. m/z: 1422.33 $[M+5H]^{5+}$, 1185.50 $[M+6H]^{6+}$, 1016.25 $[M+7H]^{7+}$, 889.25 $[M+8H]^{8+}$, MALDI-TOF m/z found: 7106.828 $[M+H]^{+}$



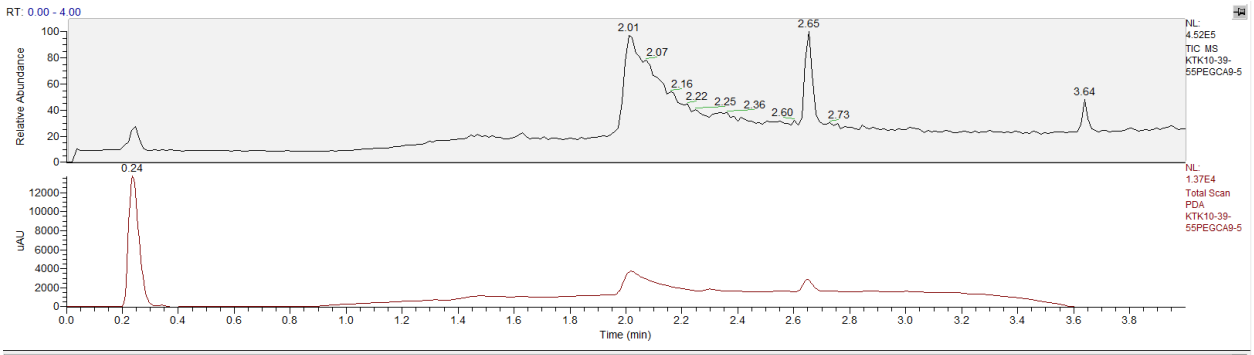


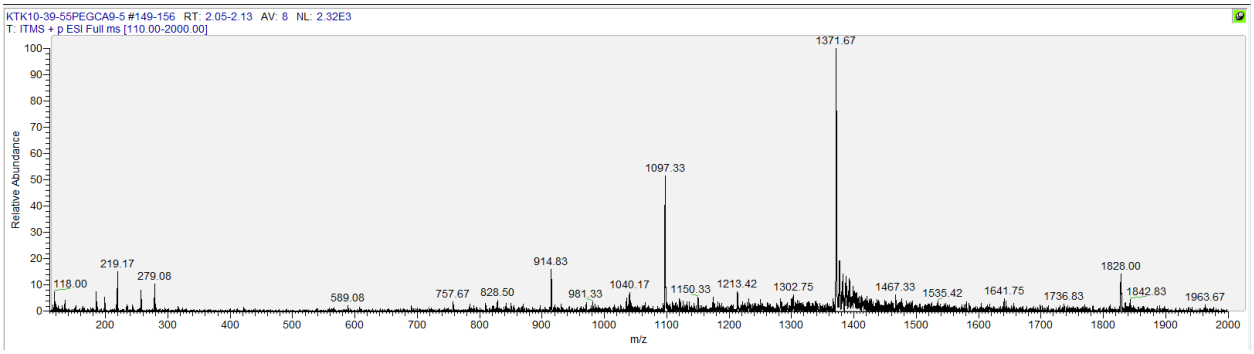
pegIF (Ac-ACTAG-CGGCA-Lys-peg(FITC)); Expected Mass for $C_{172}H_{230}N_{66}O_{53}S$: 4099.7052; isotopic mass range (green insert). LC-MS (ESI) RT= 1.77 min. m/z: 1368.00 $[M+3H]^{3+}$, 1026.50 $[M+4H]^{4+}$, 821.58 $[M+5H]^{5+}$, 684.58 $[M+6H]^{6+}$, MALDI-TOF m/z found: 4103.007 $[M+H]^{+}$

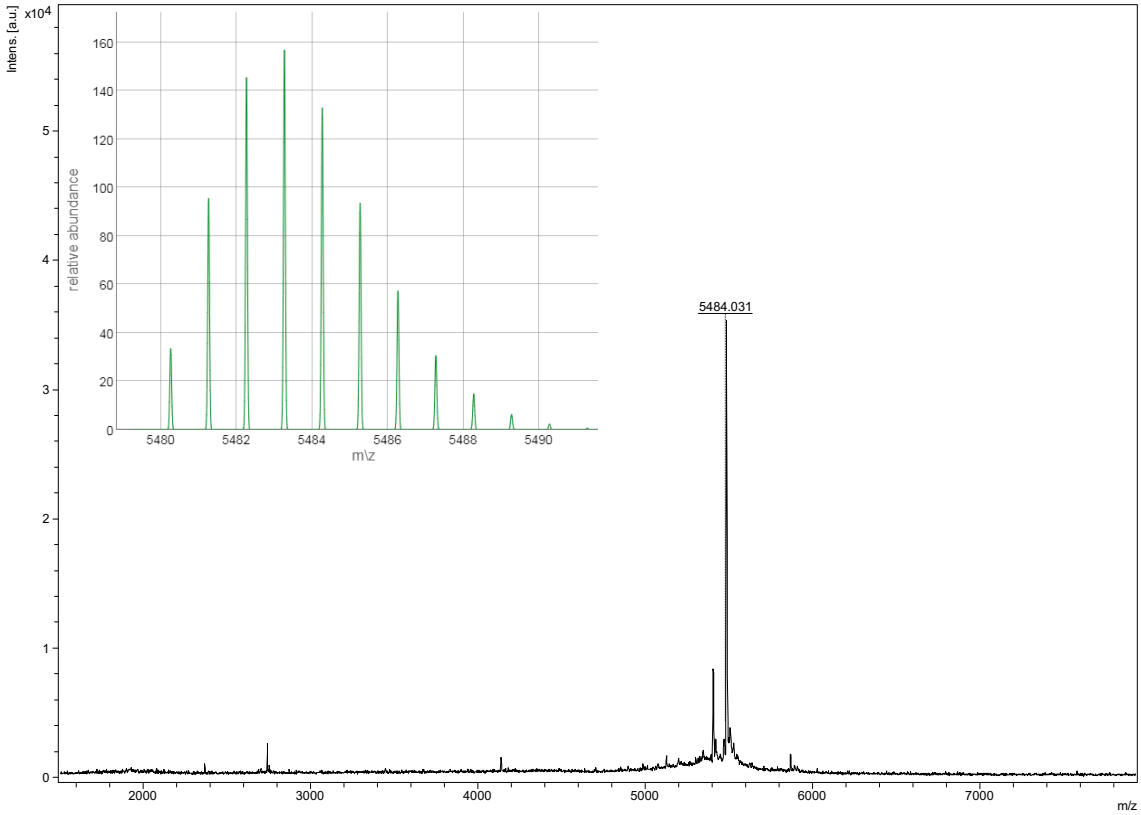




LpegI (ligand-Lys(Cy3)-peg-ACTAG-CGGCA); Expected Mass for $C_{226}H_{316}N_{87}O_{69}S_4^+$: 5480.2770; isotopic mass range (green insert). LC-MS (ESI) RT= 2.01 min. m/z: 1828.00 $[M+2H]^{3+}$, 1371.67 $[M+3H]^{4+}$, 1097.33 $[M+4H]^{5+}$, 914.83 $[M+5H]^{6+}$, MALDI-TOF m/z found: 5484.031 $[M]^+$







4. References

(1) Kim, K. T.; Angerani, S.; Chang, D.; Winssinger, N. *J. Am. Chem. Soc.* **2019**, *141*, 16288.