Supporting Information for:

Duplex stem replacement with bPNA+ triplex hybrid stems enables reporting on tertiary interactions of internal RNA domains

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S1. Materials, instrumentation and general notes

All chemicals were used without further purification from commercial sources, unless otherwise noted. DNAs were purchased from Integrated DNA Technologies (IDT) and used without further purification. SYBR gold was purchased from Thermo Fisher Scientific. DNA stock solutions were serially diluted in deionized water and concentrations were determined by measuring solution absorbance at 260 nm by Thermo Fisher Nanodrop 2000. Sample fluorescence was measured on Thermo Fisher Nanodrop 3300. RNA constructs were prepared by in vitro transcription except where noted. The promoter sequence in each DNA template is underlined and does not appear in the final RNA transcripts.

S2. General experimental procedures

S2.1. UV-melting.

UV-melting curves were measured on Cary-100 UV-vis spectrophotometer equipped with an air-circulating temperature controller. All measurements were carried out with temperature change rate of 1°C/min and monitored at 260 nm. All samples are freshly annealed in 1X PBS buffer (pH=7.4) before measurements, concentration of DNA/RNAs are 2 μM.

S2.2. Differential Scanning Calorimetry (DSC).

DSC experiments were carried out on Microcalorimeter VP-DSC. Samples were prepared to have 25 μM DNA and 25 μM XK^{2M} peptides in 1X PBS buffer (pH=7.4). Samples were scanned from 25°C to 90°C with 60°C/h scanning rate, 16s filtering period and low feedback. 1X PBS was used as reference. Background data was collected with only 25 μM DNA in the sample cell and was subtracted from all DNA/peptide sample traces.

S2.3. Fluorescence anisotropy.

A series of samples were made by mixing various concentrations of $T_6C_4T_6$ and constant concentration of FITC labelled peptides (25 nM or 50 nM) in 1X PBS. All samples were annealed at 95°C for 5 min. Experiments were carried out on Molecular Devices SpectraMax M5 with excitation wavelength at 495 nm and emission at 520 nm. Fluorescence anisotropy was converted into complex concentration using equation 1[1]:

$$[complex] = (\frac{FA - FAmin}{FAmax - FAmin}) * [peptide]$$

The concentration of complex was plotted against total DNA concentration in each sample, the data was fitted using equation 2[2]:

$$[complex] = (Kd + [DNA] + [peptide])/2 - (\sqrt{(Kd + [DNA] + [peptide])^2 - 4 * [DNA] * [peptide]}\ )/2$$

S2.4. EMSA.

A series of samples were made by mixing various concentrations of RNA1-U6 and constant concentration of FITC labelled peptides (50 nM) in 1X PBS. All samples were annealed at 95°C for 5 min and slow cooling to room temperature. Samples were then subjected to electrophoresis in 20% native acrylamide gel at 120V under ice for 3 hrs. The gel was visualized in a Typhoon FLA 9500 (GE Healthcare) and analyzed with ImageQuant TL software (GE Healthcare). The gel bands intensity were quantified and the percentage of bounded DNA was plotted. Dissociation constant (K_d) was obtained by fitting the data into the equation 2.

S2.5. FRET measurement for ColE1 system.

RNA1-U6 · Cy5-$(SK^{2M})_3$ and RNA2-U6 · Cy3-$(SK^{2M})_3$ stocks were prepared by adding RNA and peptide in a 1 : 1 ratio. RNA-peptide complexes were heat at 90°C for 5 min. The final concentration of RNA2-U6 · Cy3-$(SK^{2M})_3$ was 500 nM and RNA1-U6 · Cy5-$(SK^{2M})_3$ ranged from 0 μM to 1.5 μM. RNA-peptides were mixed and diluted

into 1x kissing buffer and incubated at least 30 mins. The fluorescence experiment was performed on a Thermo Scientific NanoDrop 3300 fluorospectrometer by exciting Cy3 at 550 nm and monitoring at 564 nm and 666 nm.

S2.6. ColE1 EMSA assay

Cy-modified (SK^{2M}) peptides were added in a 1:1 ratio to the corresponding RNAs and incubated for 10 mins. RNA-peptide complexes were mixed, annealed at 95ºC for 5 min and then snap cooled for 5 min on ice and diluted into 1x kissing buffer (20 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, and 300 mM NaCl). Rop was added as necessary after RNA and incubated for 20 minutes on ice. Rop concentrations are reported as dimer concentration unless otherwise stated. After incubation on ice for 30 min, glycerol was added to a final concentration of 10% v/v and samples were loaded onto a 18.5% w/v acrylamide gel (8.3 cm X 7.3 cm X 1.0 mm) composed of 75:1 acrylamide to bis-acrylamide which had be pre-run for 2 h at 120 volts. Gels were electrophoresed for 10 h at 90 volts in 1x TBM buffer(89 mM Tris, 89 mM boric acid, and 10 mM $MgCl_2$). Gels were imaged on a GE Healthcare Typhoon FLA 9500 using the preset laser excitation and emission setting for Cy5 and Cy3 fluorophores.

S2.7. Fluorescence measurement for ColE1 system.

RNA1 U6 · Cy5-$(SK^{2M})_3$ and RNA2 WT were prepared as ColE1 EMSA assay. RNA2 WT was titrated into solutions containing 300 nM RNA1 U6 · Cy5-$(SK^{2M})_3$ and diluted into 1x kissing buffer. For Rop turn on assay, RNA1 U6 · Cy5-$(SK^{2M})_3$ and RNA2 WT were mixed in 1x kissing buffer at 300 nM with addition of different concentration of Rop. After incubation at room temperature for 30 min, the fluorescence was measured on Cary eclipse fluorescence spectrophotometer (Agilent) by exciting at 610 nm and monitoring at 667 nm. The apparent K_d was fitted into equation 3: $Y=B_{max}*X/(K_d + X)$

S2.8. FRET measurement for TL/TLR system.

TL/TLR RNA was incubated in distilled water at 90ºC for 5 mins and slow cooling for 2 hrs. Cy3-$(SK^{2M})_3$ and Cy5-$(SK^{2M})_3$ were added sequentially to the RNA in a 1:1:1 ratio with a final concentration of 500 nM, using a 20 min incubation time for each peptide addition step in 10x folding buffer (500 mM HEPES pH 7.5, 1M NaCl), and an appropriate concentration of Mg^{2+} ranged from 0 - 20 mM was added to the solution and incubated for 30 mins. The fluorescence experiment was performed on a Thermo Scientific NanoDrop 3300 fluorospectrometer by exciting Cy3 at 550 nm and monitoring at 564 nm and 666 nm.

S3. Templates and transcripts

● DNAs for bPNA(+) binding measurements

dT6C4T6: 5′- TTT TTT CCC CTT TTT T -3'

● ColE1 RNA kissing loop complex

RNA1-U6

Template:

5'- GGC AGC AAA AAA CTA CCA AAA AAA AGC TGC CTA TAG TGA GTC GTA TTA ATT TC -3'

Transcript:

5'- GGC AGC UUU UUU UUG GUA GUU UUU UGC UGC C -3'

RNA2-WT

Template

5'- GCA CCG TTG GTA GCG GTG CTA TAG TGC GTC GTA TTA ATT TC -3

Transcript:

5'- GCA CCG CUA CCA ACG GUG C -3'

RNA2-U6

Template:

5'- GGC ACC GAA AAA ATT GGT AGA AAA AAC GGT GCC TAT AGT GAG TCG TAT TAA TTT C -3'

Transcript:

5'- GGC ACC GUU UUU UCU ACC AAU UUU UUC GGU GCC -3'

● TL/TLR

Template:

5'- GAA CGC AGT CAC AGT CCA TAT CAA AAA AGC GTT TGC AAA AAA GAC TTA GGA CTG AGA CTA CGC TGC AAC CGT AGT TGC AAT TGA CAC AAA AAA GTT TCC AAA AAA GTG CCT ATA GTG AGT CGT ATT AAT TTC -3'

Transcript:

5'- GGC ACU UUU UUG GAA ACU UUU UUG UGU CAA UUG CAA CUA CGG UUG CAG CGU AGU CUC AGU CCU AAG UCU UUU UUG CAA ACG CUU UUU UGA UAU GGA CUG UGA CUG CGU UC -3'

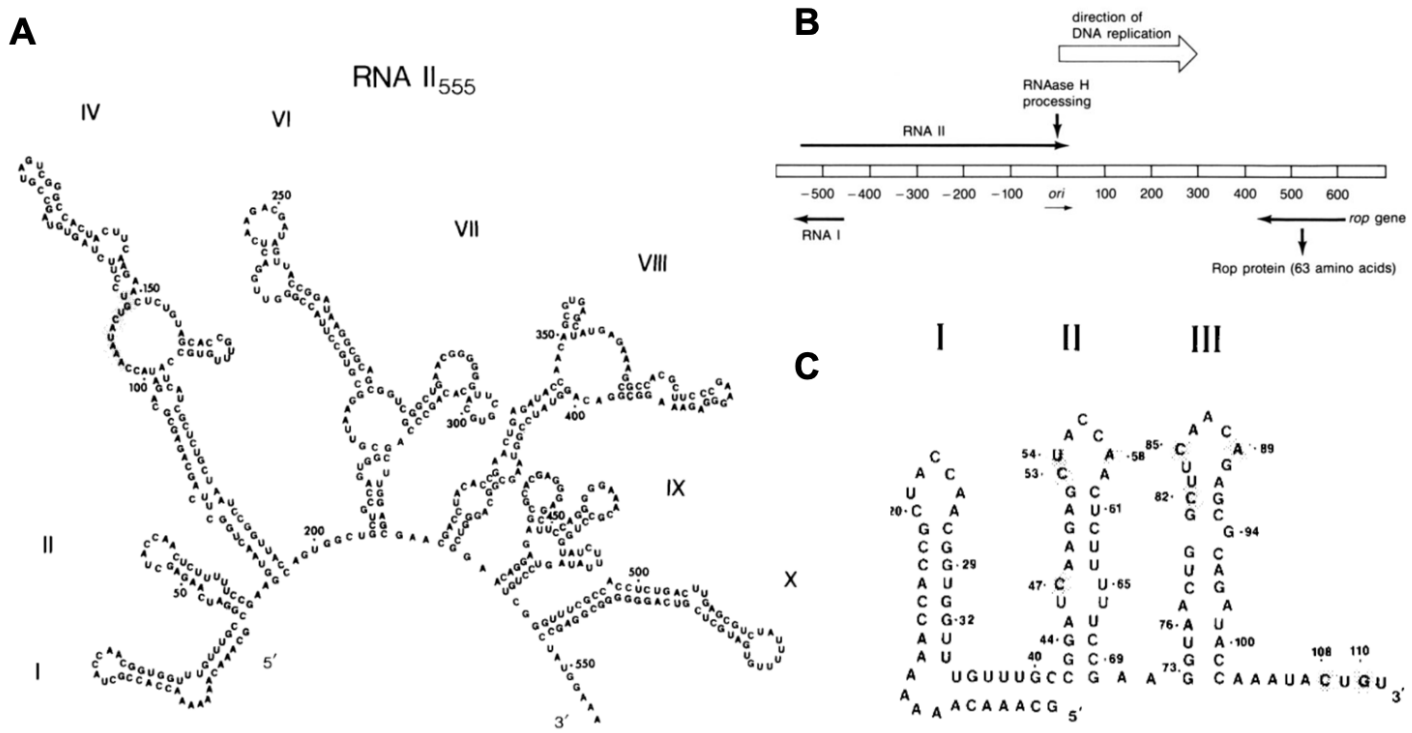


Figure S3.1. (A) The predicted secondary structure of RNA 2_{555}. (B) Scheme for ColE1 two RNA transcripts and the process. (C) RNA 2_{110} from 5′ end, which is antisense strand of RNA 1.[1]

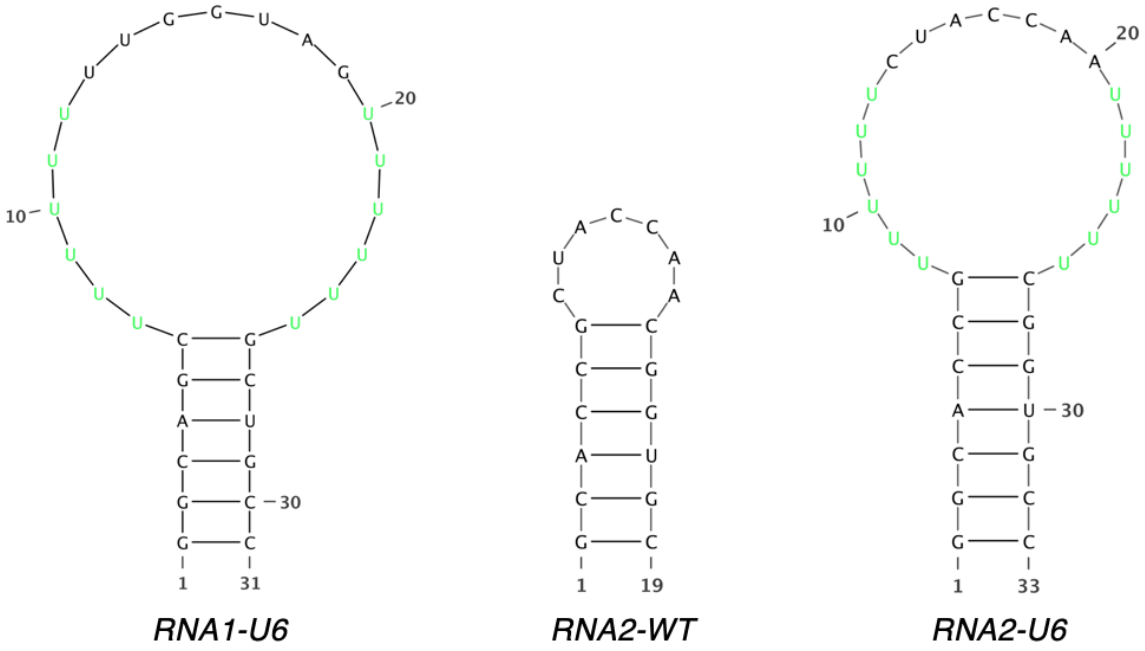


Figure S3.2. Secondary structure of RNAs designed for ColE1 kissing loop complex system. The loop sequence of RNA1: UUGGUAC and RNA2: CUACCAA are the same as the native transcripts.

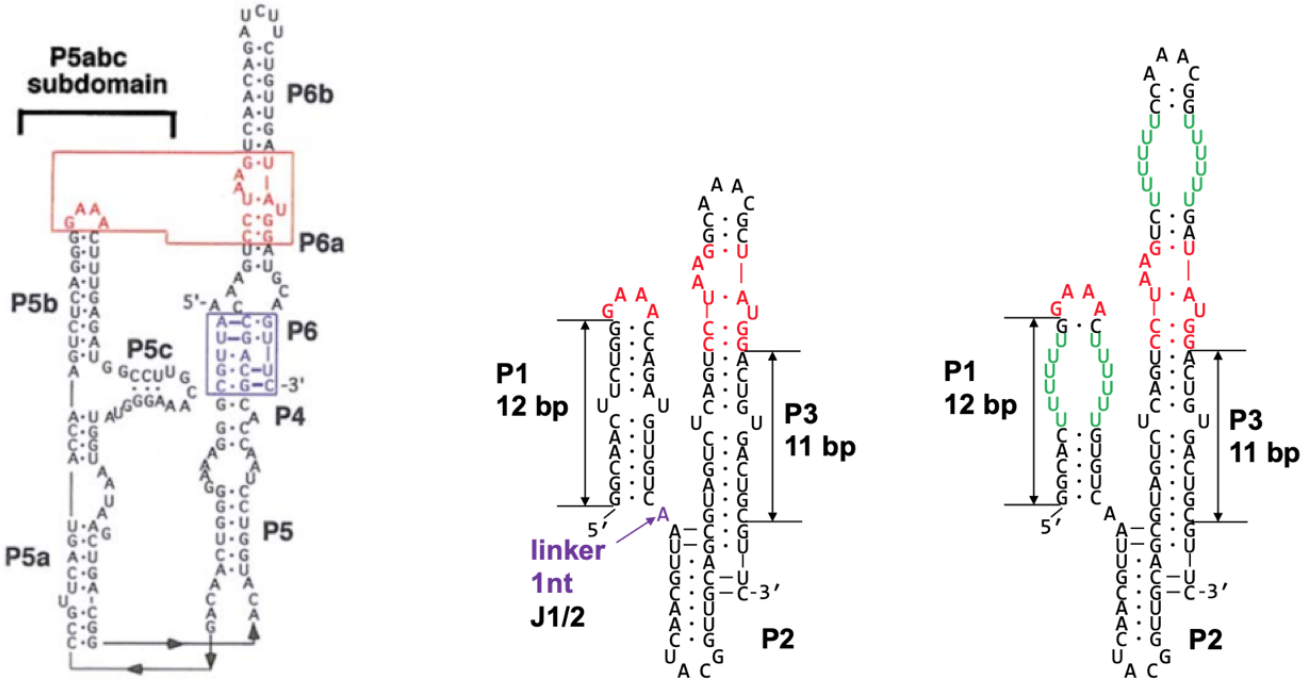


Figure S3.2. (Left) Secondary structure of P4-P6 domain of the *Tetrahymena* group I ribozyme. (Middle) TL/TLR self-folding RNA.[2] (Right) Designed intramolecular TL/TLR system with internal bPNA(+) binding sites for probe placement.

S4. RNA transcription and purification

RNA constructs were made via T7 runoff transcription. RNA transcription buffer 10x: 1M HEPES-KOH pH 7.5, 20 mM Spermidine-HCl, 400 mM DTT. Transcription was performed in the following concentrations: 1x Buffer with an additional 10 mM DTT, 35 mM $MgCl_2$, 20 mM rNTP, 500 nM DNA duplex, 2 µL of 100 U/mL Inorganic yeast Pyrophosphatase (New England Biolabs), and 5 µL of in house produced T7 polymerase stock (per 100 µl of transcription reaction solution, conditions subject to optimization based on sequences). Transcription was incubated at 37ºC for 4 hours, and then treated with DNase 1 (New England Biolabs) for 30 min at 37ºC. 1.5 equivalents of EDTA was added to the mixture to quench the reaction, and an extraction was conducted utilizing Phenol/Chloroform/Isoamyl Alcohol (25:24:1 Mixture, pH 6.7/8.0, Liq.) (Fisher BioReagents) An equal volume of 2x TBE/urea loading buffer was added. The sample was heated at 95ºC for 10 minutes and put on ice, and loaded onto 20% acrylamide (19:1 acrylamide:bisacrylamide) denaturing gel (8.3cm X 7.3cm X 1.5mm) with 8M urea as the denaturant. The RNA was visualized using UV shadowing and desired gel bands were cut out of the gel and placed in Spectra/Por® 3 Standard RC Dry Dialysis Tubing (Spectrum Laboratories) filled with 1X TBE buffer. Electroelution was set up at 120 V for 3 hrs to make the RNA fragment to leave the gel slices. The solution was removed from the tubing and placed in a microfuge tube to be precipitated with NH_4OAc (2.5M final concentration) and 2.5 volumes of 200 proof ethanol at -20ºC for at least 2 hrs. RNA was centrifuged down at high speed for 20 minutes at 4ºC and the pellet was washed with 500 µl of cold 70% ethanol in water and centrifuged again at high speed for 10 minutes at 4ºC. RNA concentration was determined by utilizing absorbance as measured on a Nanodrop and extinction coefficients as determined by the IDT OligoAnalyzer. Sequence length and purity were determined using 20% polyacrylamide gel electrophoresis (PAGE) TBE-Urea gels.

S5. Additional biophysical data for bPNA(+) hybrid structures

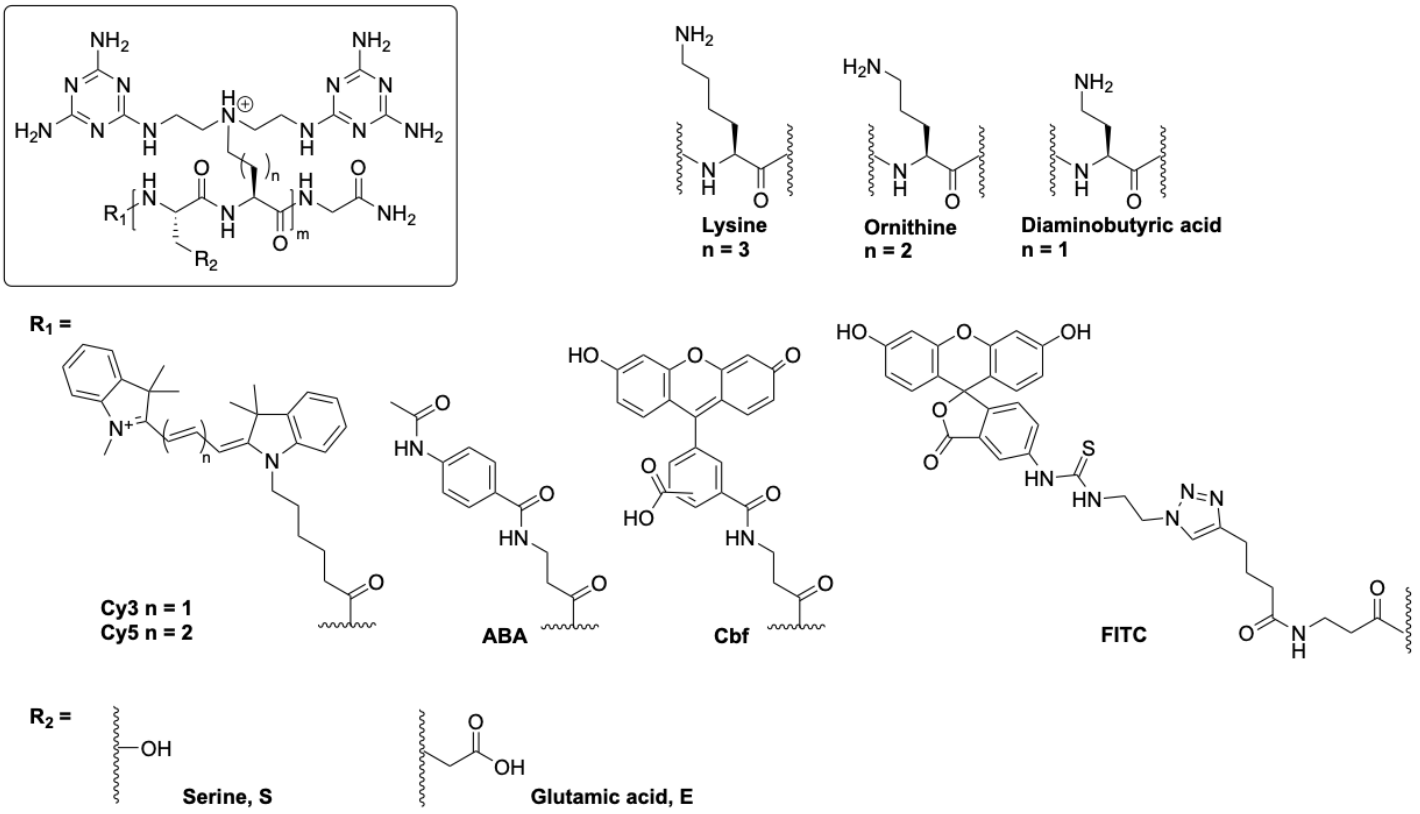
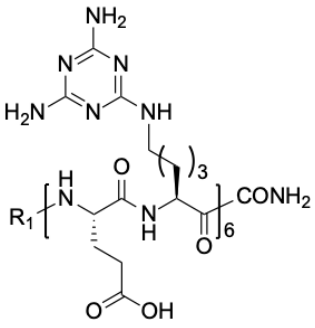
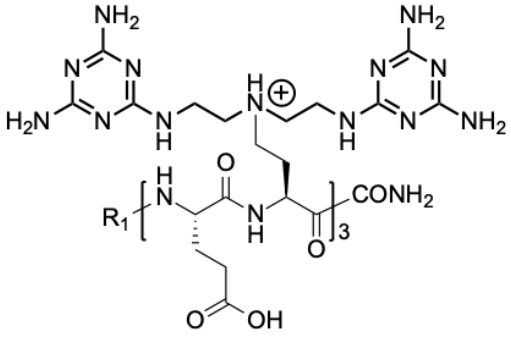
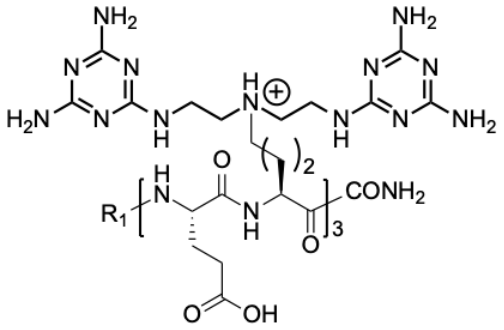
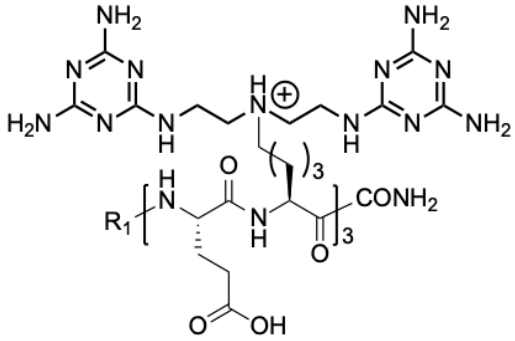
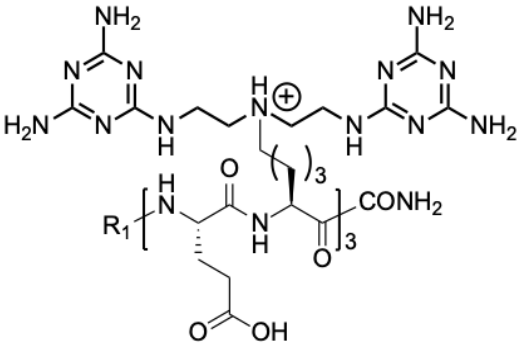
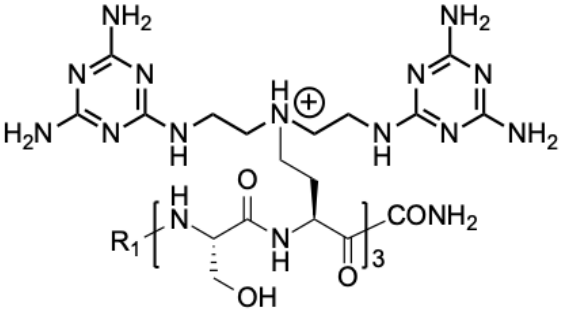
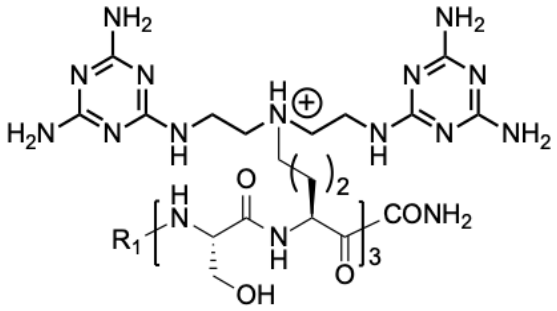
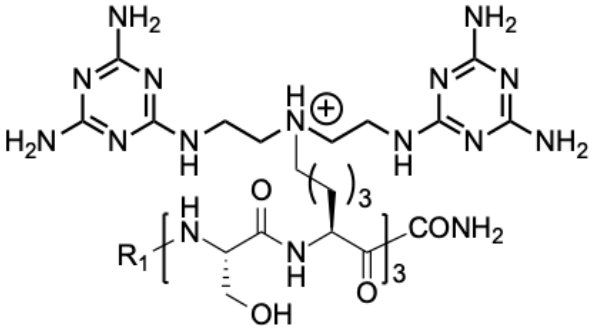
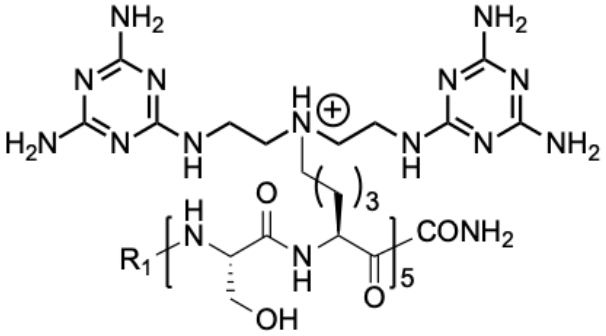


Figure S5.1. Structure of bPNA(+) and derivatives.

Table S5.1. Structure of bPNA(+) and derivatives

Peptide Abbreviation	Structure
$(EK^{M})_6$	
$(EB^{2M})_3$	

$(EO^{2M})_3$	
$(EK^{2M})_3$	
$(EK^{2M})_5$	
$(SB^{2M})_3$	

$(SO^{2M})_3$	
$(SK^{2M})_3$	
$(SK^{2M})_5$	

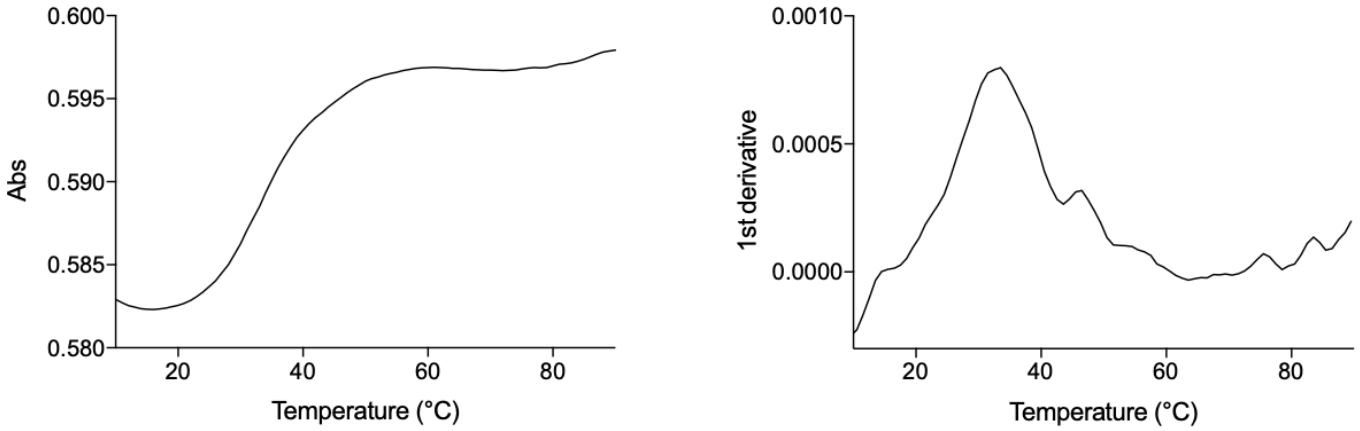


Figure S5.2. UV-melting of $(EK^{2M})_2$ peptides with DNA $T_4C_4T_4$. Samples were prepared in 1X PBS, both DNA and peptide concentrations were 2 μM. T_m for $T_4C_4T_4$ complex was 34°C.

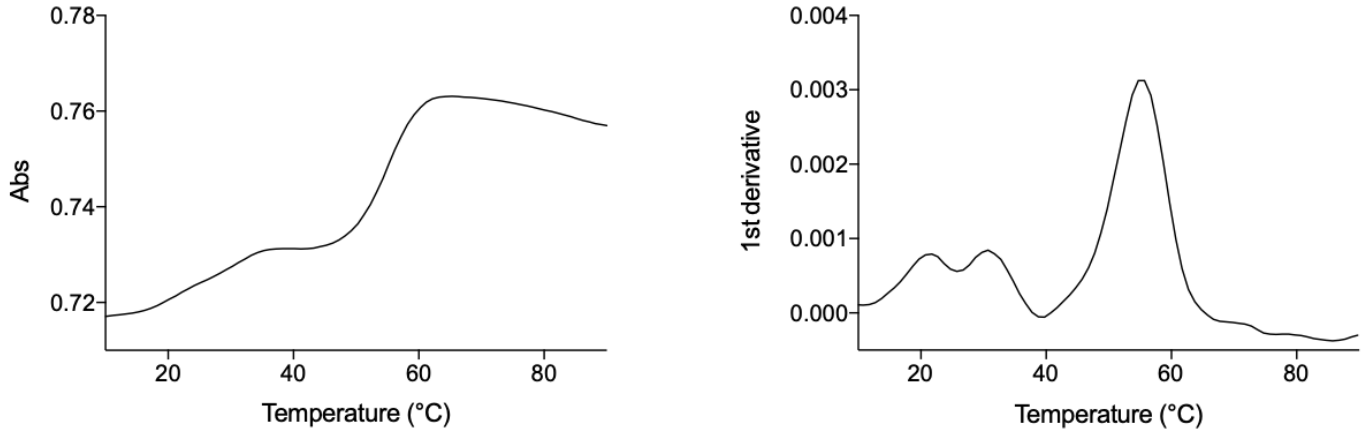


Figure S5.3. UV-melting of $(EK^{2M})_2$ peptides with DNA $T_{10}C_4T_{10}$. Samples were prepared in 1X PBS, DNA concentrations were 2 μM, peptide concentrations was 4 μM. T_m for $T_{10}C_4T_{10}$ complex was 54°C.

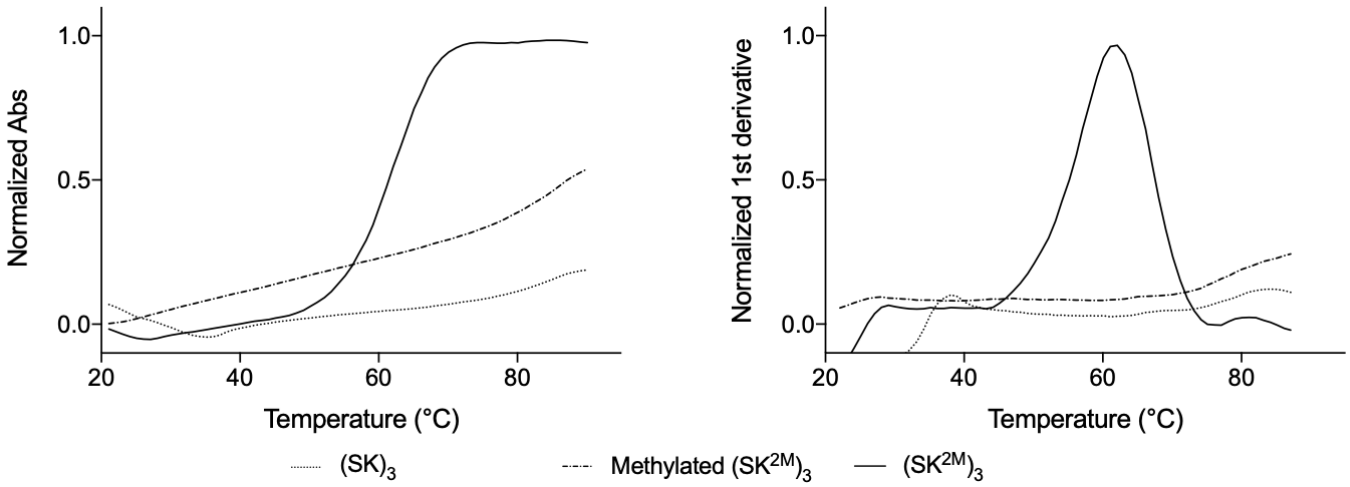


Figure S5.4. UV-melting of $T_6C_4T_6$ and $(SK^{2M})_3$ control peptides. Samples were prepared in 1X PBS, both DNA and peptide concentrations were 2 μM.

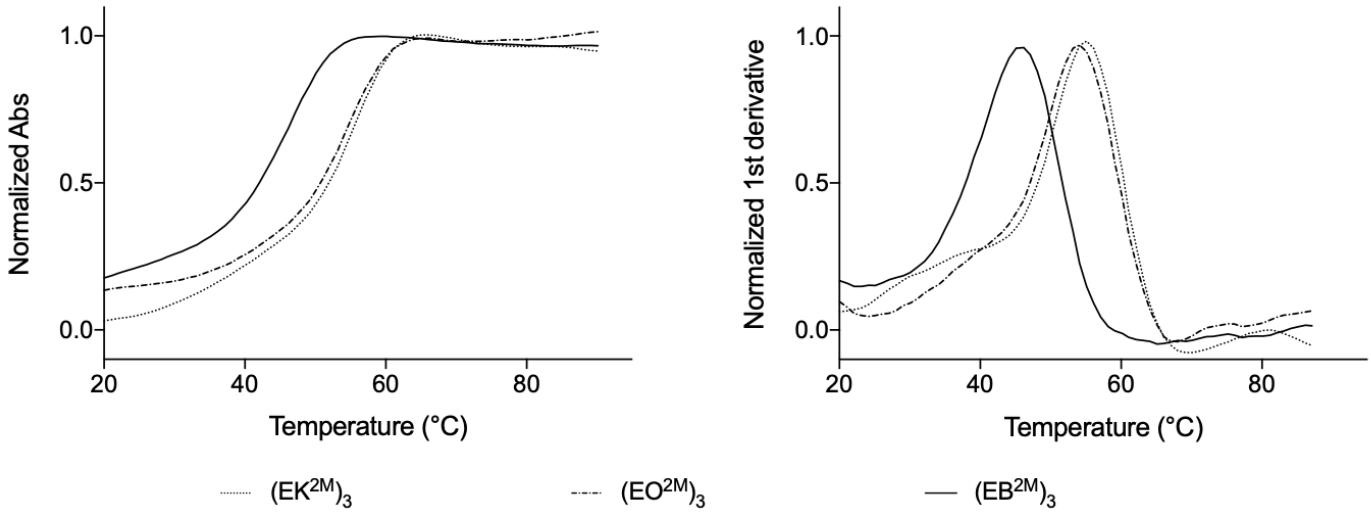


Figure S5.5. UV-melting of $T_6C_4T_6$ and E series peptides. Samples were prepared in 1X PBS, both DNA and peptide concentrations were 2 μM.

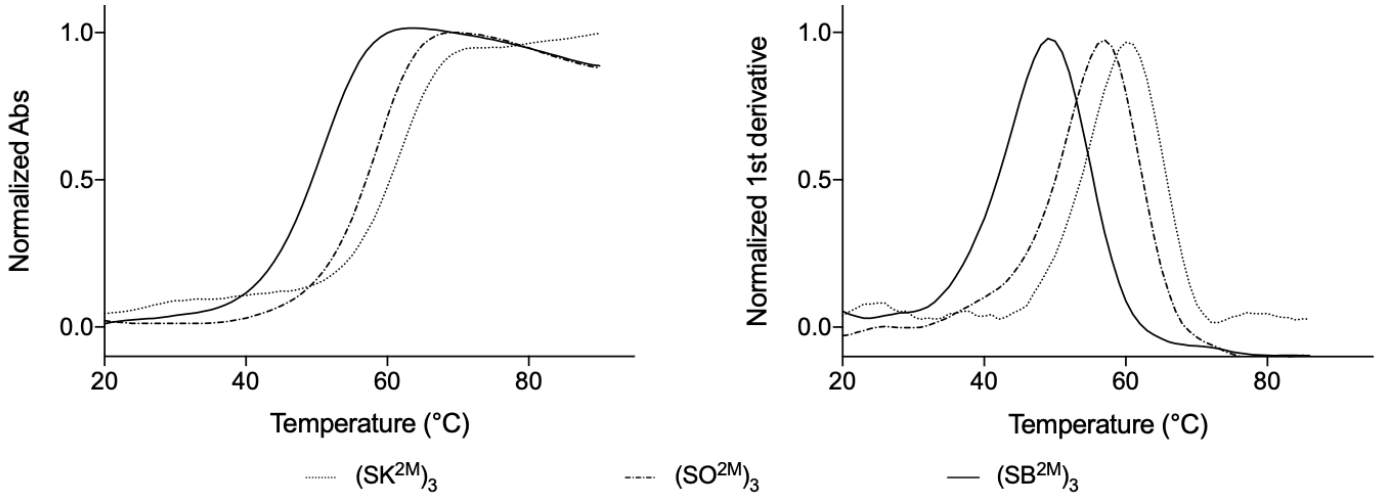


Figure S5.6. UV-melting of $T_6C_4T_6$ and S series peptides. Samples were prepared in 1X PBS, both DNA and peptide concentrations were 2 μM.

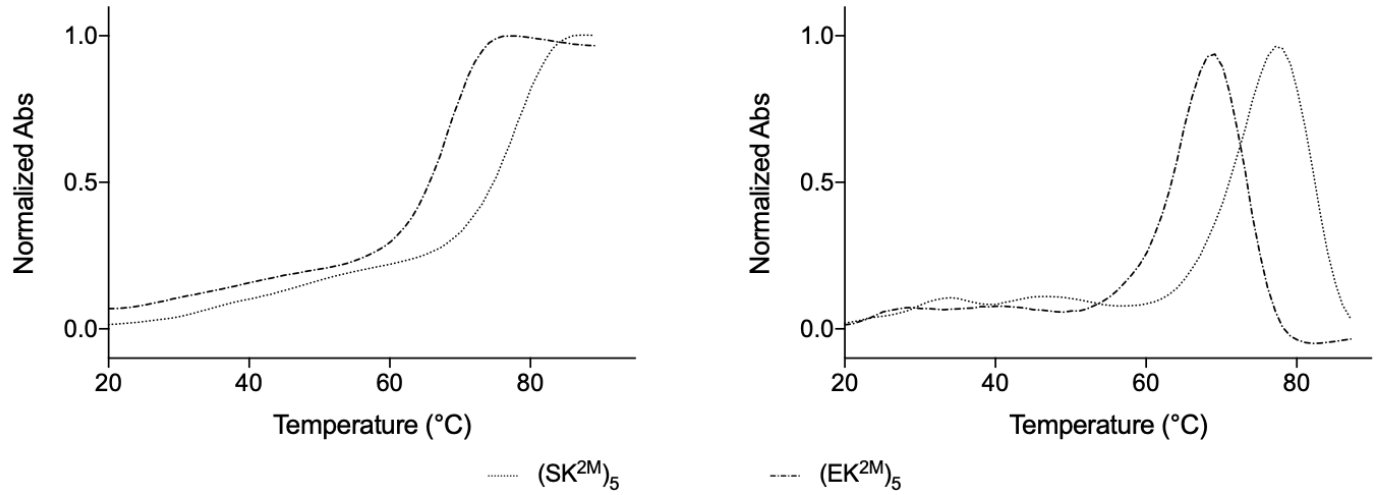


Figure S5.7. UV-melting of $T_{10}C_4T_{10}$ and $(EK^{2M})_5$ and $(SK^{2M})_5$ peptides. Samples were prepared in 1X PBS, both DNA and peptide concentrations were 2 μM.

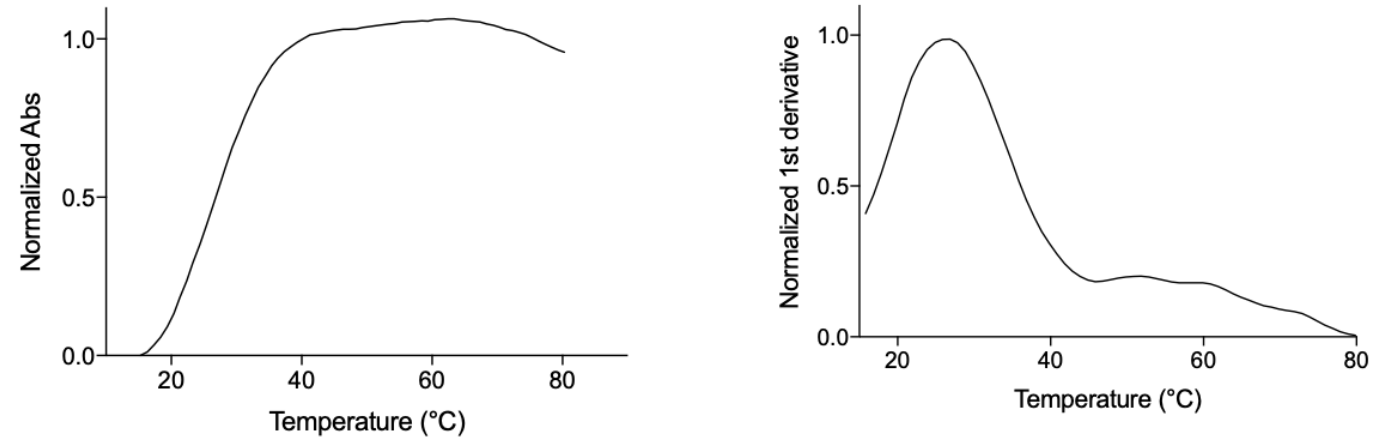


Figure S5.8. UV-melting of U6C4U6 RNA with $(SK^{2M})_3$ peptide. Samples were prepared in 1X PBS, both RNA and peptide concentrations were 2 μM. T_m for $U_6C_4U_6$ complex was 25°C.

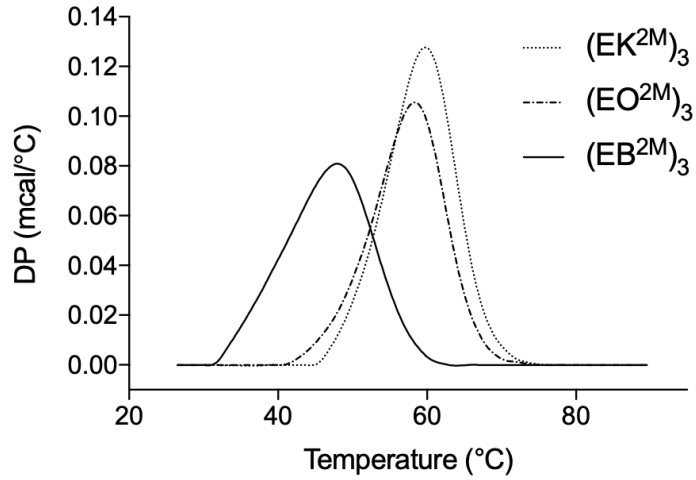


Figure S5.9. DSC curve of $T_6C_4T_6$ with E series T2M peptides.

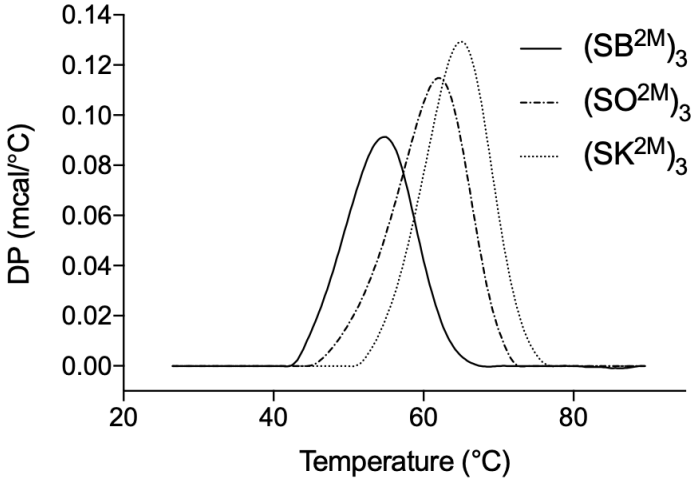


Figure S5.10. DSC curve of $T_6C_4T_6$ with S series T2M peptides.

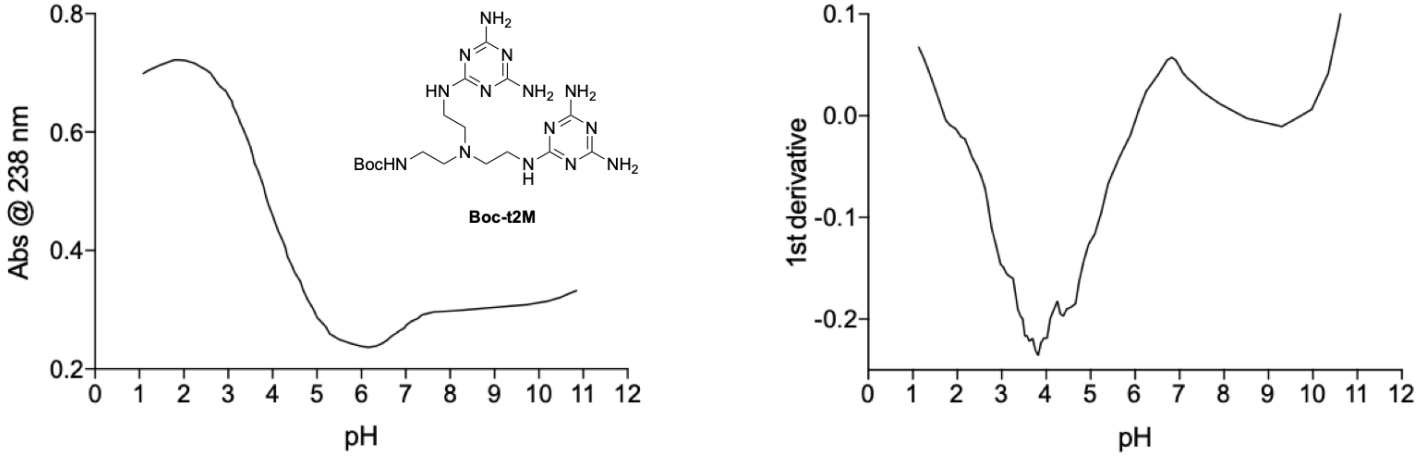


Figure S5.11. Measurement of pK_A of Boc-t2M as surrogate for K^{2M}. Absorbance (UV-Nanodrop instrument) of the protonated ring is more intense, yielding the pH-dependent curve shown. The absorbance curve indicates the pK_A of melamine is 3.82.

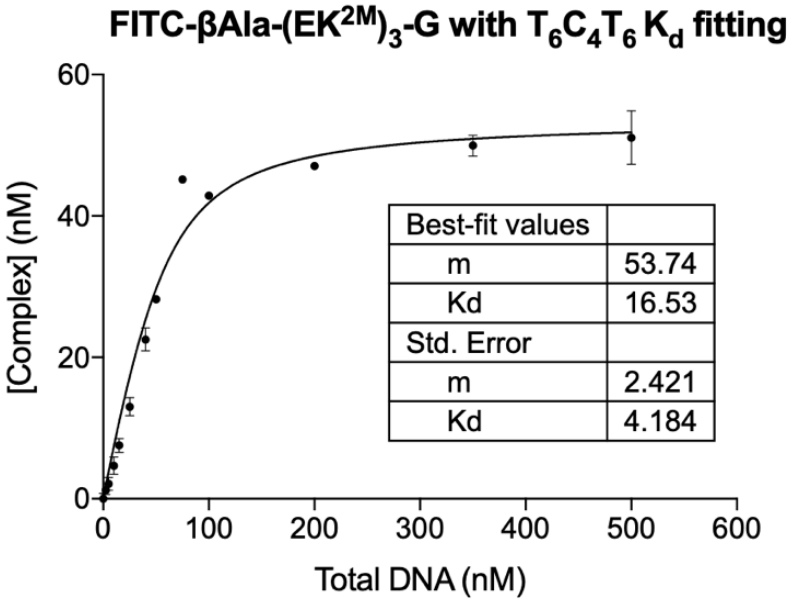


Figure S5.12. Binding isotherm obtained by fluorescence anisotropy of $T_6C_4T_6$ titrated into 50 nM FITC-βAla-$(EK^{2M})_3$-G. Data fitted using 1:1 binding model.

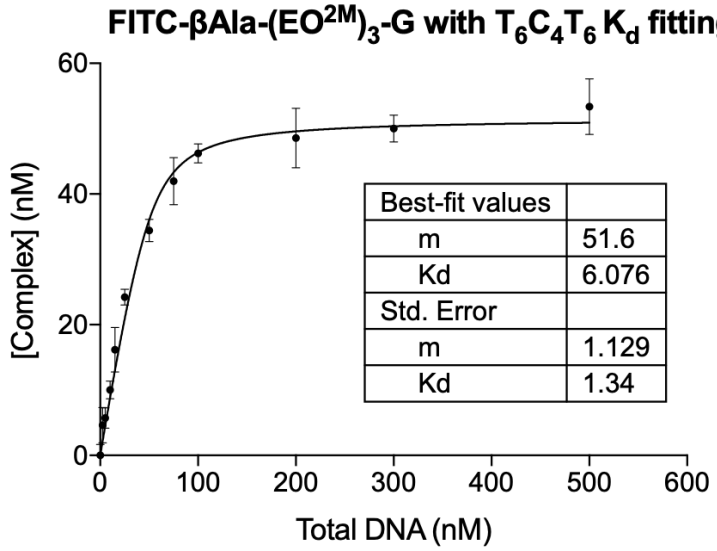


Figure S5.13. Binding isotherm obtained by fluorescence anisotropy of $T_6C_4T_6$ titrated into 50 nM FITC-βAla-$(EO^{2M})_3$-G. Data fitted using 1:1 binding model.

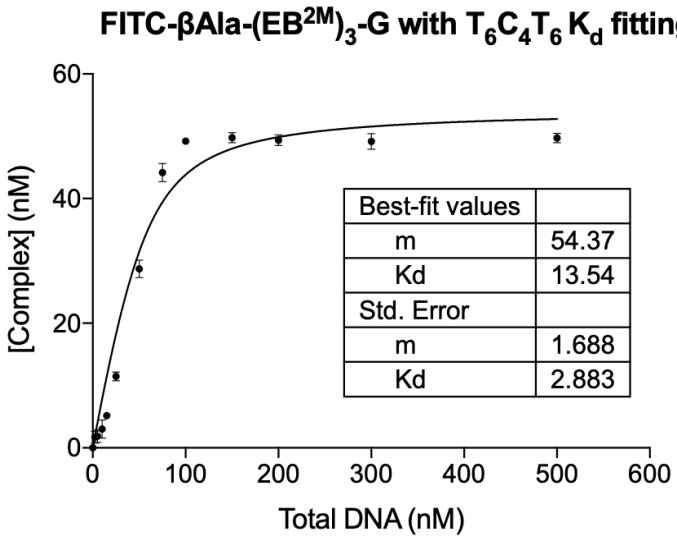


Figure S5.14. Binding isotherm obtained by fluorescence anisotropy of $T_6C_4T_6$ titrated into 50 nM FITC-βAla-$(EB^{2M})_3$-G. Data fitted using 1:1 binding model.

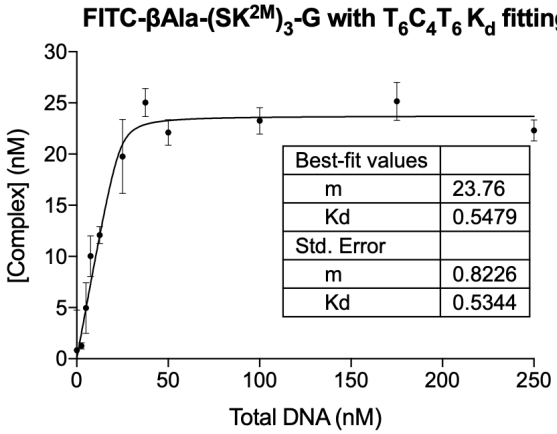


Figure S5.15. Binding isotherm obtained by fluorescence anisotropy of $T_6C_4T_6$ titrated into 25 nM FITC-βAla-$(SK^{2M})_3$-G. Data fitted using 1:1 binding model.

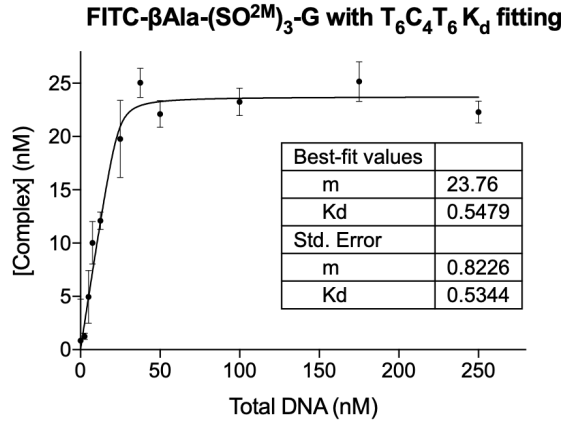


Figure S5.16. Binding isotherm obtained by fluorescence anisotropy of $T_6C_4T_6$ titrated into 25 nM FITC-βAla-$(SO^{2M})_3$-G. Data fitted using 1:1 binding model.

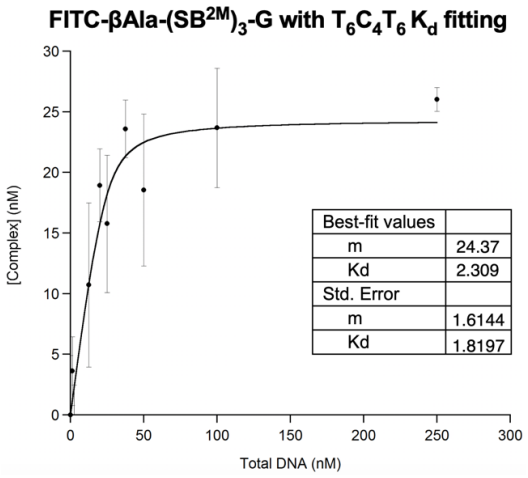


Figure S5.17. Quantification of gel data binding to FITC-βAla-$(SB^{2M})_3$-G RNA1-U6. Binding curve data fitted using 1:1 binding model with equation 2.

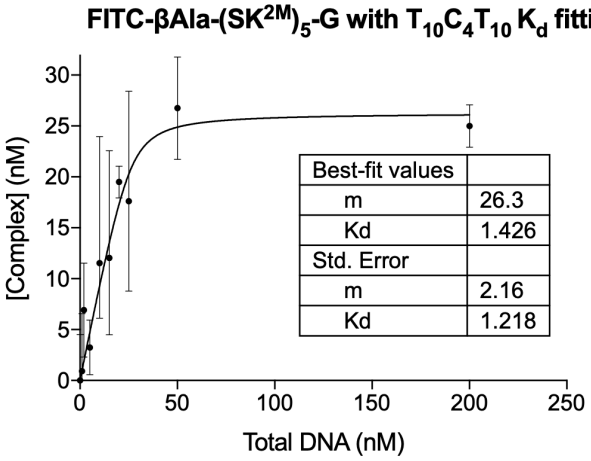


Figure S5.18. Binding isotherm obtained by fluorescence anisotropy of $T_{10}C_4T_{10}$ titrated into 25 nM FITC-βAla-$(SK^{2M})_5$-G. Data fitted using 1:1 binding model.

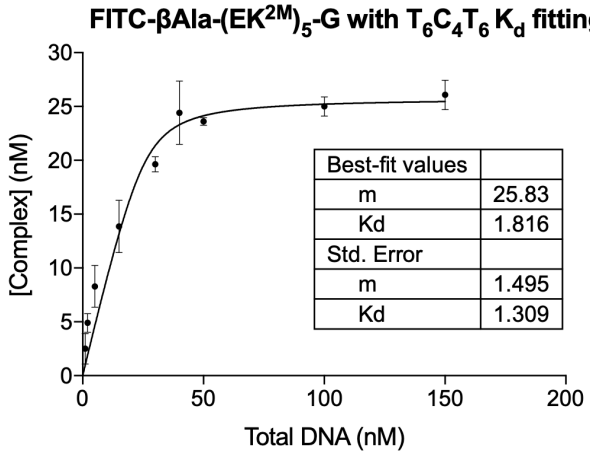


Figure S5.19. Binding isotherm obtained by fluorescence anisotropy of $T_{10}C_4T_{10}$ titrated into 25 nM FITC-βAla-$(EK^{2M})_5$-G. Data fitted using 1:1 binding model.

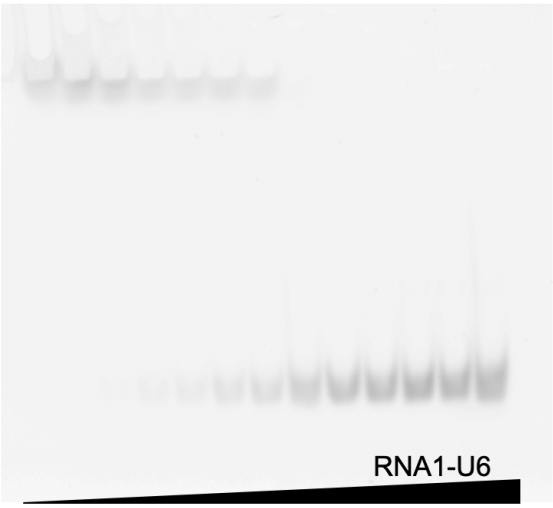


Figure S5.20. Example of EMSA assay for RNA1-U6 titration into 50 nM FITC-βAla-$(SK^{2M})_3$-G.

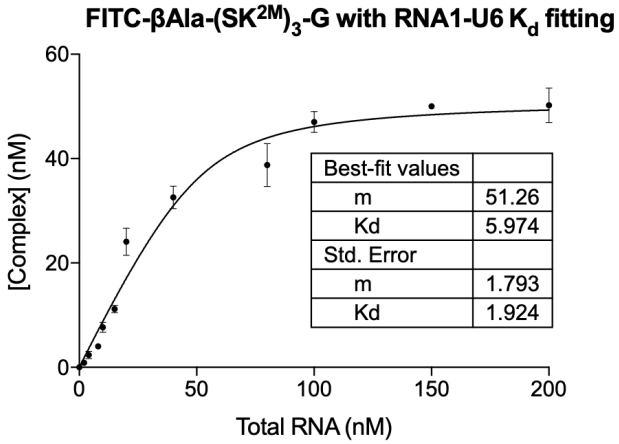


Figure S5.21. Quantification of gel data binding to FITC-βAla-$(SK^{2M})_3$-G RNA1-U6. Binding curve data fitted using 1:1 binding model with equation 2.

S6. Fluorescence and FRET data

S6.1. Fluorescence turn on for ColE1 system

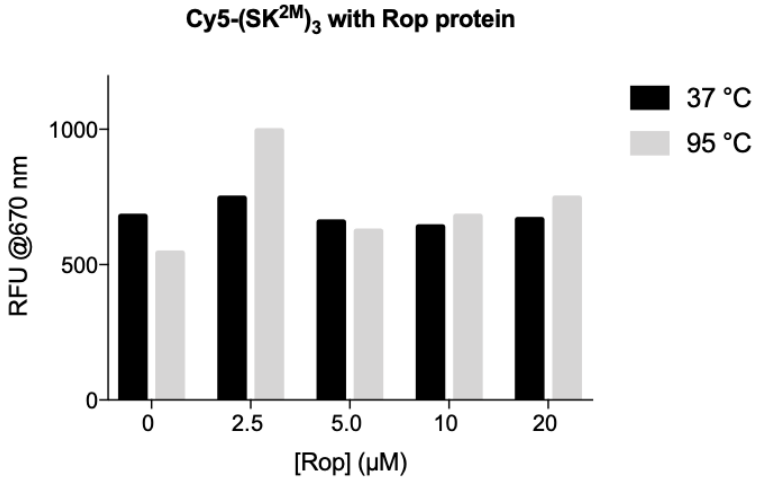


Figure S6.1. Control experiment. Rop did not change fluorescence of Cy5-$(SK^{2M})_3$ in the absence of kissing loop RNAs.

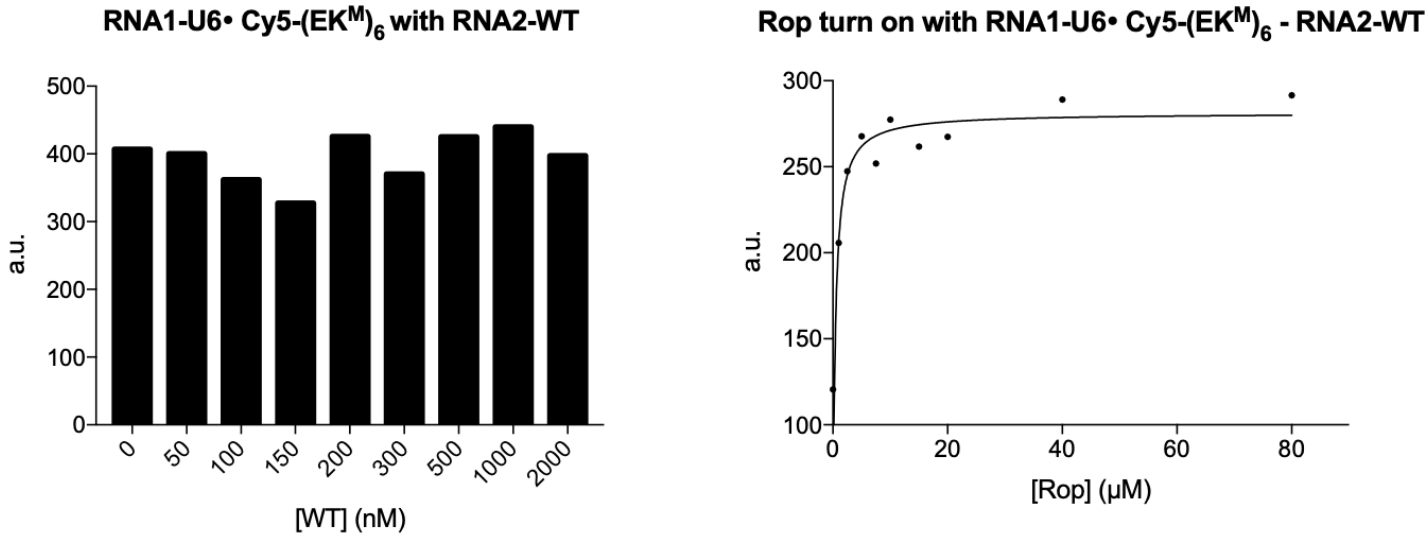


Figure S6.2. Fluorescence change with the 1st generation of bPNA(+), Cy5-$(EK^{M})_6$. No change was observed when RNA1 WT was titrated in and ~2-fold increase was observed with addition of Rop.

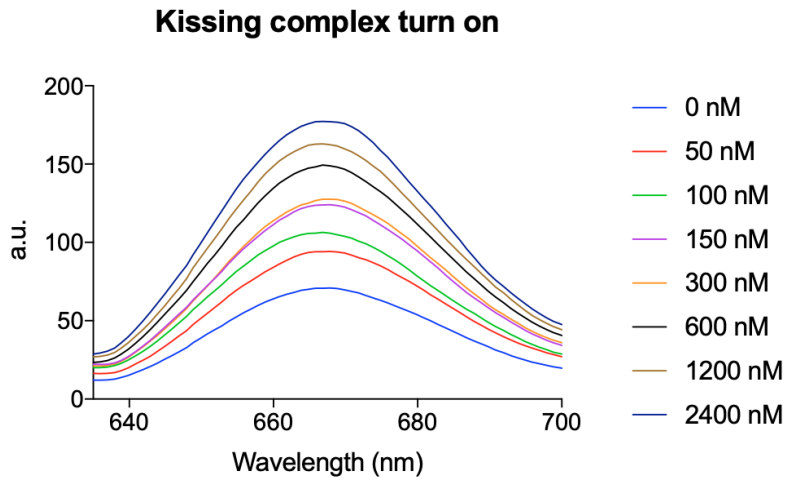


Figure S6.3. Representative emission spectra of kissing loop complex formation as a function of RNA2 WT concentration.

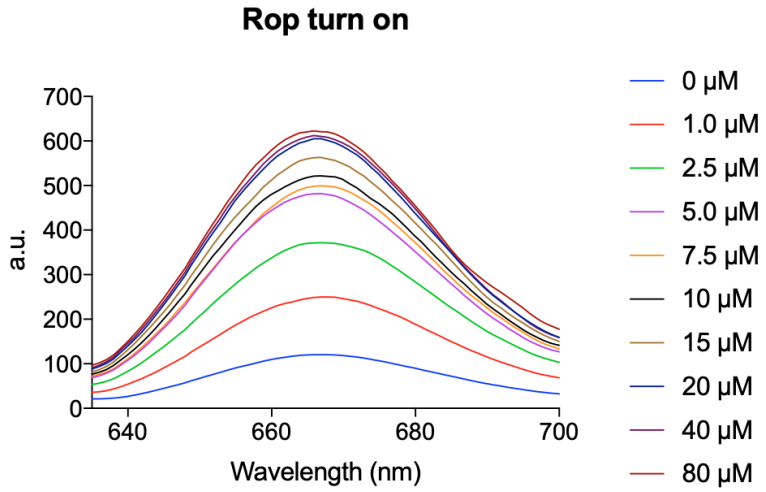


Figure S6.4. Representative emission spectra of labeled kissing loop complex as a function of Rop concentration.

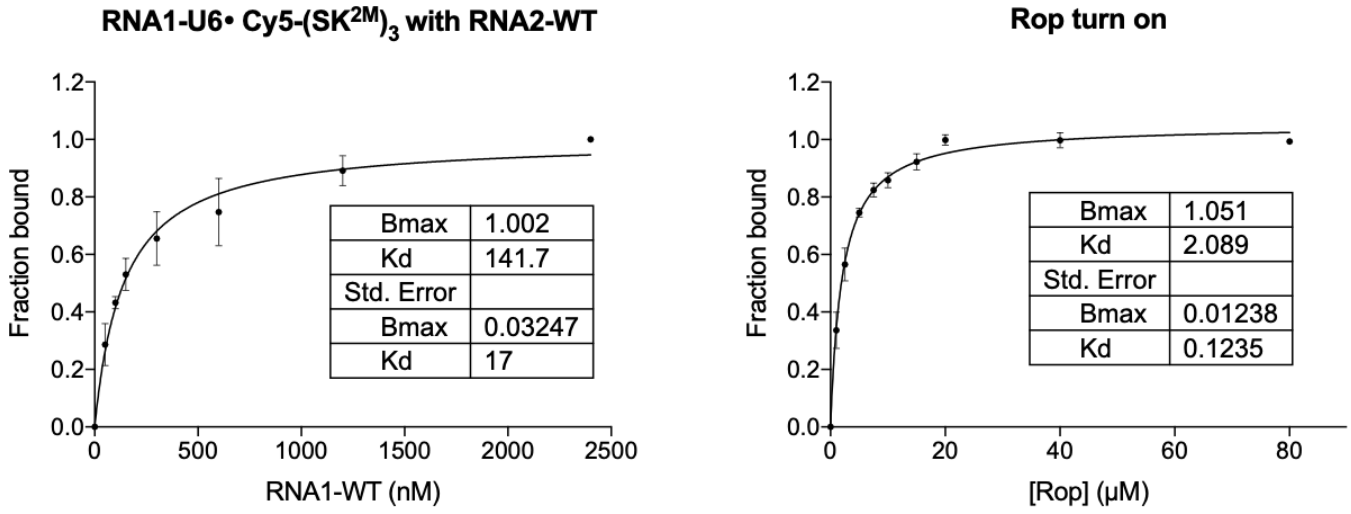


Figure S6.5. Fluorescence change of Cy5-$(SK^{2M})_3$ complexes as labeled. Data fit to 1:1 binding model (equation 3).

S6.2. ColE1 system FRET

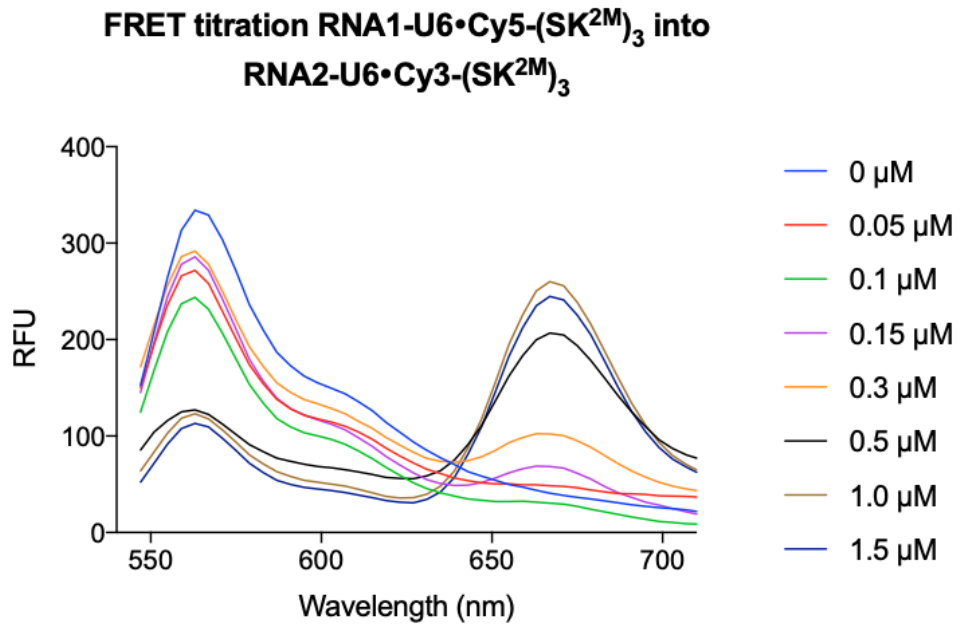


Figure S6.6. FRET titration assay, RNA1 U6 · Cy5-$(SK^{2M})_3$ was titrated into constant 0.3 μM RNA2 U6 · Cy3-$(SK^{2M})_3$ solution.

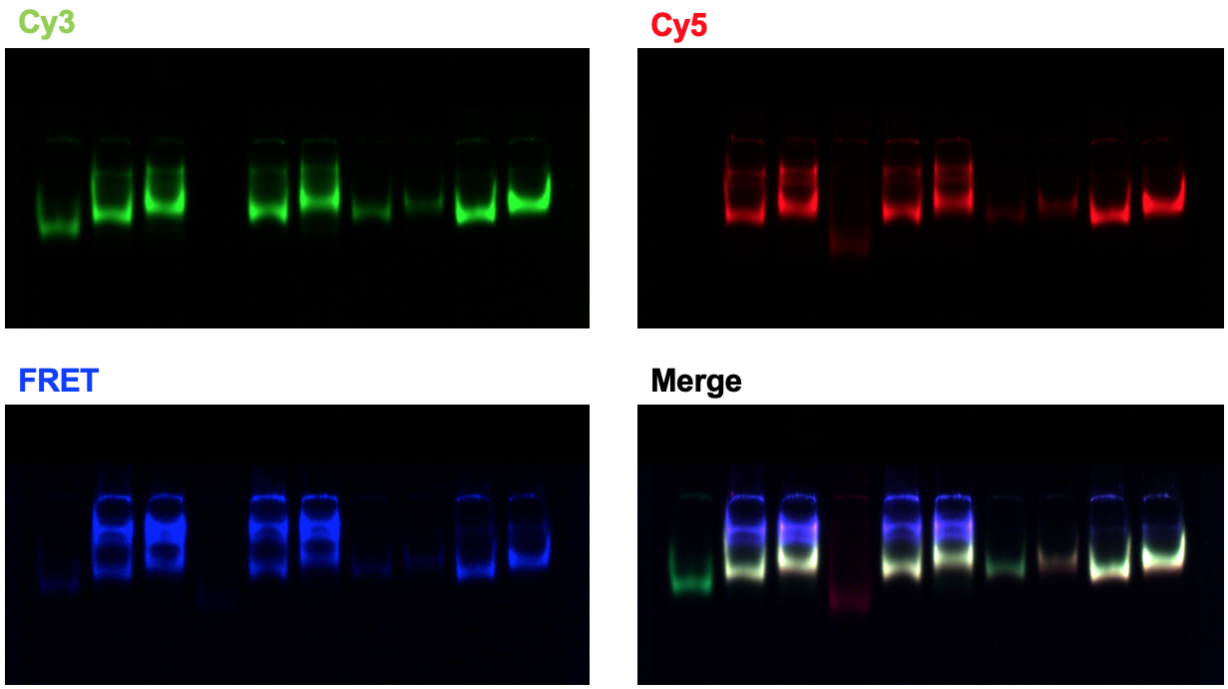


Figure S6.7. FRET EMSA gel assay. Lane 1: RNA2 U6 · Cy3-$(SK^{2M})_3$, Lane 2, 5: +RNA1-U6 · Cy5-$(SK^{2M})_3$, Lane 3, 6:+ RNA1-U6 · Cy5-$(SK^{2M})_3$ and Rop. Lane 4: RNA1 U6 · Cy5-$(SK^{2M})_3$. (Lane 2, 3, 4, 5: RNA-peptide complex was heated at 95ºC for 5 mins and snap cool for 5 mins on ice, mixed, and diluted into 1x kissing buffer) Lane 7: RNA1-U6, RNA2-U6, Cy3-$(SK^{2M})_3$ and Cy5-$(SK^{2M})_3$ were heated at 95ºC for 5 mins and snap cool for 5 mins on ice separately in water and mixed. Lane 8: + Rop. Lane 9: RNA1-U6, RNA2-U6, Cy3-$(SK^{2M})_3$ and Cy5-$(SK^{2M})_3$ were mixed together and heated at 95ºC for 5 mins and put on ice, add Mg^{2+} to the final concentration of 10 mM. Lane 10: +Rop.

S6.3. TL/TLR system FRET

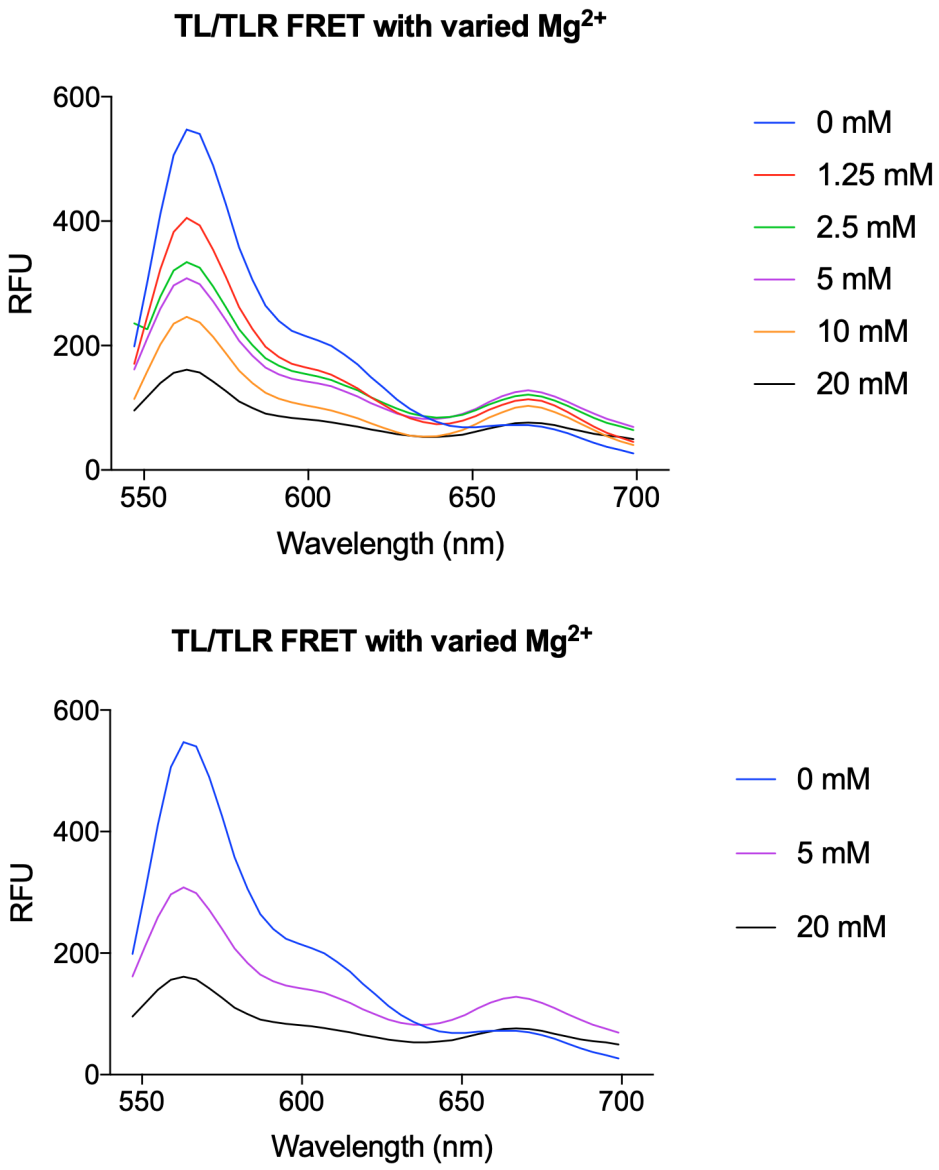
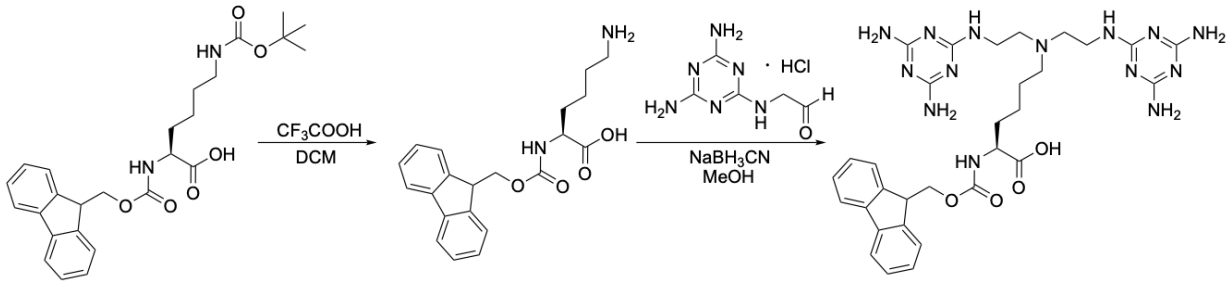


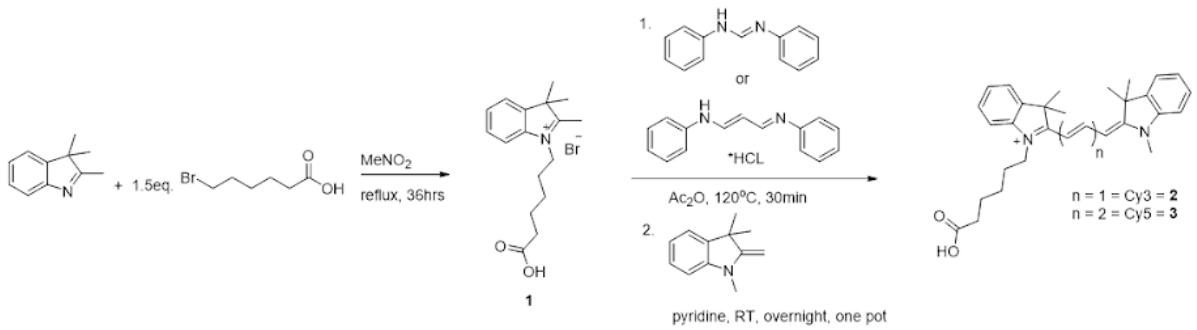
Figure 6.8. Representative emission spectra for the TL/TLR RNA bound with Cy3 and Cy5 labeled bPNA+ as a function of Mg^{2+}, with Cy3 excitation. (Top) Full range of Mg^{2+} concentrations and (Lower) selected Mg^{2+} concentrations for clarity.

S7. Synthetic procedures



Scheme S7.1. Synthesis of Fmoc-K^{2M}-OH from commercially available Fmoc-Lys(Boc)-OH.

Fmoc-K^{2M}-OH. Fmoc-Lys(Boc)-OH (1 g, 2.1 mmol) was dissolved in 20 mL dichloromethane and 4 mL of trifluoroacetic acid was added. The reaction was stirred for 1 h and the solution was condensed to syrup under a stream of N_2. Dichloromethane (10 mL) was added to dissolve the syrup and was removed by a stream of N_2. The syrup was dissolved in 30 mL methanol and the pH was adjusted to 5 with solid $NaHCO_3$. Melamine aldehyde (0.95 g, 4.62 mmol) and $NaBH_3CN$ (0.293 g, 4.62 mmol) was aliquoted into 4 portions, respectively. To the reaction solution, 2 portions of melamine aldehyde were added and was stirred and incubated at 50°C for 30 min. Then the reaction was taken out to cool to room temperature and 1 portion of $NaBH_3CN$ was added. The reaction was stirred at room temperature for another 30 min. Then 1 portion of aldehyde was added, incubated at 50°C for 30 min and 1 portion of $NaBH_3CN$ was added and incubated at room temperature for 30 min. These addition of aldehyde and reductant steps were repeated until all 4 portions of aldehyde and 3 portions of $NaBH_3CN$ were added. The last portion of $NaBH_3CN$ was added to the reaction and stirred for 30 min at room temperature. The reaction was monitored by HPLC. The remaining monoadduct (Fmoc-K^{M}-OH) was reacted by adding 0.23 g of melamine aldehyde, incubating at 50°C for 30 min and 0.072 g $NaBH_3CN$ was added to finish the reaction. Methanol was reduced to 5 ml and was discarded after centrifugation, yielding white solid as crude product after drying. The reaction was quenched by adding 2 mL 1N hydrochloric acid and the solid was triturated. The hydrochloric acid was discarded after centrifugation. Acetone (5 mL) was added to the residue and the residue was triturated. The acetone was removed by centrifugation. The acetone wash was repeated 3 times and ethanol was added for trituration instead of acetone. After removing ethanol by centrifugation, the product (1 g, 70%) was obtained as white solid. **^{1}H NMR** (DMSO-d6): 7.89 (2H, d); 7.72 (2H, d); 7.62 (1H, d); 7.41 (2H, t); 7.32 (2H, t); 6.83 (2H, t); 6.43 (8H, d); 4.28 (2H, d); 4.23 (1H, t); 3.93 (1H, m); 3.45 (4H, q); 2.98 (4H, t); 2.86 (2H, s); 1.44-1.80 (4H, m); 1.33 (2H, m). **^{13}C NMR** (DMSO-d6): 174.36; 164.75; 156.65; 144.28; 141.20; 128.12; 127.56; 125.76; 120.58; 66.07; 54.28; 53.60; 52.62; 47.13; 36.30; 30.90; 23.45. **HRMS** (ESI): calculated for $[M+H^+]$=673.3429, found $[M+H^+]$=673.3421, $[M+2H^+]$=337.1729.



Scheme S7.2. Synthesis of cyanine dye derivatives based on a previously reported procedure.[3]

1-(5-carboxypentyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (1). (Scheme S7.2) In a round bottom flask was added 18.8 mmol (3 g) of 1,3,3-Trimethyl-2-methyleneindoline in 10 ml of nitromethane. To this was added 21 mmol (4.13 g) of 6-bromohexanoic acid and the solution was refluxed for 36 h. Upon completion, the reaction was cooled to room temperature and slowly added to 150 ml of ethyl acetate with vigorous stirring. The product was collected by vacuum filtration and washed with 3 x 50 ml of ethyl acetate to yield 15.3 mmol (5.39 grams, 81% yield) of **1** as a purple powder. Calculated [M+]: 274.1802 Found: 274.1803

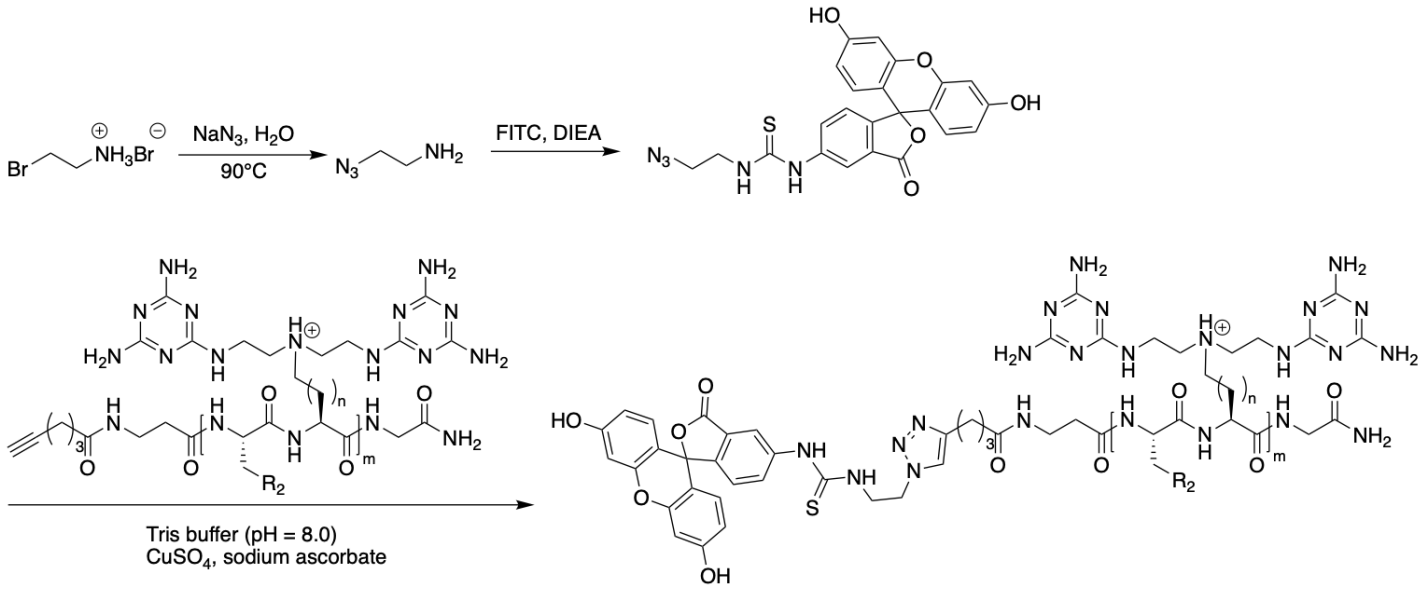
Cy3-acid (2). (Scheme S7.2) To a round bottom flask was added 0.5 grams of **1** (1.4 mmol), 340 mg of *N*,*N*′-diphenylformamidine, and 7.5 ml of acetic anhydride. This solution was heated at 120ºC for 0.5 h. The solution was then cooled to room temperature followed by the addition of 310 mg of 1,3,3-trimethyl-2-methyleneindoline and 7.5 ml of pyridine. The solution was stirred overnight at room temperature. The following day, 200 ml of ethyl ether was added and the product precipitated overnight at -20ºC. The precipitate was collected by centrifugation at 3500 rpm for 10 min, dried with a nitrogen stream, and purified by flash column chromatography using 100% EtOAc, then a gradient of methanol/dichloromethane gradient of 2%-5%-7% yielding 358 mg of **2** as a dark red foam. R_f = 0.3 (10% MeOH in DCM). Calculated [M+]: 457.2850 Found: 457.2853

Cy5 acid (3). (Scheme S7.2) To a round bottom flask was added 0.5 grams of **1** (1.4 mmol), 450 mg of N-(3-(phenylamino)-2-propenylidene)aniline hydrochloride, and 7.5 ml of acetic anhydride. This solution was heated at 120ºC for 0.5 h. The solution was then cooled to room temperature followed by the addition of 310 mg of 1,3,3-trimethyl-2-methyleneindoline and 7.5 ml of pyridine. The solution was stirred overnight at room temperature. The following day, 200 ml of diethyl ether was added and the product precipitated overnight at -20ºC. The precipitate was collected by centrifugation, dried with a nitrogen stream, and purified by flash column chromatography using 100% EtOAc, then a gradient of methanol/dichloromethane gradient of 2%-5%-7% yielding 413 mg of **3** as a dark blue foam. R_f = 0.3 (10% MeOH in DCM). Calculated [M+]: 483.3006 Found: 483.3002.

S8. Solid phase peptide synthesis and characterization

Peptide synthesis was performed manually using Rink Amide resin (100-200 mesh, loading 0.3 mmol/g) employing standard Fmoc chemistry. 0.25 M of Amino acids were coupled with 0.25 M of HOBt and 0.25 M DIC in 2 ml NMP/DMSO (1:1). Cyanine and Fluorescein dyes were coupled using three equivalents of dye, 3.3 equivalents of HBTU, and 3.3 equivalents of DIEA in 2 ml DMF. Dye and coupling reagents were allowed to react for fifteen min before addition to resin. Peptides were cleaved from the solid support and tert-Butyl protective groups removed using 95% trifluoroacetic acid (TFA) in H_2O for 4 h. Cold diethyl ether (Et_2O) was added to precipitate the peptide and the crude pellet was washed with cold Et_2O two times and dried over vacuum. Crude peptides were then dissolved in solvent A and purified by HPLC on a semi-prep C18 reversed phase column at 8 mL/min using the following gradient: 5% solvent B in 0-5 min, 5-42% solvent B in 5-20min, 42% solvent B in 20-25 min, 42-100% solvent B in 25-27 min, 100% solvent B in 27 min-32 min, 100-5% solvent B in 32-34 min, 5% solvent B in 34-40 min (solvent A=0.1%TFA in water, solvent B=0.07% TFA in 90% acetonitrile, 10% water). The UV detector was set at 230 nm. The purified peptides were lyophilized to dryness. The identity of peptide was checked by MALDI-TOF and purity checked by analytical HPLC on a C18 column.

**Ionization of the FITC peptides, in particular in MALDI-MS, less so for ESI, was prone to loss of the FITC moiety, though HPLC indicated clean product isolation. This was observed for all FITC bPNA+, which showed well behaved fluorescence otherwise. The alkyne MS is shown when it was not possible to cleanly ionize the FITC coupled product. ABA peptides all ionized as expected, supporting the notion that the FITC moiety itself is labile under ionization.



Scheme S8.1. Synthesis of FITC labeled bPNA+.

FITC-N_3 was synthesized according to literature procedure.[4] bPNA+ alkyne was prepared using the methods described above. To an aqueous solution of bPNA+ alkyne (18mM, 10µl) was added FITC-N_3 in DMSO (300mM, 10µl) and 50µl of 1M Tris-Cl (pH=8.0). Then $CuSO_4$(0.1M, 10µl) and sodium ascorbate(0.1M, 20µl) solution was added. The mixture was then reacted for 1h at room temperature before quenched by addition of 1N HCl. Product was purified by HPLC on C18 column to yield FITC-bPNA+ (6.4mM, 50µl, 89%) using the following gradient 5% solvent B in 0-5min, 5-25% solvent B in 5-20 min, 25% solvent B in 20-25 min, 25-100% solvent B in 25-27 min, 100% solvent B in 27-32 min, 100-5% solvent B in 32-34 min, 5% solvent B in 34-40 min.

S9. Compound characterization

S9.1. NMR

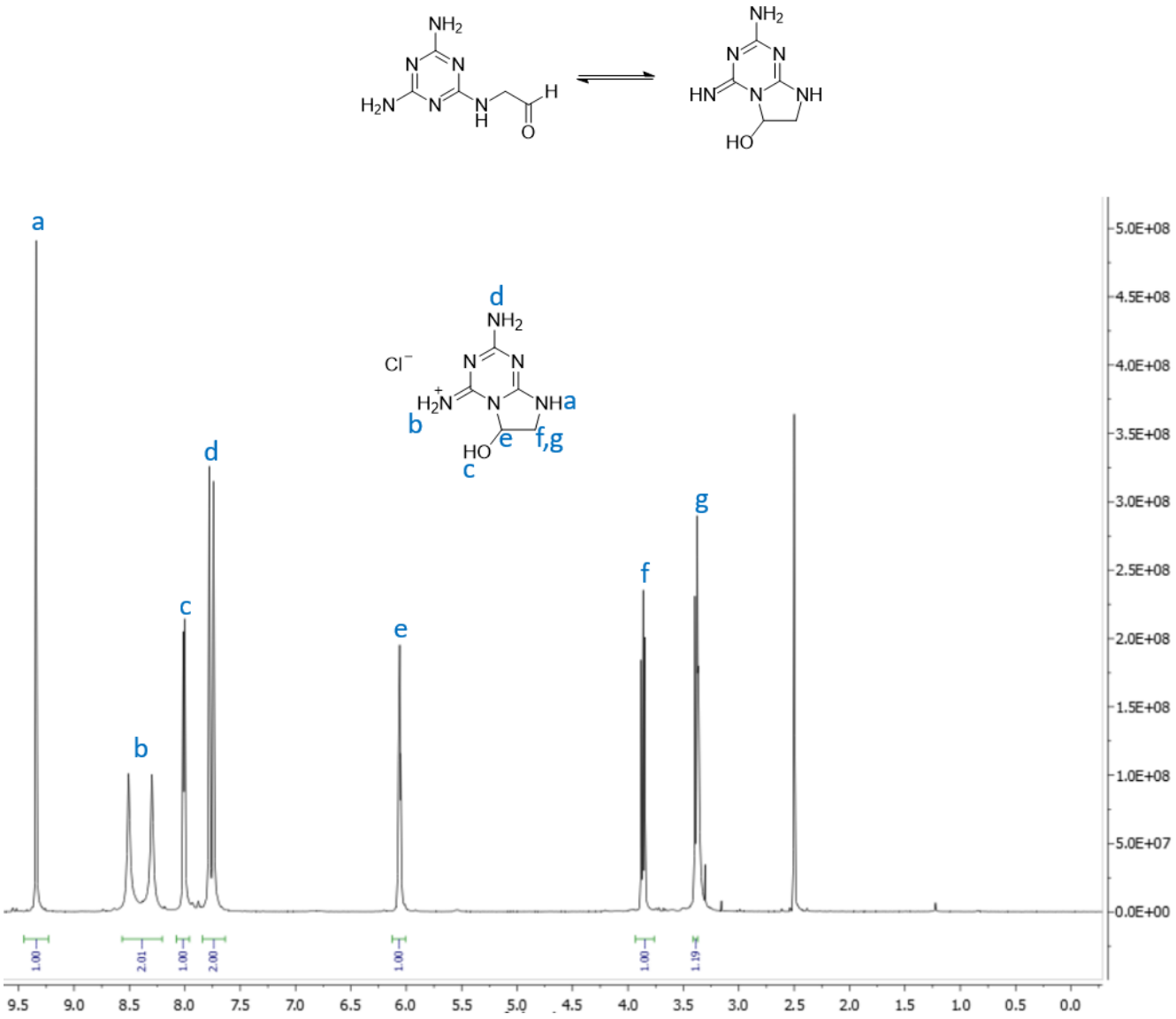


Figure S9.1. ^{1}H NMR of melamine acetaldehyde in DMSO-d6. The aldehyde is in equilibrium with hemiaminal**.**

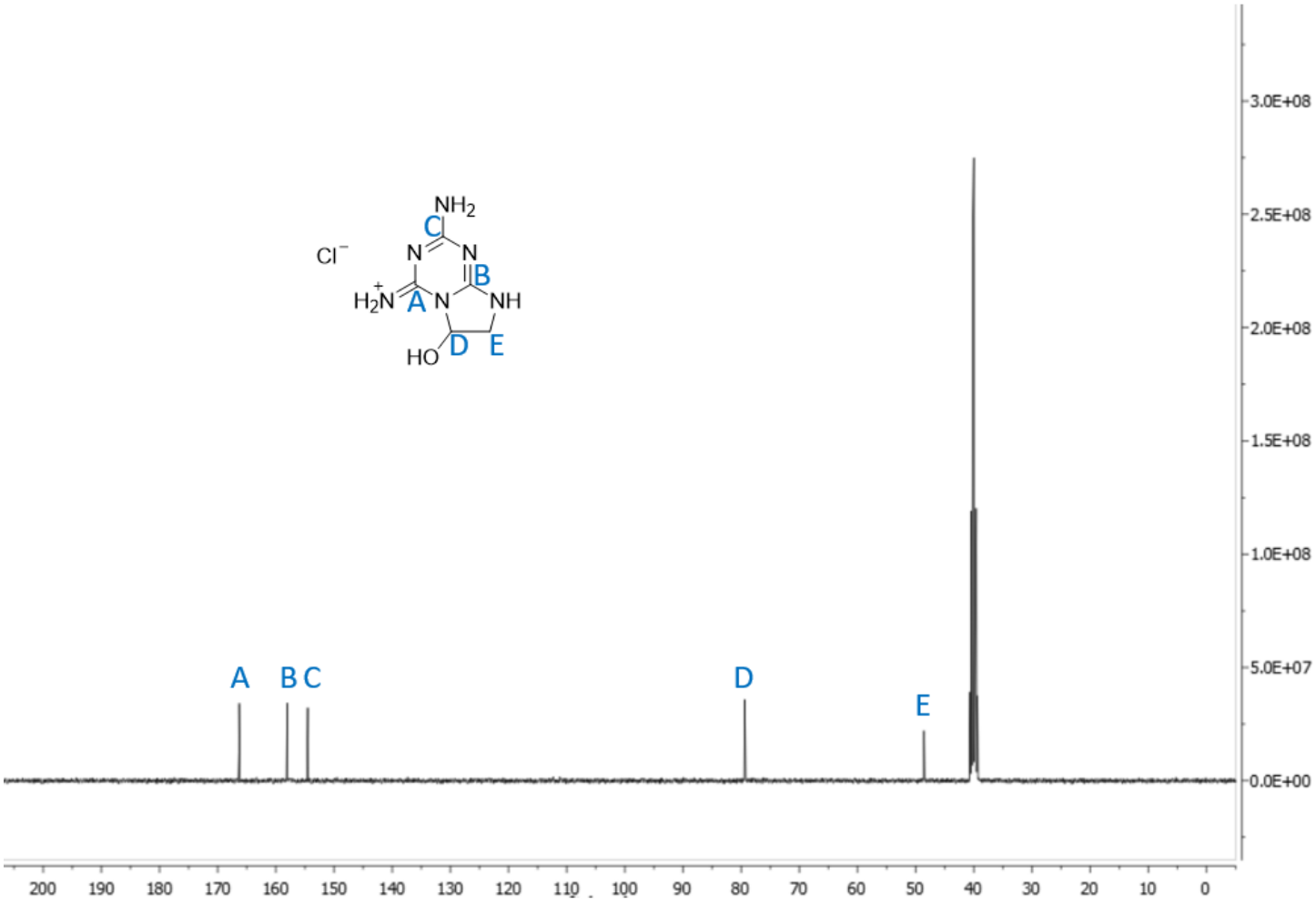


Figure S9.2. ^{13}C NMR of melamine acetaldehyde

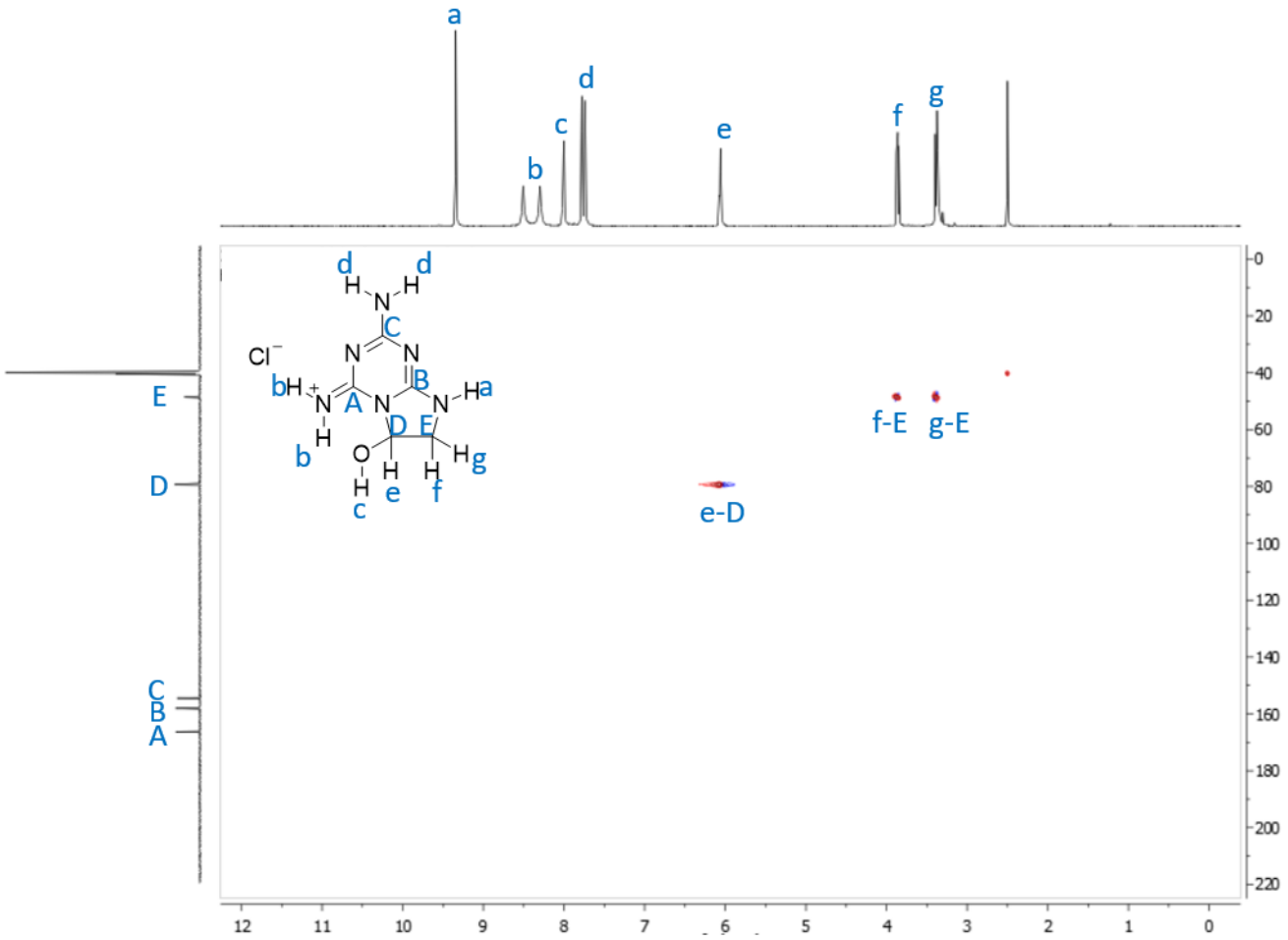


Figure S9.3. ^{1}H-^{13}C HSQC NMR of melamine acetaldehyde

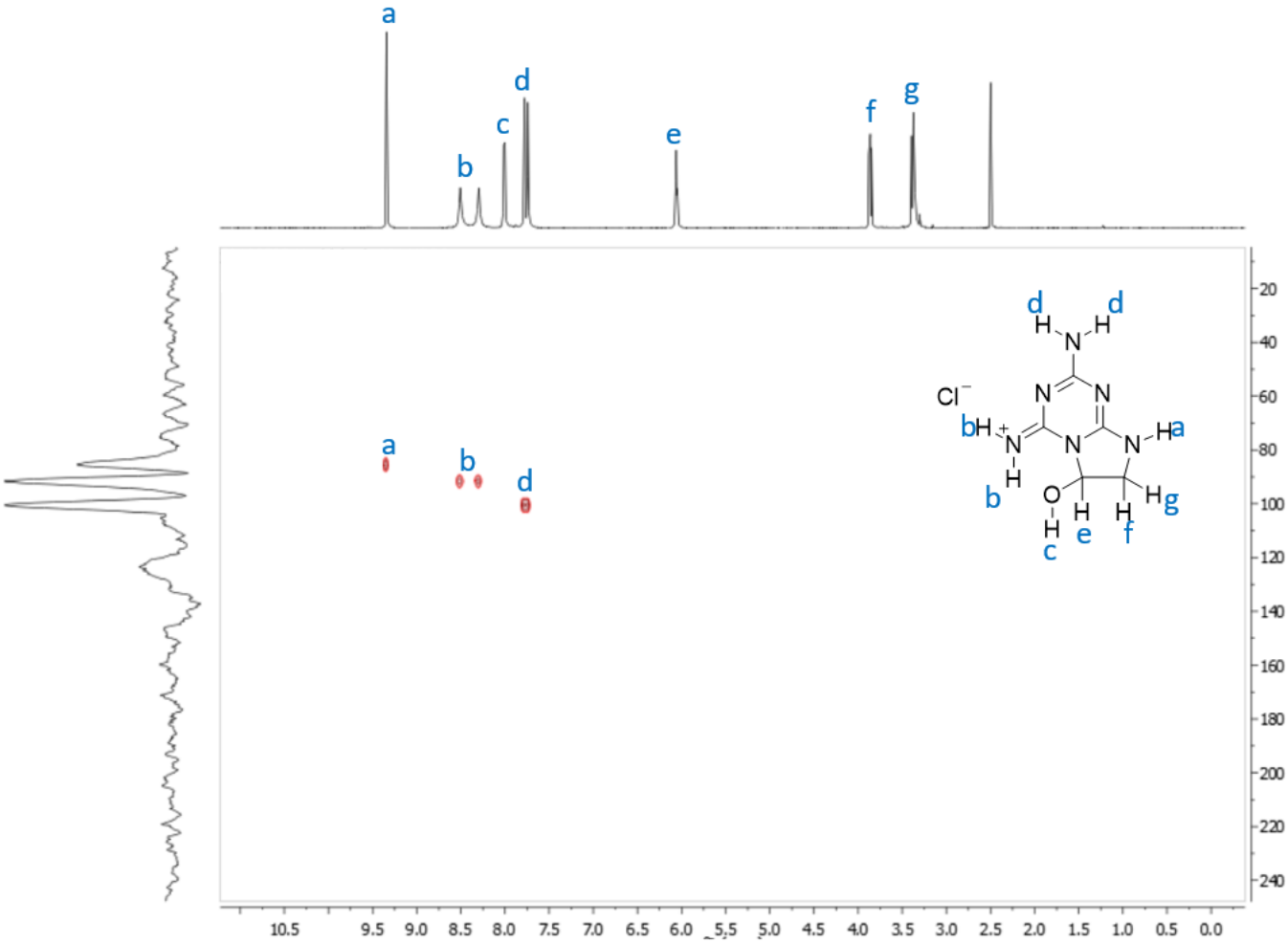


Figure S9.4. ^{1}H-^{15}N HSQC NMR of melamine acetaldehyde

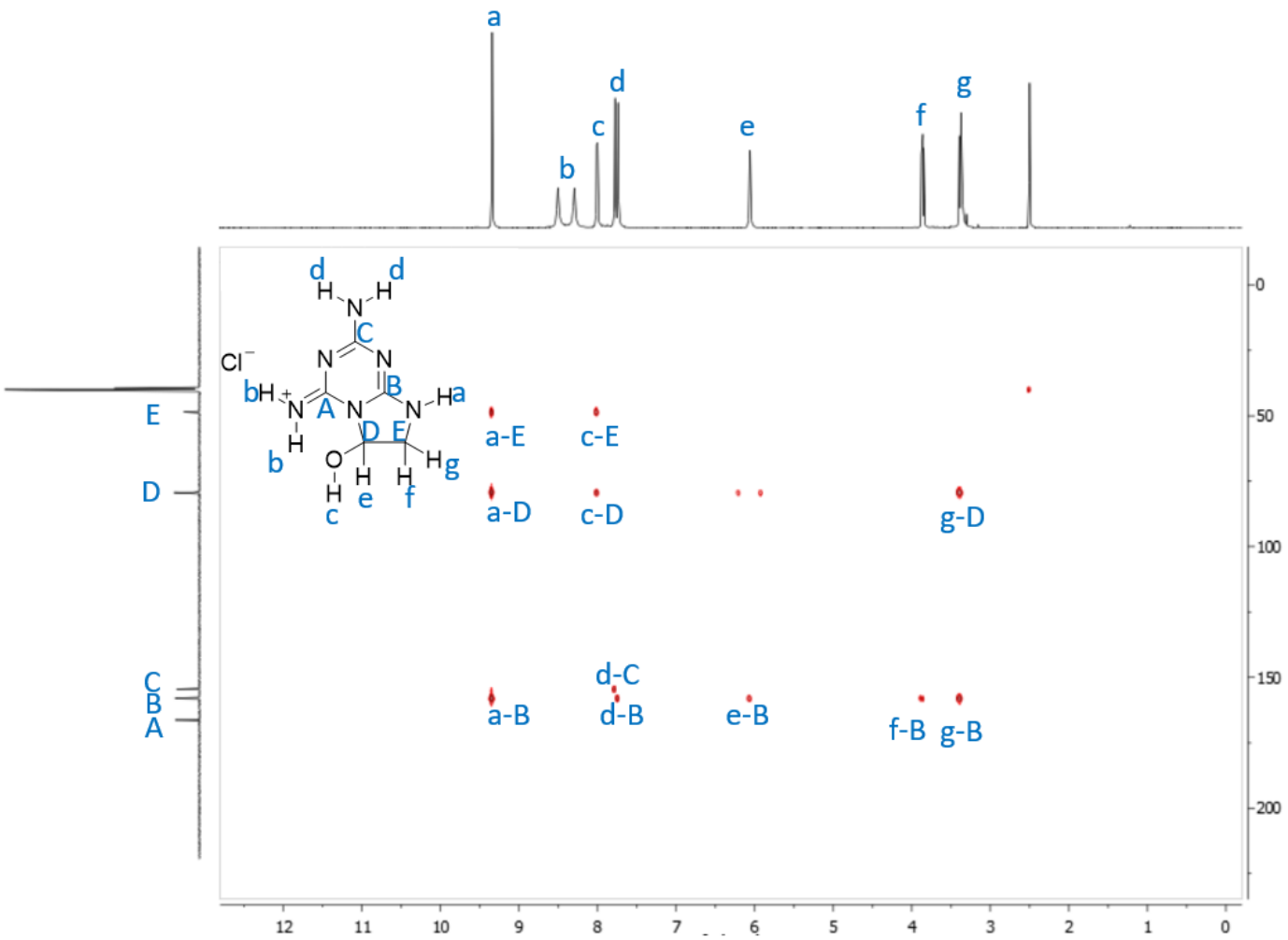


Figure S9.5. HMBC NMR of melamine acetaldehyde. Coupling of e-B indicating the structure is cyclic.

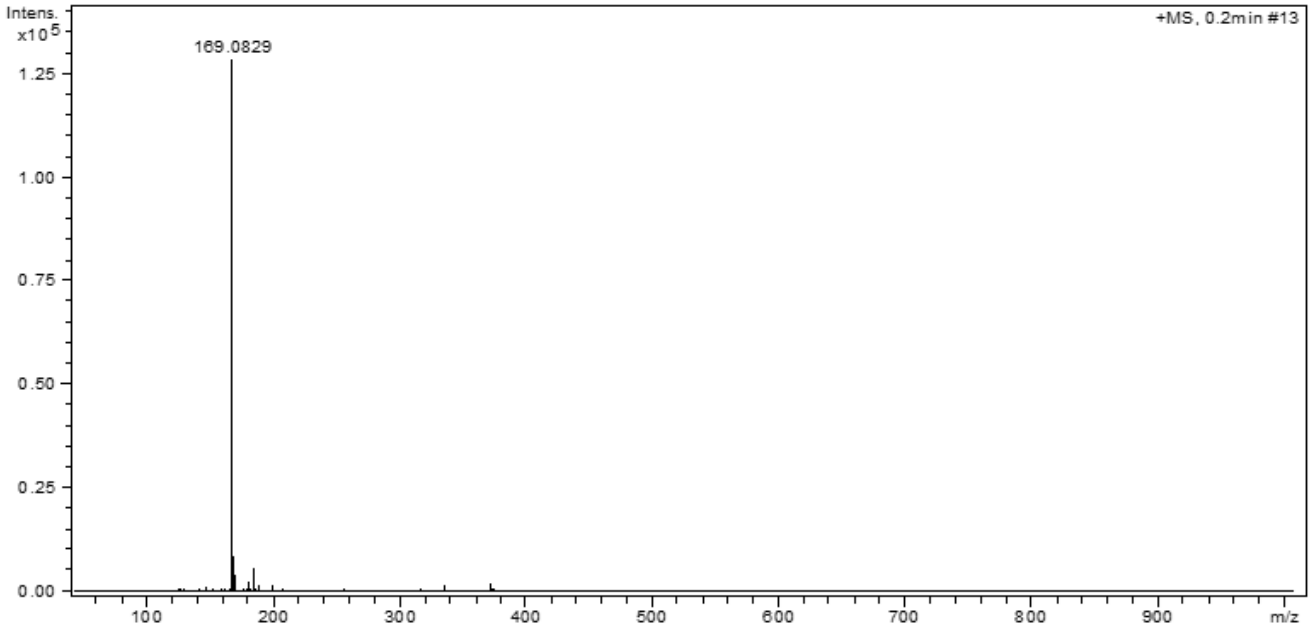


Figure S9.6. ESI of melamine acetaldehyde. Mass calculated [M+H]=169.0832. Mass found [M+H]=169.0829.

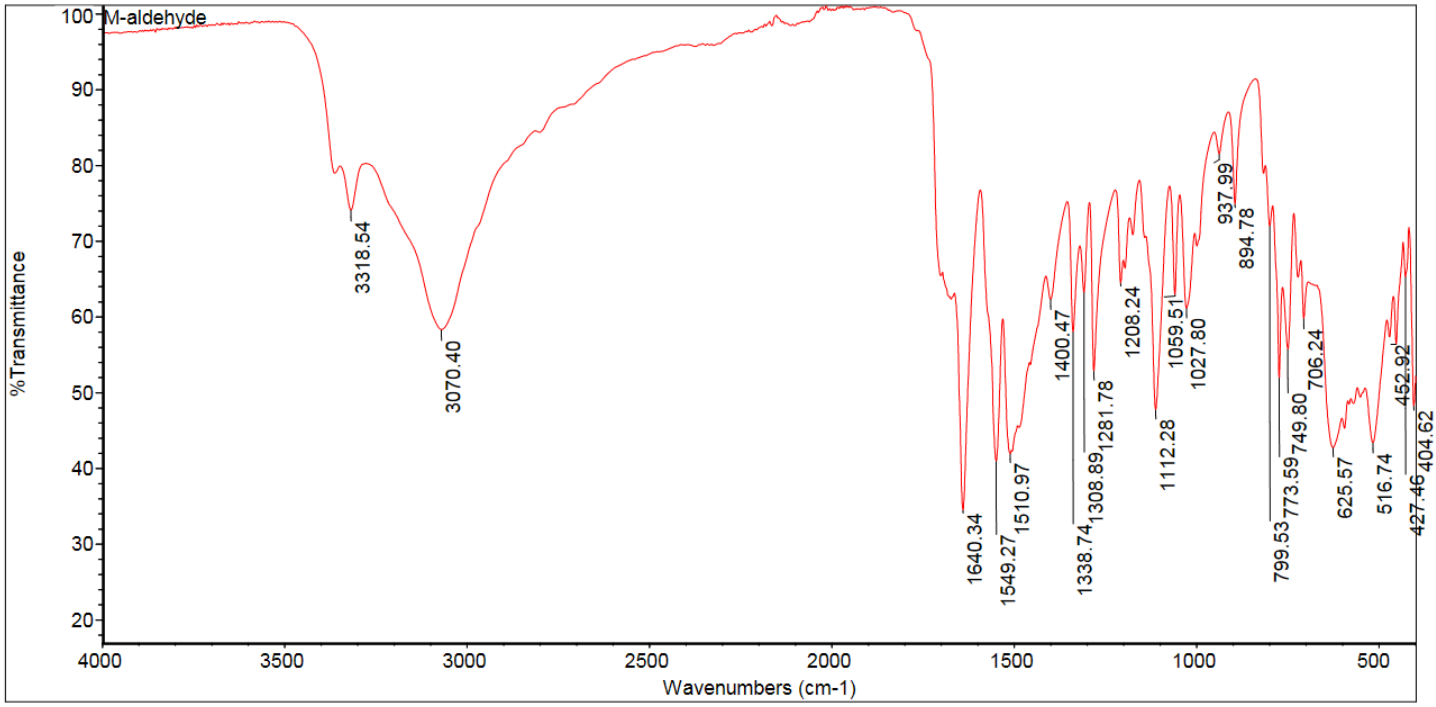


Figure S9.7. IR of solid melamine acetaldehyde

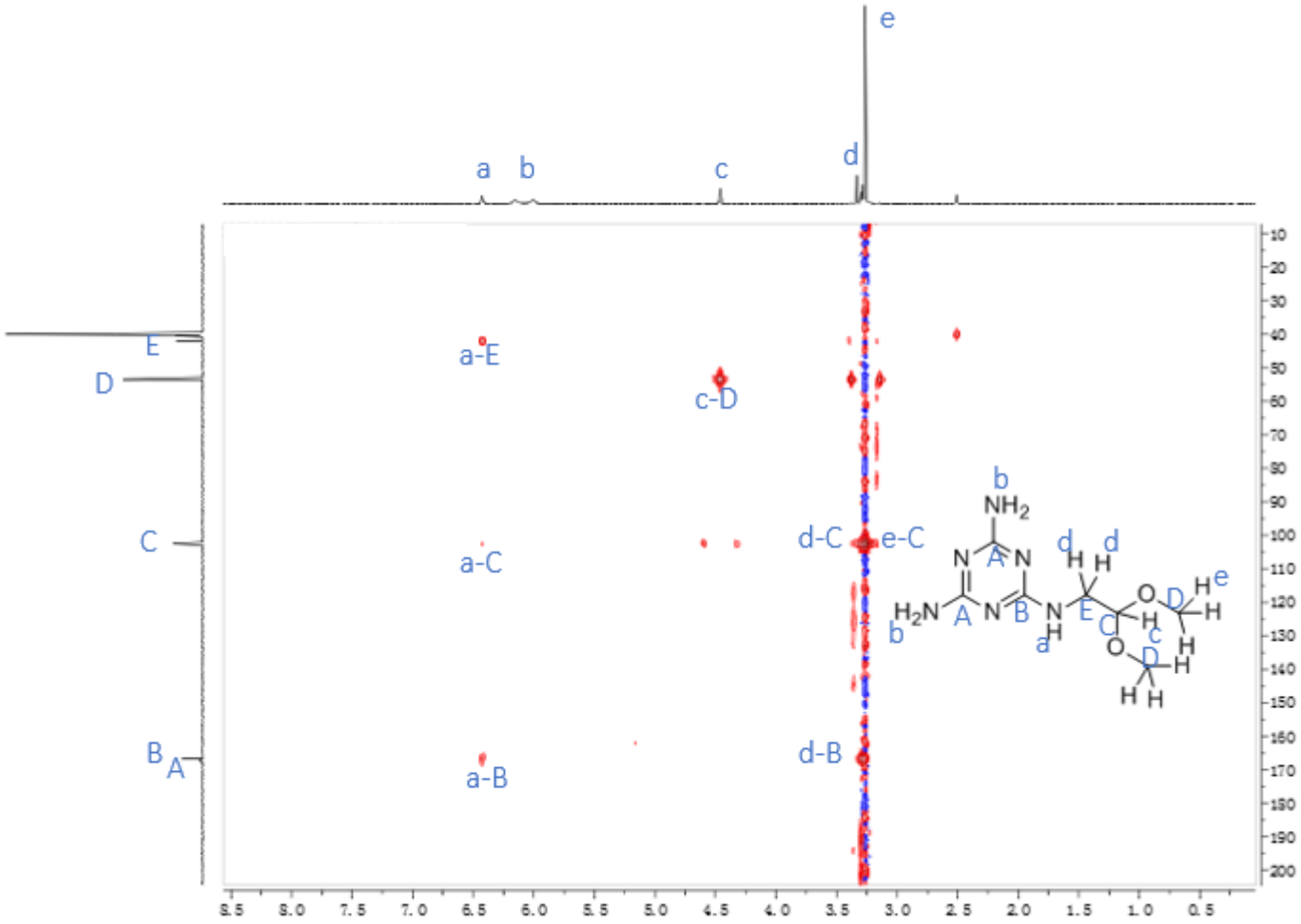


Figure S9.8. HSQC of melamine acetaldehyde dimethyl acetal. No c-B coupling indicating the structure is not cyclic.

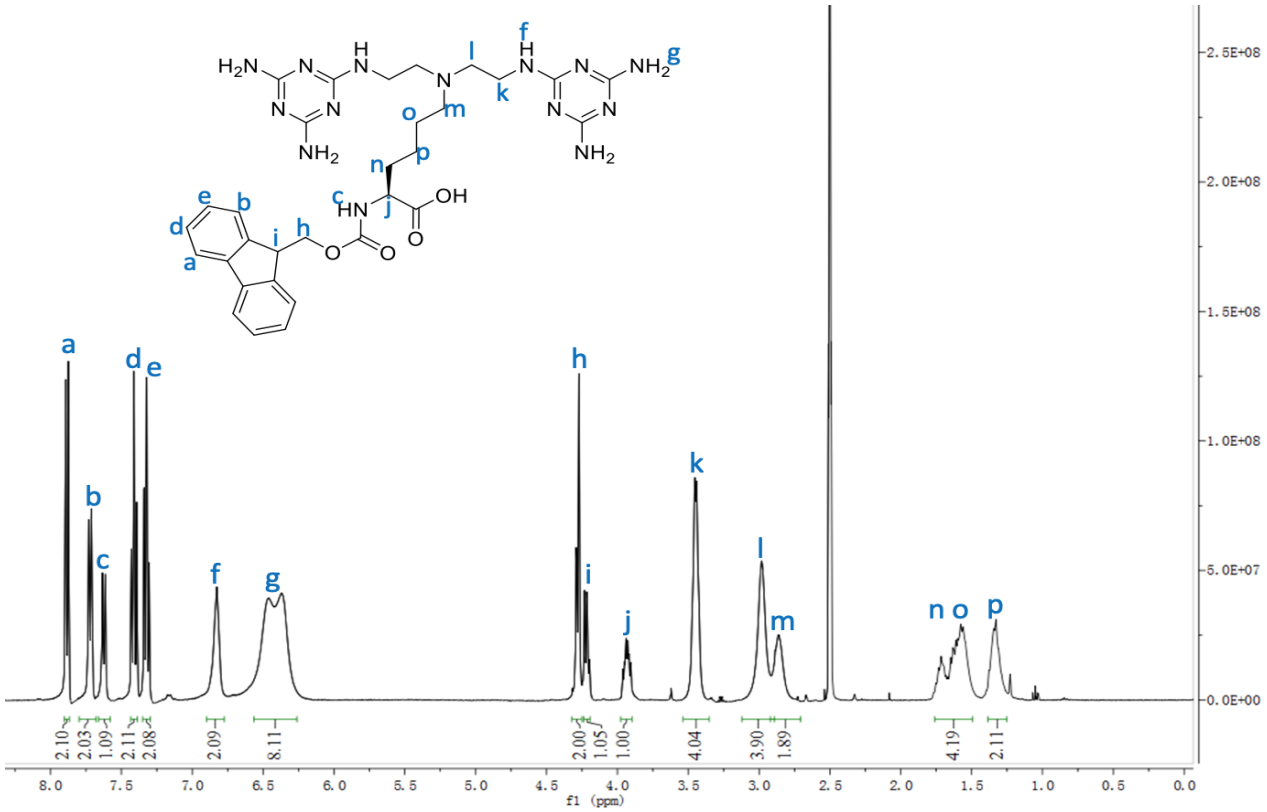


Figure S9.9. ^{1}H NMR of Fmoc-K^{2M}-OH

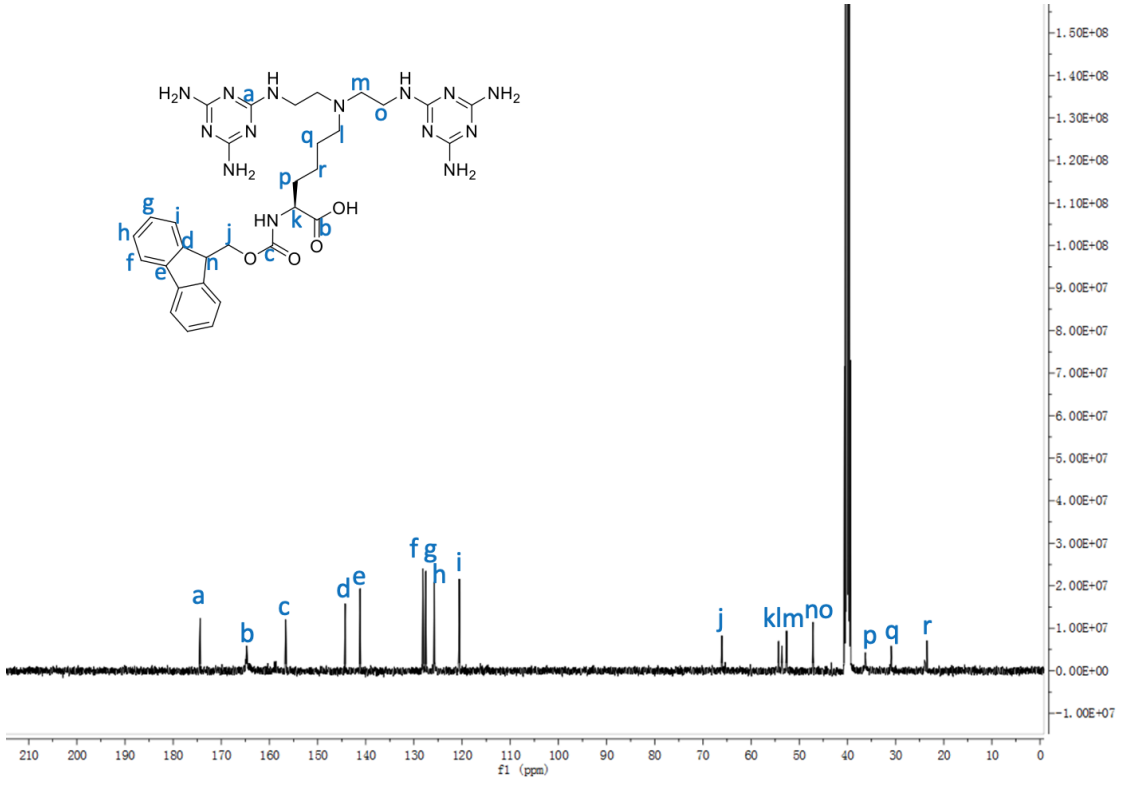


Figure S9.10. ^{13}C NMR of Fmoc-K^{2M}-OH

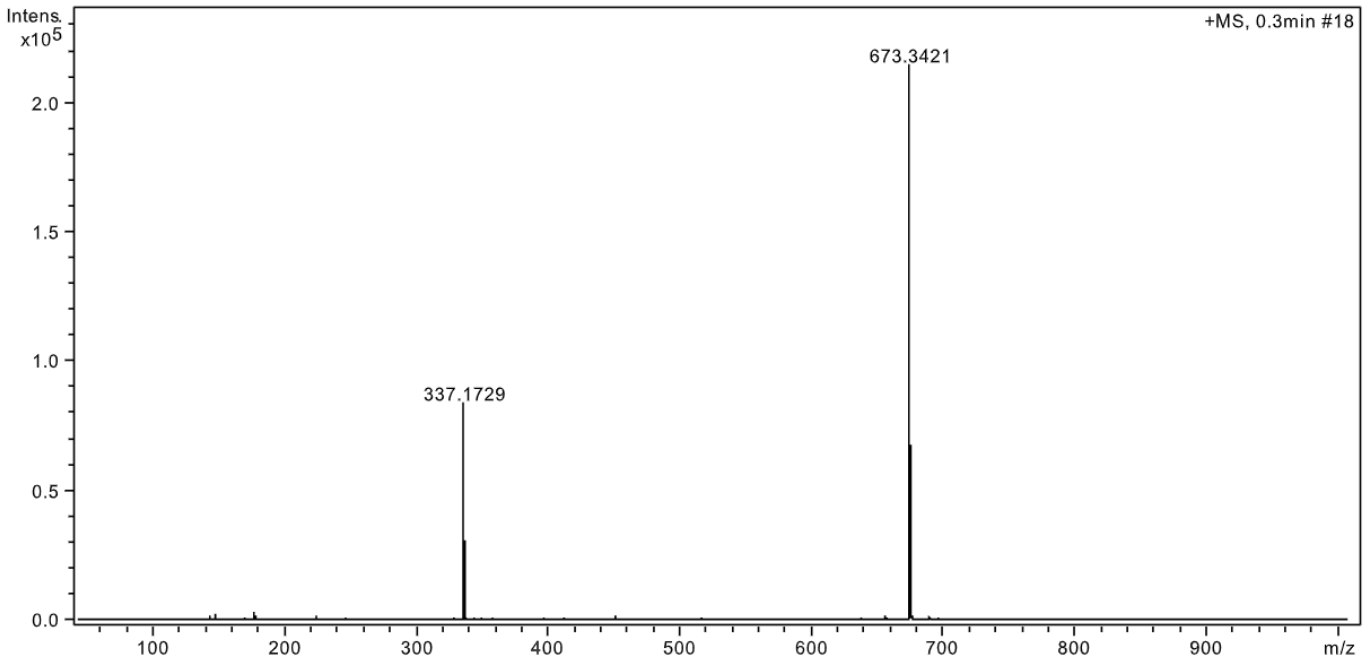


Figure S9.11. ESI of Fmoc-K^{2M}-OH. Mass found: [M+H] 673.3421, [M+2H] 337.1729. Mass calculated [M+H]: 673.3429.

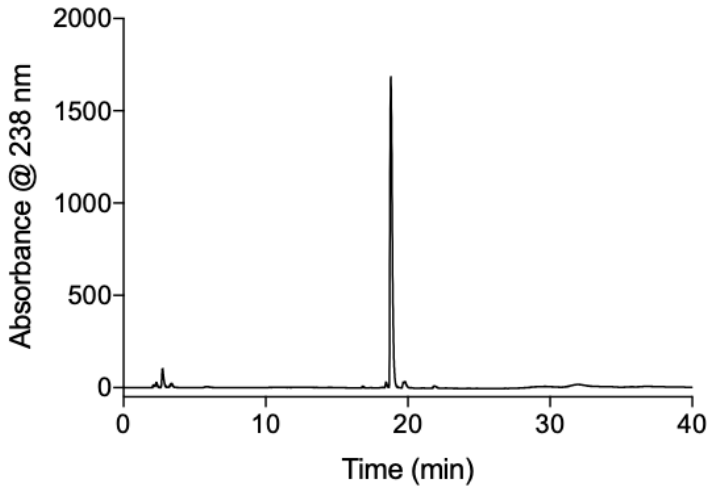


Figure S9.12. The purity was checked by HPLC on a C18 reversed phase column using the following gradient: 5% solvent B in 0-5 min, 5-55% solvent B in 5-15 min, 55% solvent B in 15-20 min, 55-100% solvent B in 20-22 min, 100% solvent B in 22 min-27 min, 100-5% solvent B in 27-30 min, 5% solvent B in 30-35 min (solvent A=0.1%TFA in water, solvent B=0.07% TFA in 90% acetonitrile, 10% water).

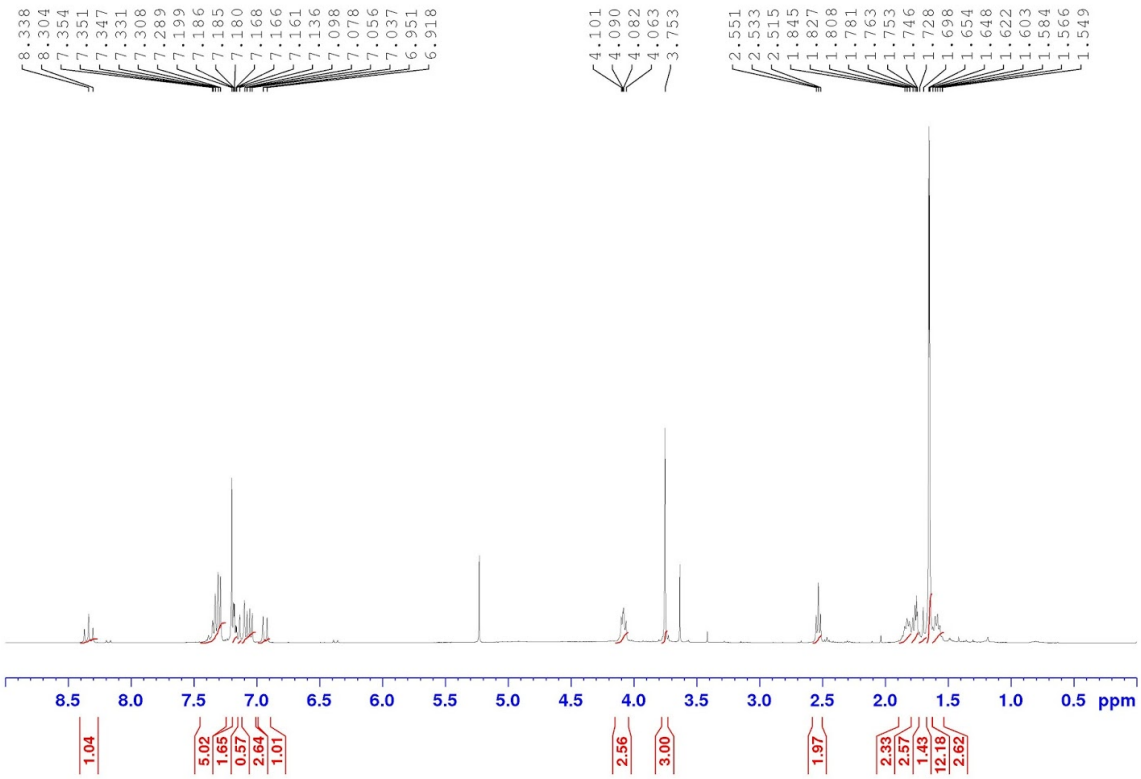


Figure S9.13. ^{1}H NMR (400 MHz, $CDCl_3$) Cy3 acid (**2**)

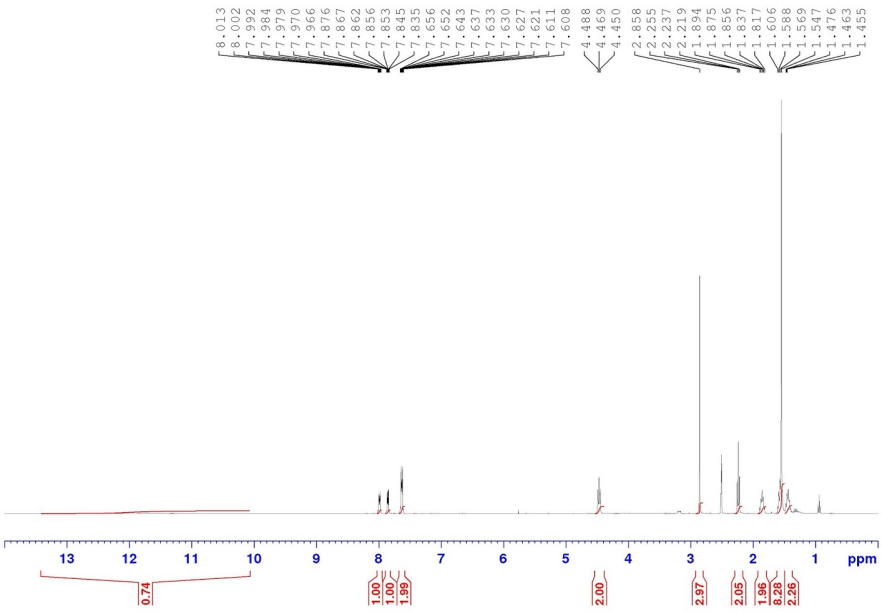


Figure S9.14. ^{1}H NMR (400 MHz, DMSO-D6) of **1**.

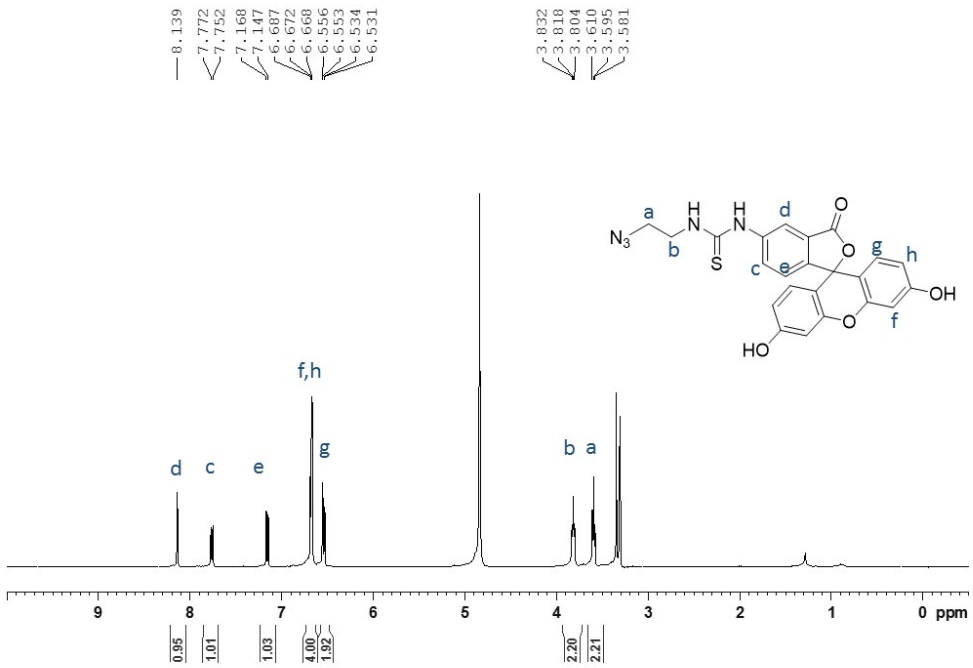


Figure S9.15. ^{1}H NMR(400 MHz, d_4-MeOD) of FITC N3.

S9.2. HPLC and MALDI-MS

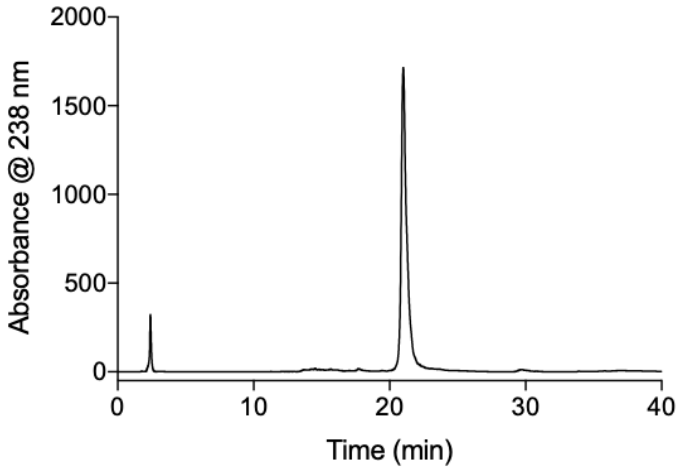


Figure S9.16. HPLC trace of Cy3-$(SK^{2M})_3$-G.

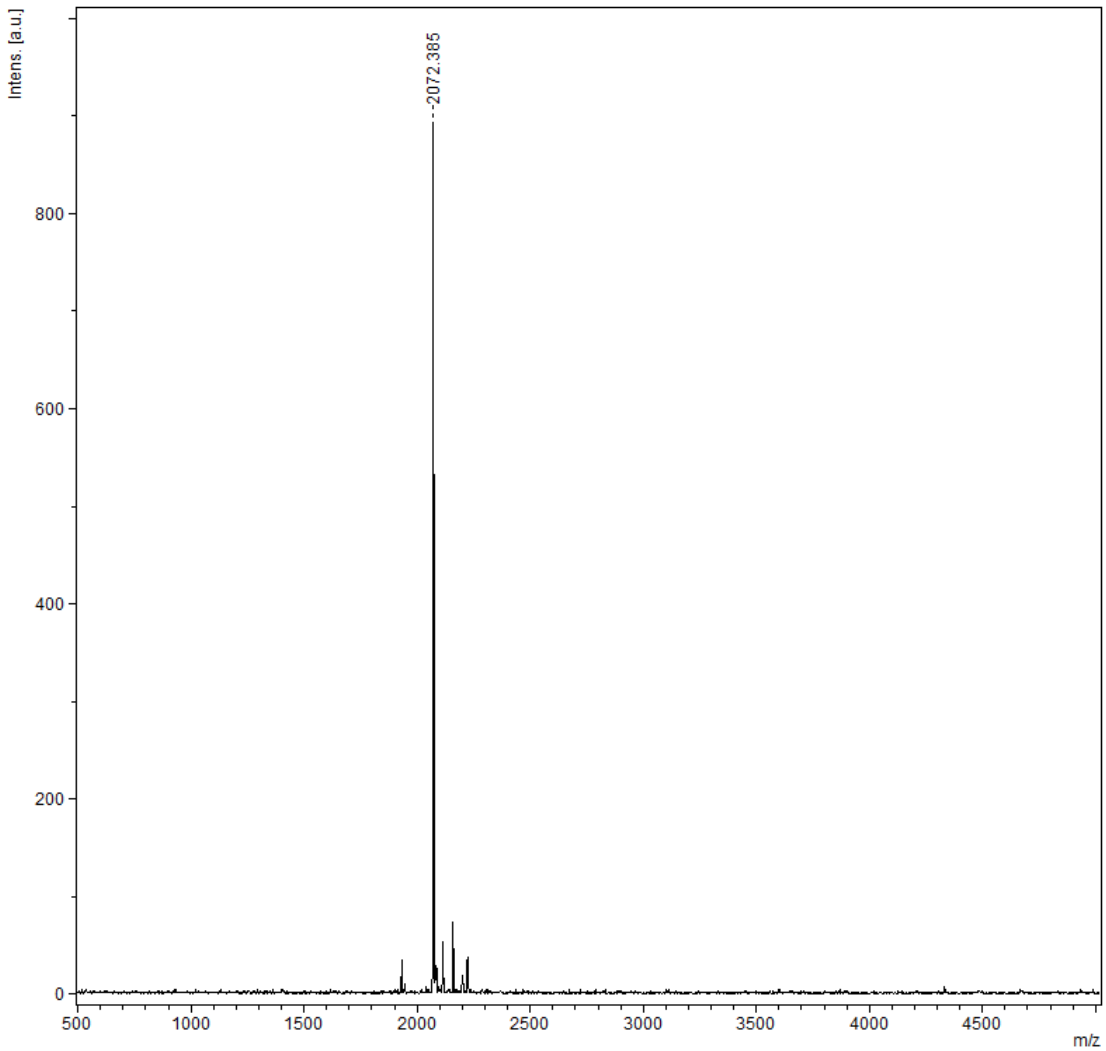


Figure S9.17. MALDI spectrum of Cy3-$(SK^{2M})_3$-G. Mass found: 2072.385, mass calculated: 2072.430.

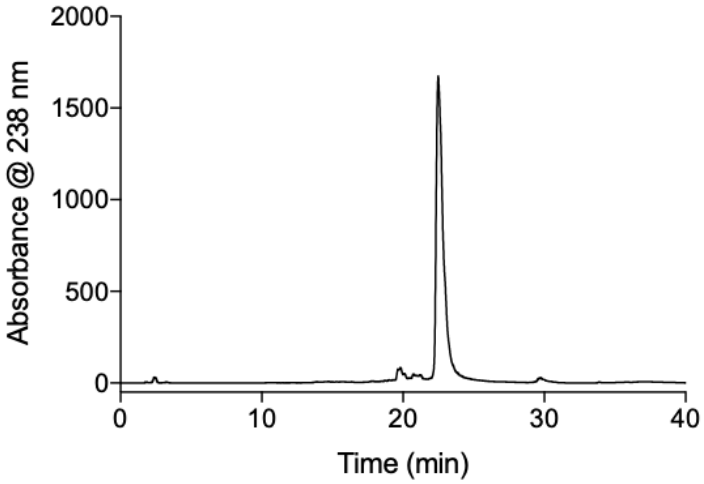


Figure S9.18. HPLC trace of Cy5-(SK^{2M})$_3$-G.

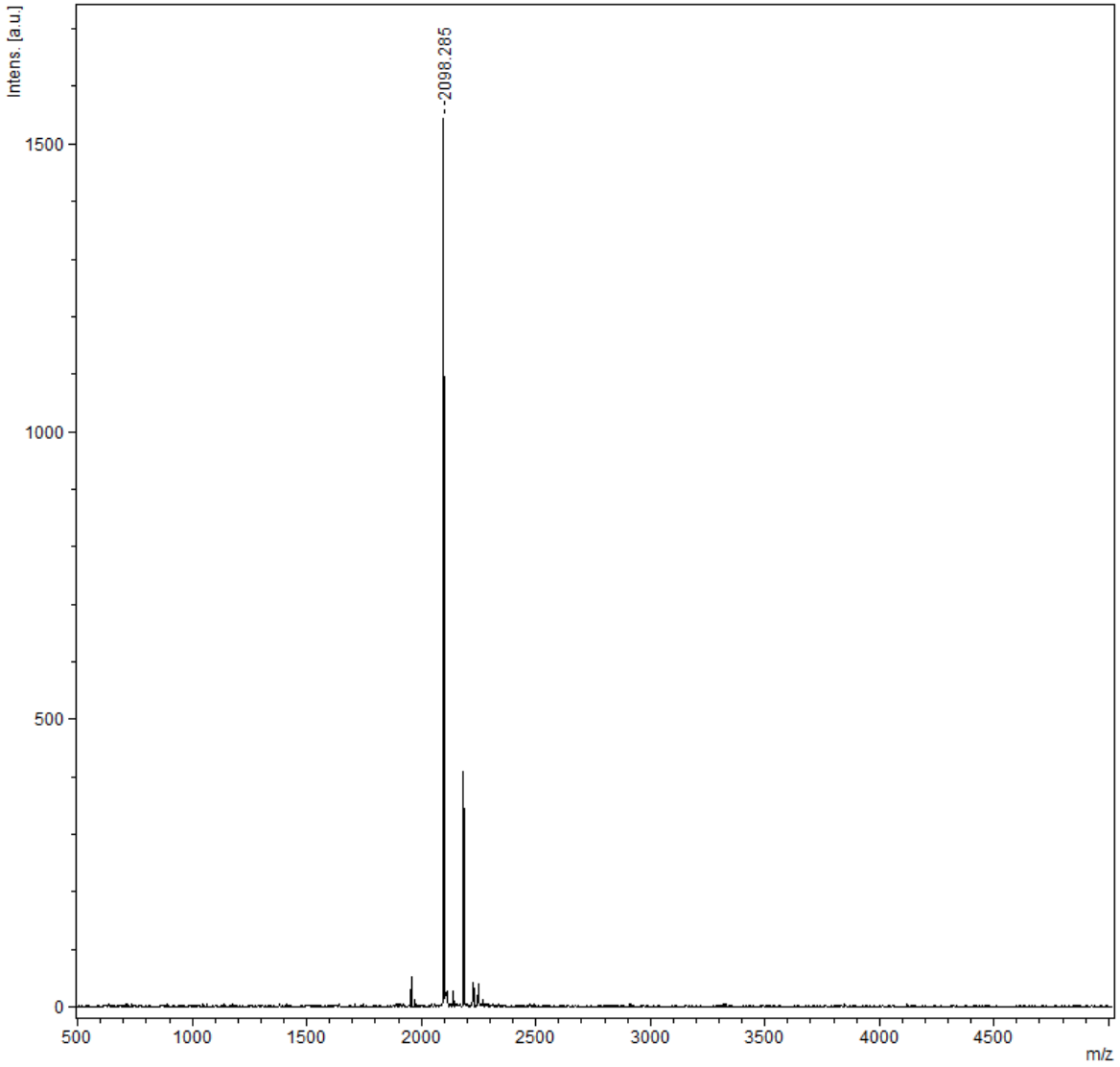


Figure S9.19. MALDI spectrum of Cy5-(SK^{2M})$_3$-G. Mass found: 2098.285, mass calculated: 2098.468.

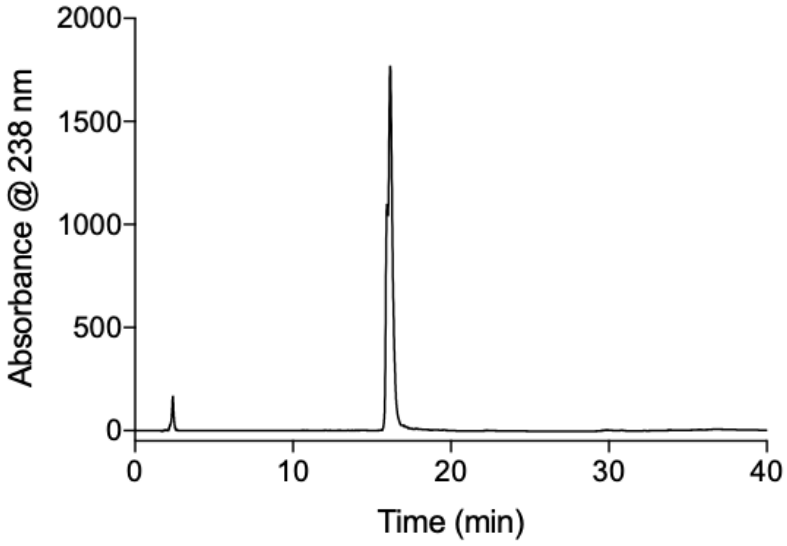


Figure S9.20. HPLC trace of Cbf-βAla-$(SK^{2M})_3$-G.

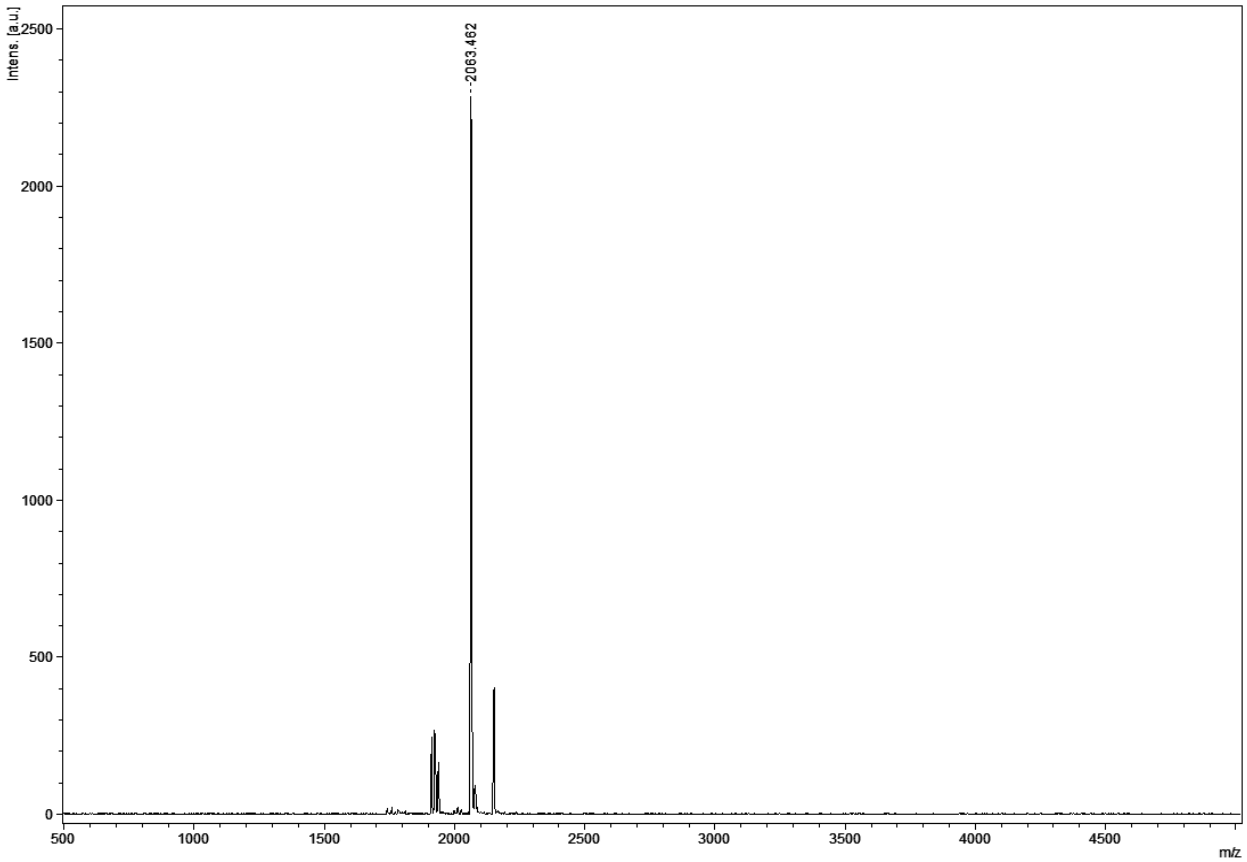


Figure S9.21. MALDI spectrum of Cbf-βAla-$(SK^{2M})_3$-G. Mass found: 2063.462, mass calculated: 2062.192.

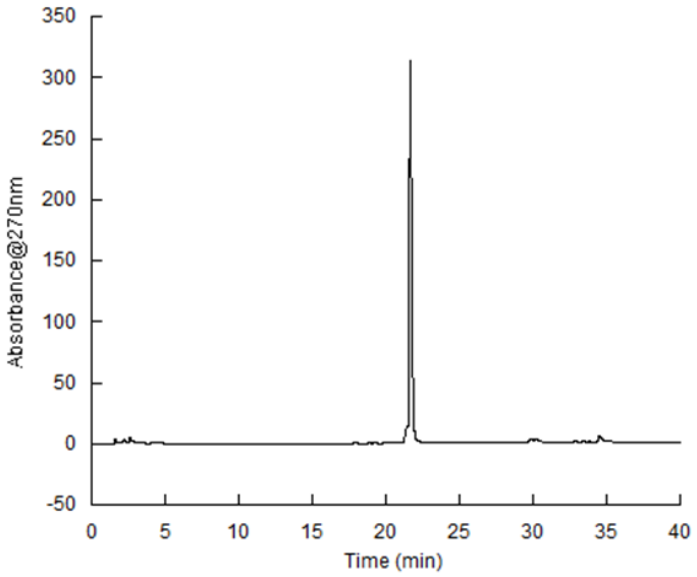


Figure S9.22. HPLC trace of ABA-βAla-$(EK^{2M})_3$-G.

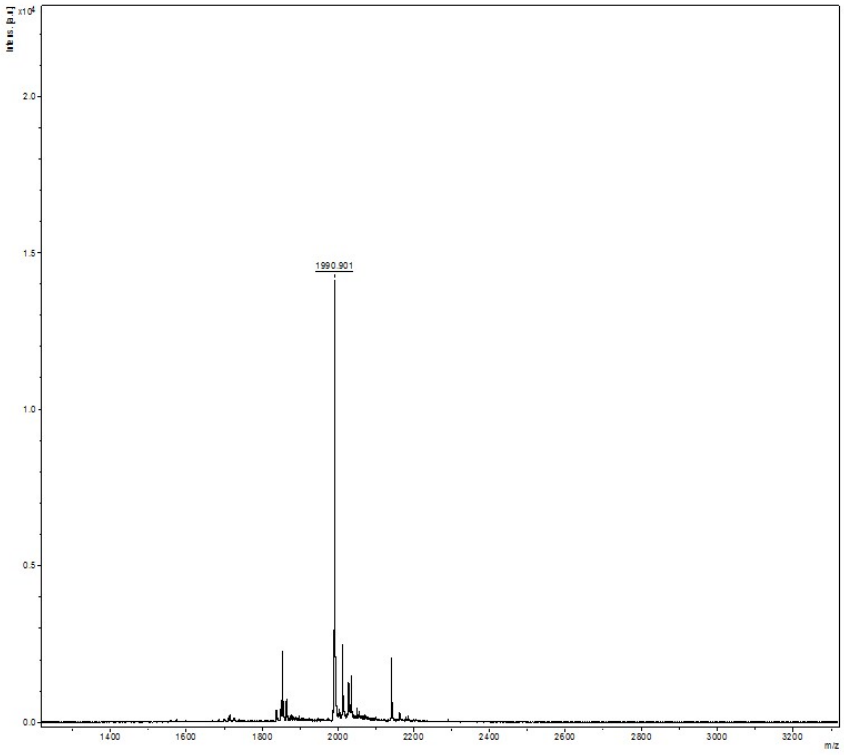


Figure S9.23. MALDI spectrum of ABA-βAla-$(EK^{2M})_3$-G. Mass found: 1990.901, mass calculated: 1991.039.

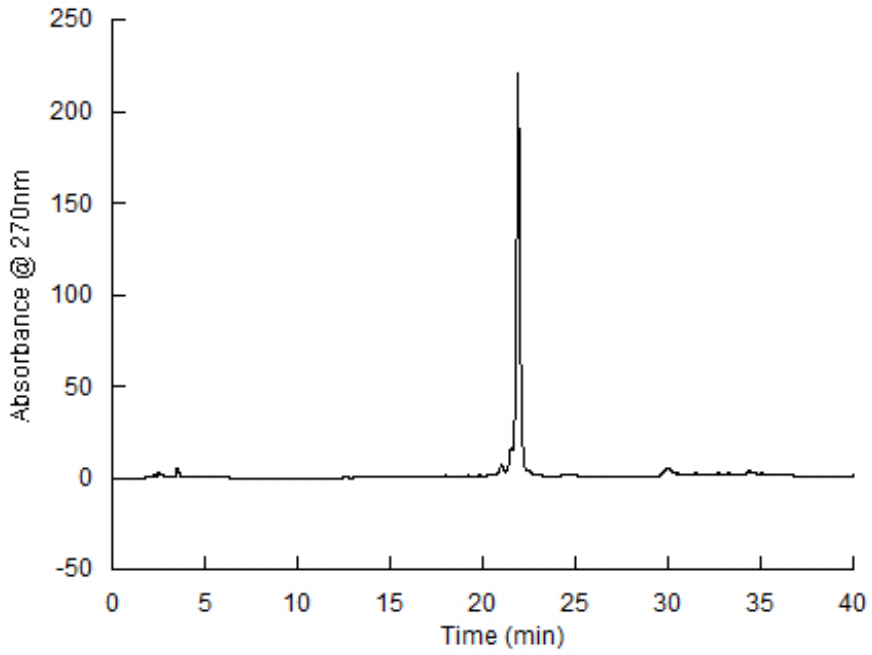


Figure S9.24. HPLC trace of ABA-βAla-$(EO^{2M})_3$-G.

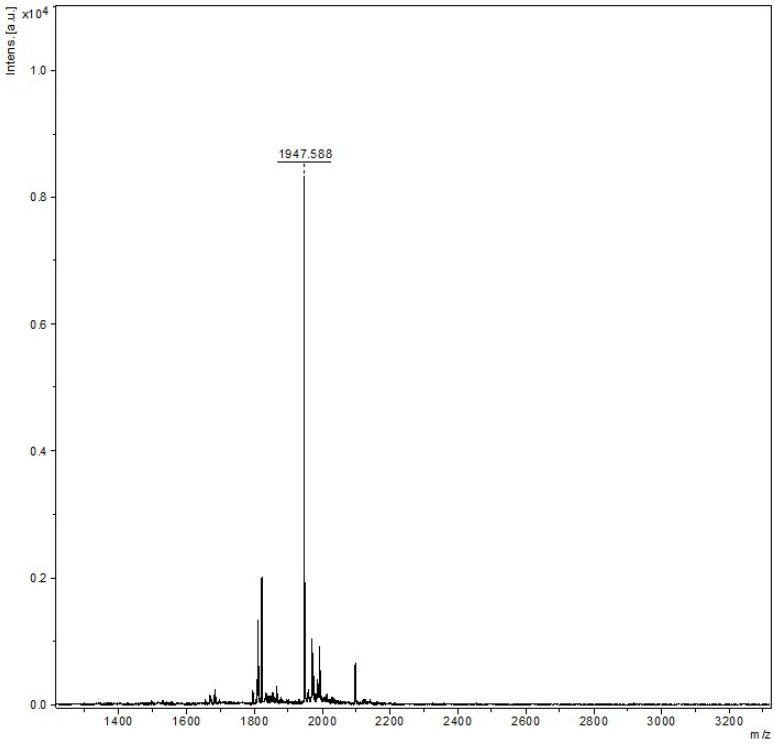


Figure S9.25. MALDI spectrum of ABA-βAla-$(EO^{2M})_3$-G. Mass found: 1947.588, mass calculated: 1948.992.

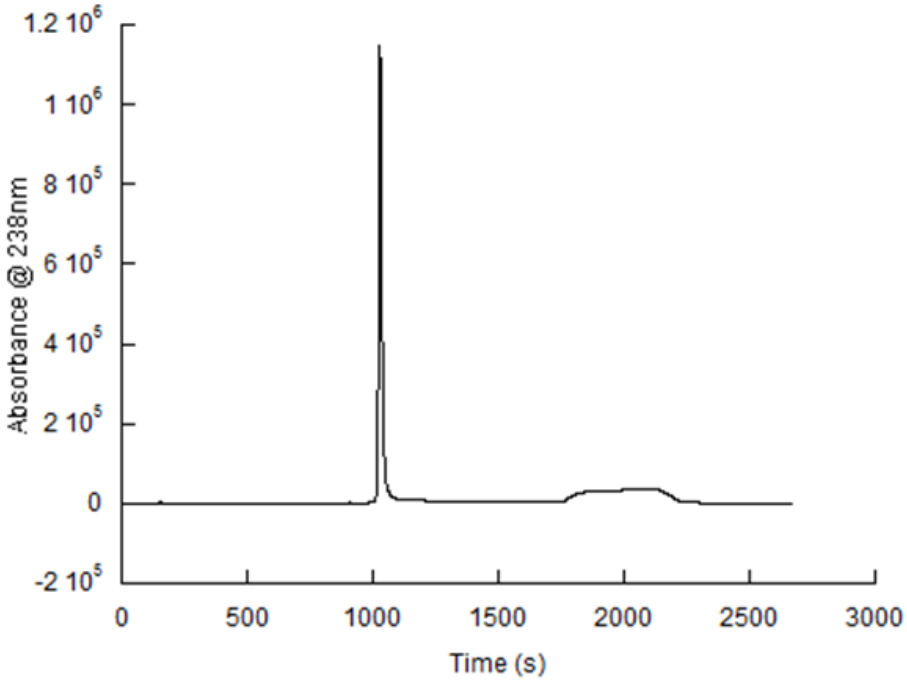


Figure S9.26. HPLC trace of ABA-βAla-$(EB^{2M})_3$-G.

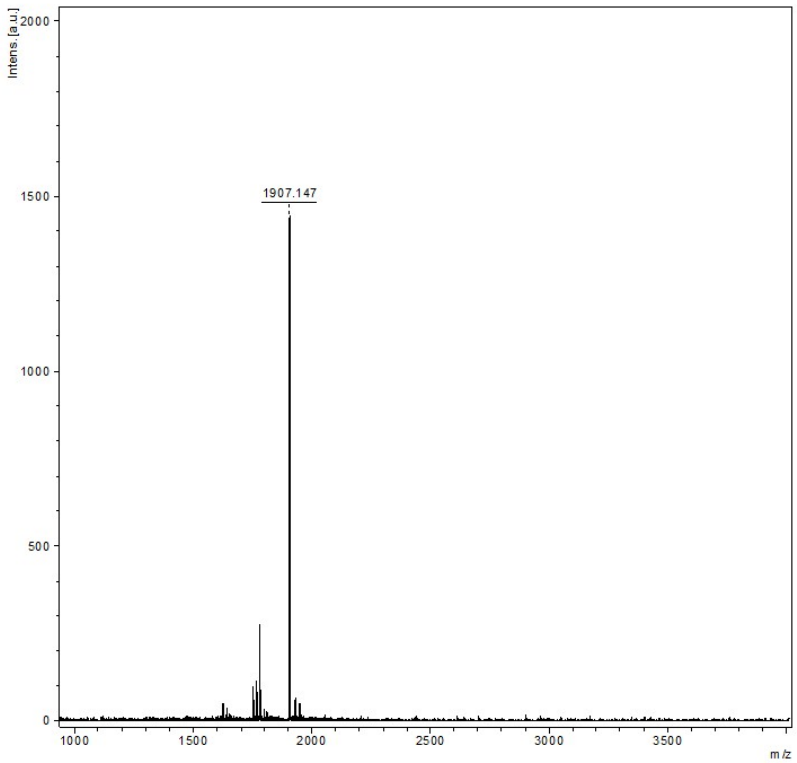


Figure S9.27. MALDI spectrum of ABA-βAla-$(EB^{2M})_3$-G. Mass found: 1907.147, mass calculated: 1906.945.

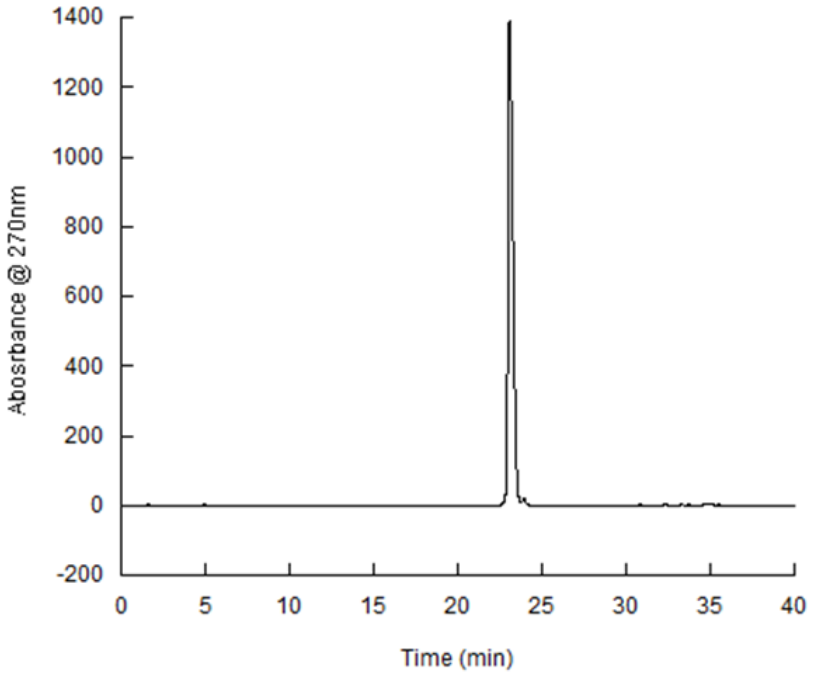


Figure S9.28. HPLC trace of ABA-βAla-(SK^{2M})3-G.

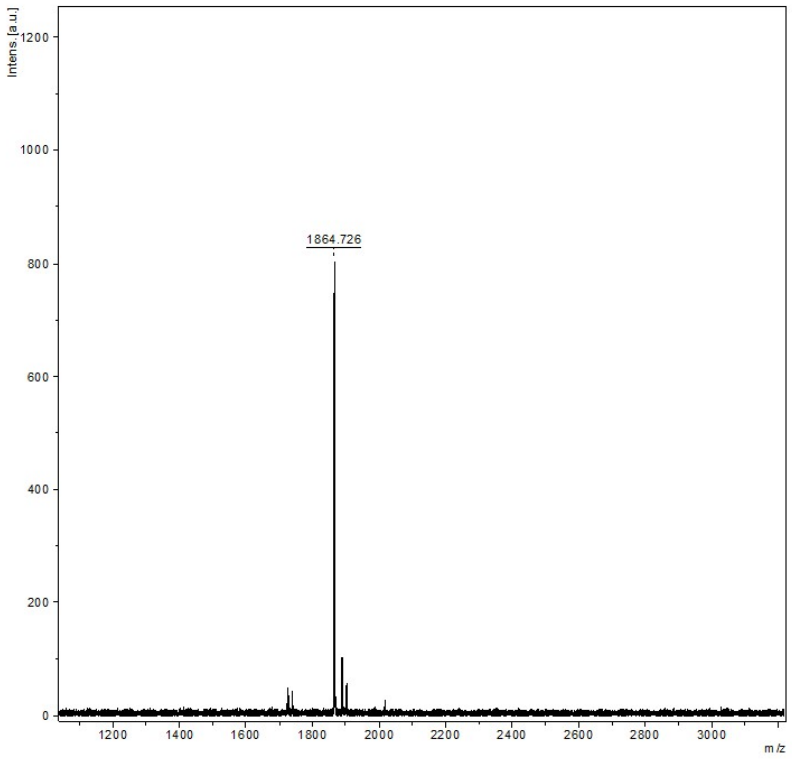


Figure S9.29. MALDI spectrum of ABA-βAla-(SK^{2M})$_3$-G. Mass found: 1864.726, mass calculated: 1865.136.

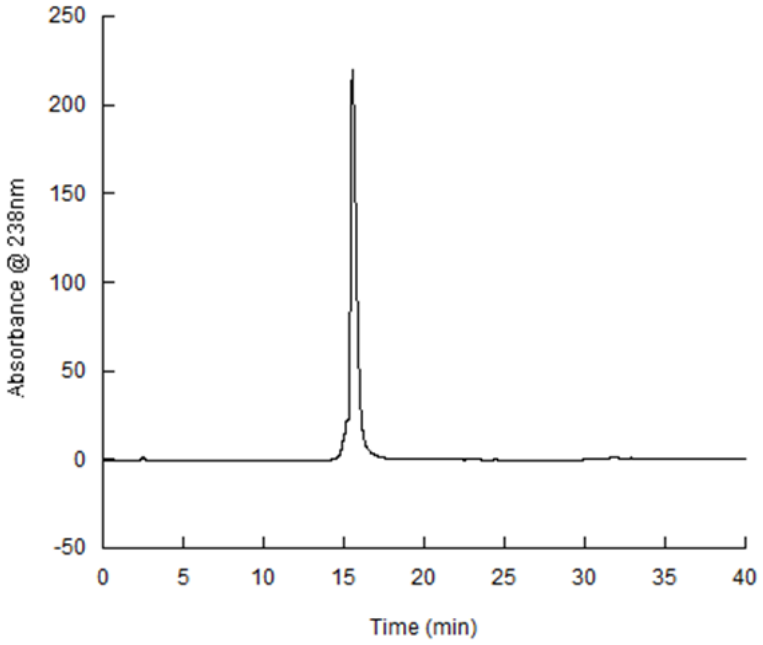


Figure S9.30. HPLC trace of ABA-βAla-$(SO^{2M})_3$-G.

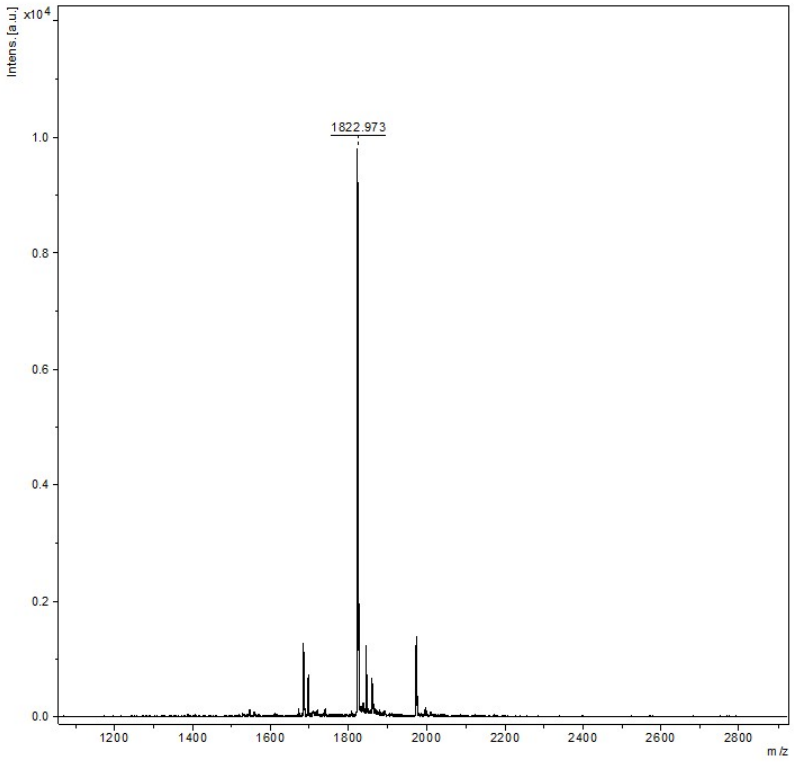


Figure S9.31. MALDI spectrum of ABA-βAla-$(SO^{2M})_3$-G. Mass found: 1822.973, mass calculated: 1822.973.

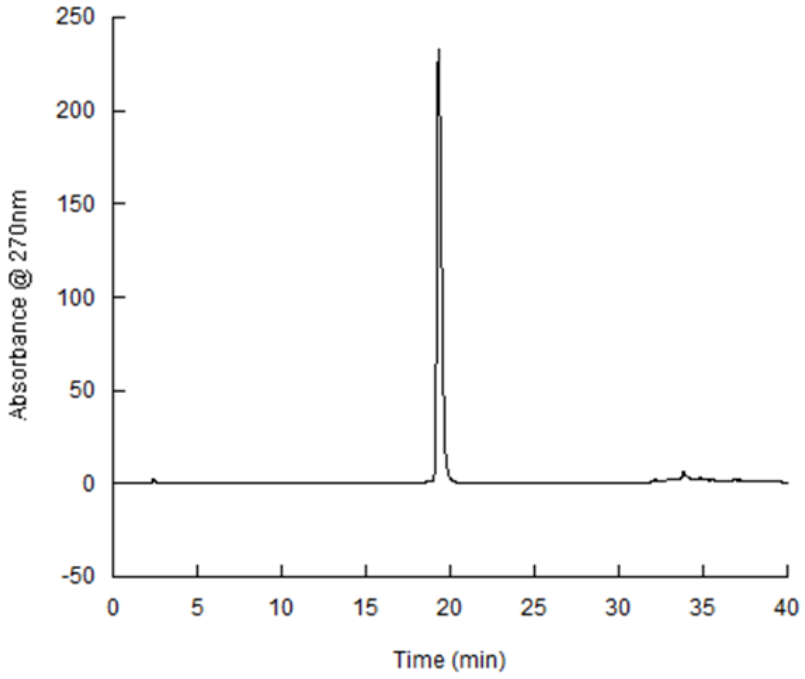


Figure S9.32. HPLC trace of ABA-βAla-$(SB^{2M})_3$-G.

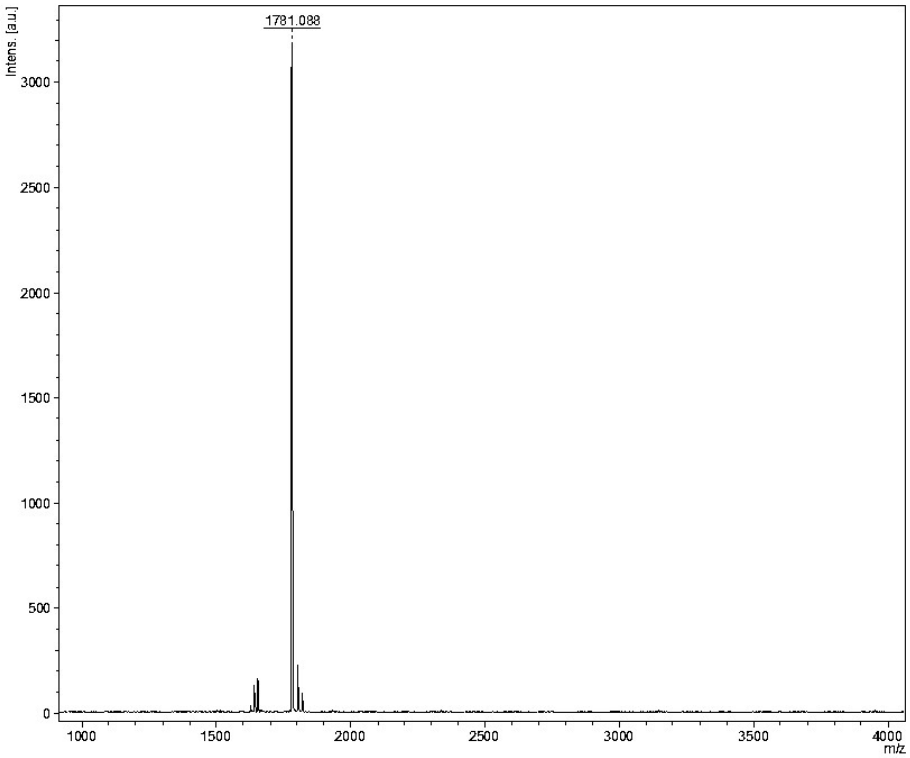


Figure S9.33. MALDI spectrum of ABA-βAla-$(SB^{2M})_3$-G. Mass found: 1781.088, mass calculated: 1780.913.

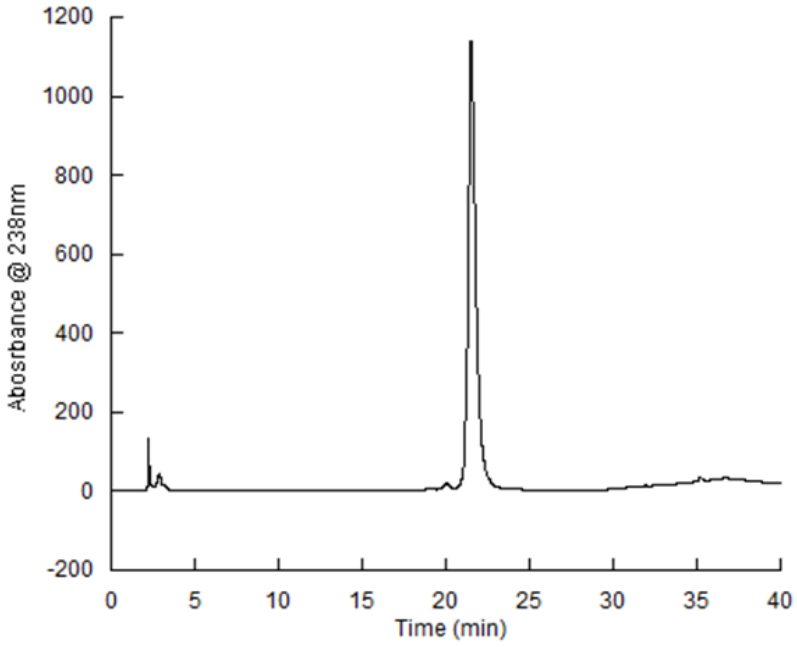


Figure S9.34. HPLC trace of ABA-βAla-$(EK^{2M})_5$-G.

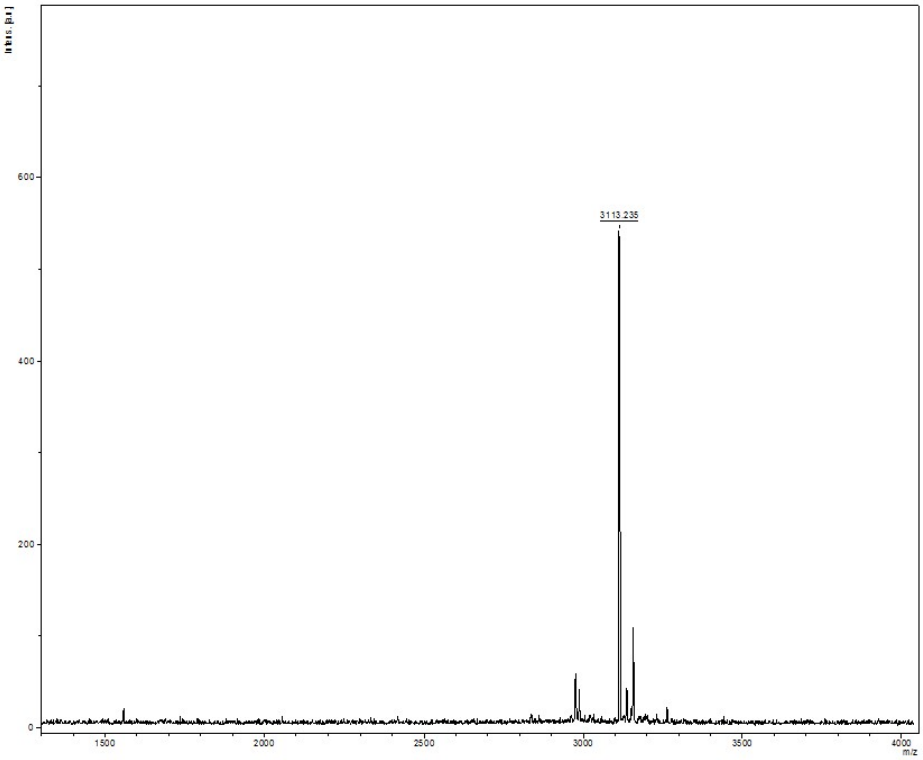


Figure S9.35. MALDI spectrum of ABA-βAla-$(EK^{2M})_5$-G. Mass found:3113.235, mass calculated: 3113.638.

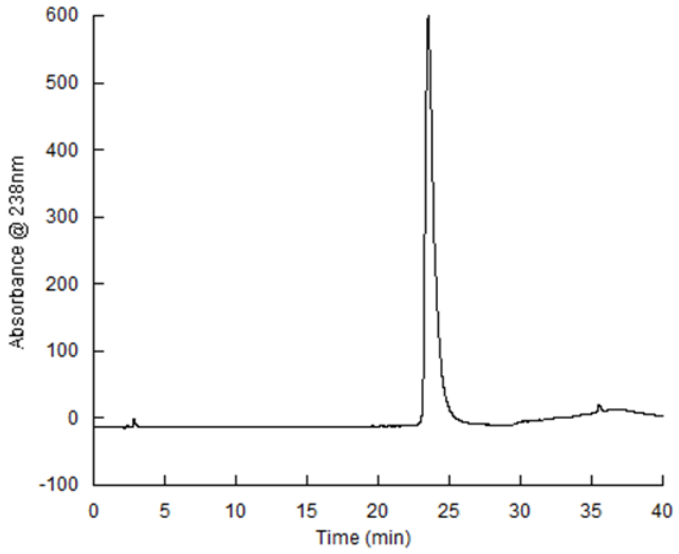


Figure S9.36. HPLC trace of ABA-βAla-$(SK^{2M})_5$-G.

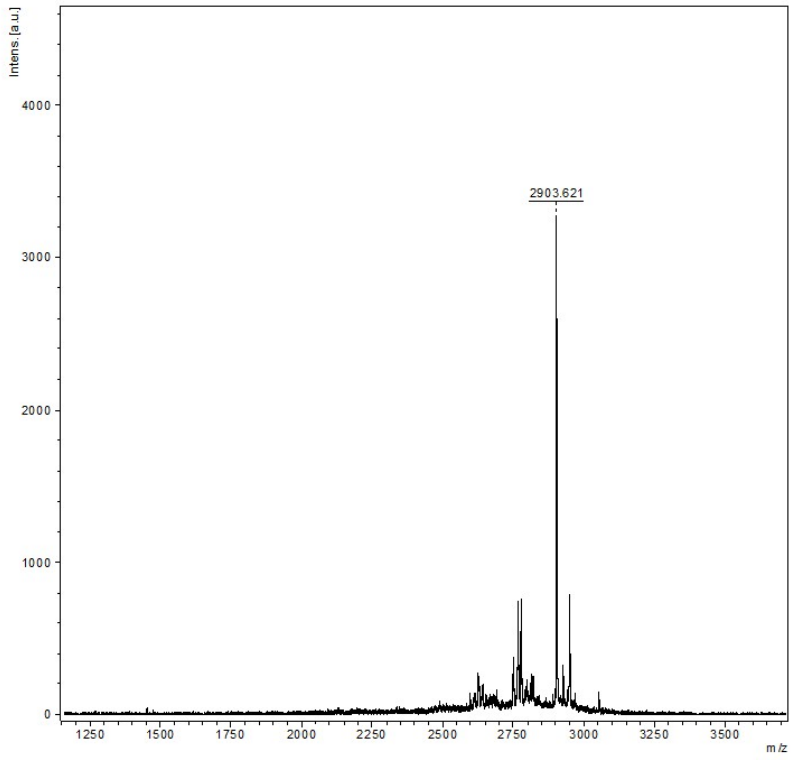
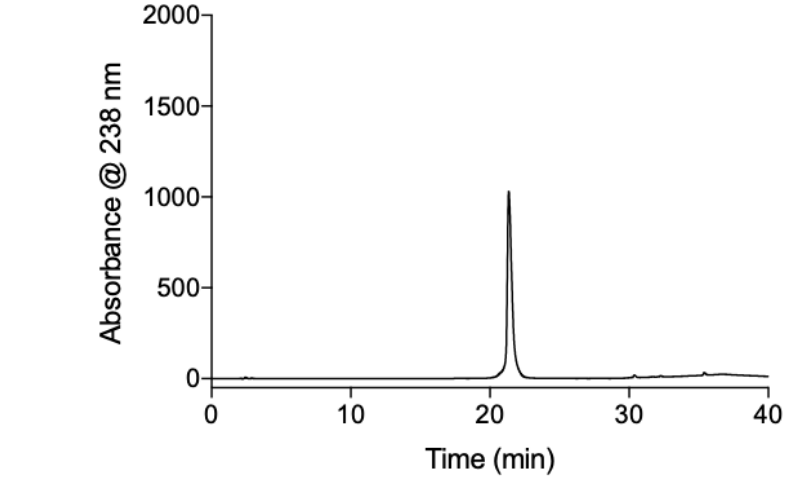
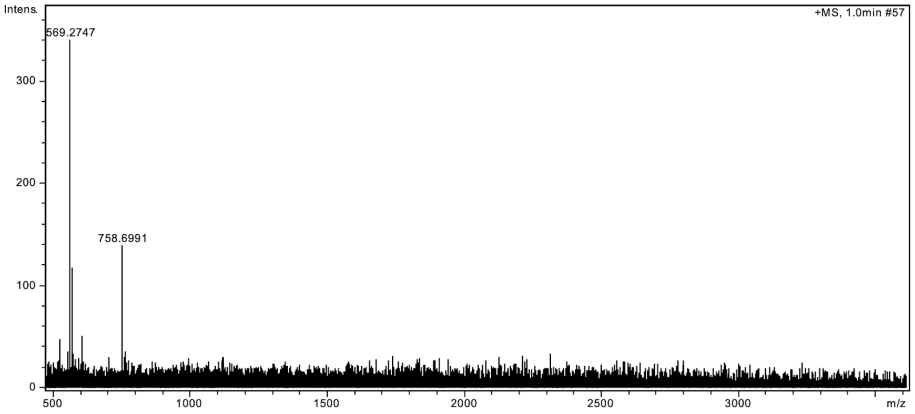


Figure S9.37. MALDI spectrum of ABA-βAla-$(SK^{2M})_5$-G. Mass found: 2903.621, mass calculated: 2903.585.





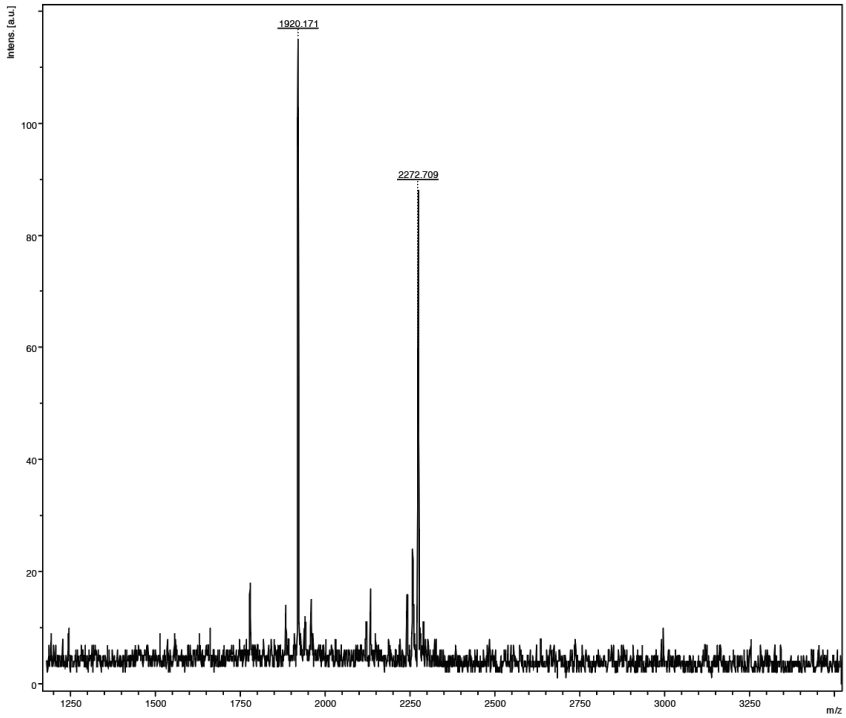


Figure S9.38. HPLC, ESI and MALDI of FITC-βAla-$(SK^{2M})_3$-G. ESI: Mass found: [M+4H] 569.2747, [M+3H] 758.6991, mass calculated: [M+4H] 569.2804, [M+3H] 758.7048. MALDI: Mass found:1920.171(fragmentation), 2272.709, mass calculated 2272.0893.

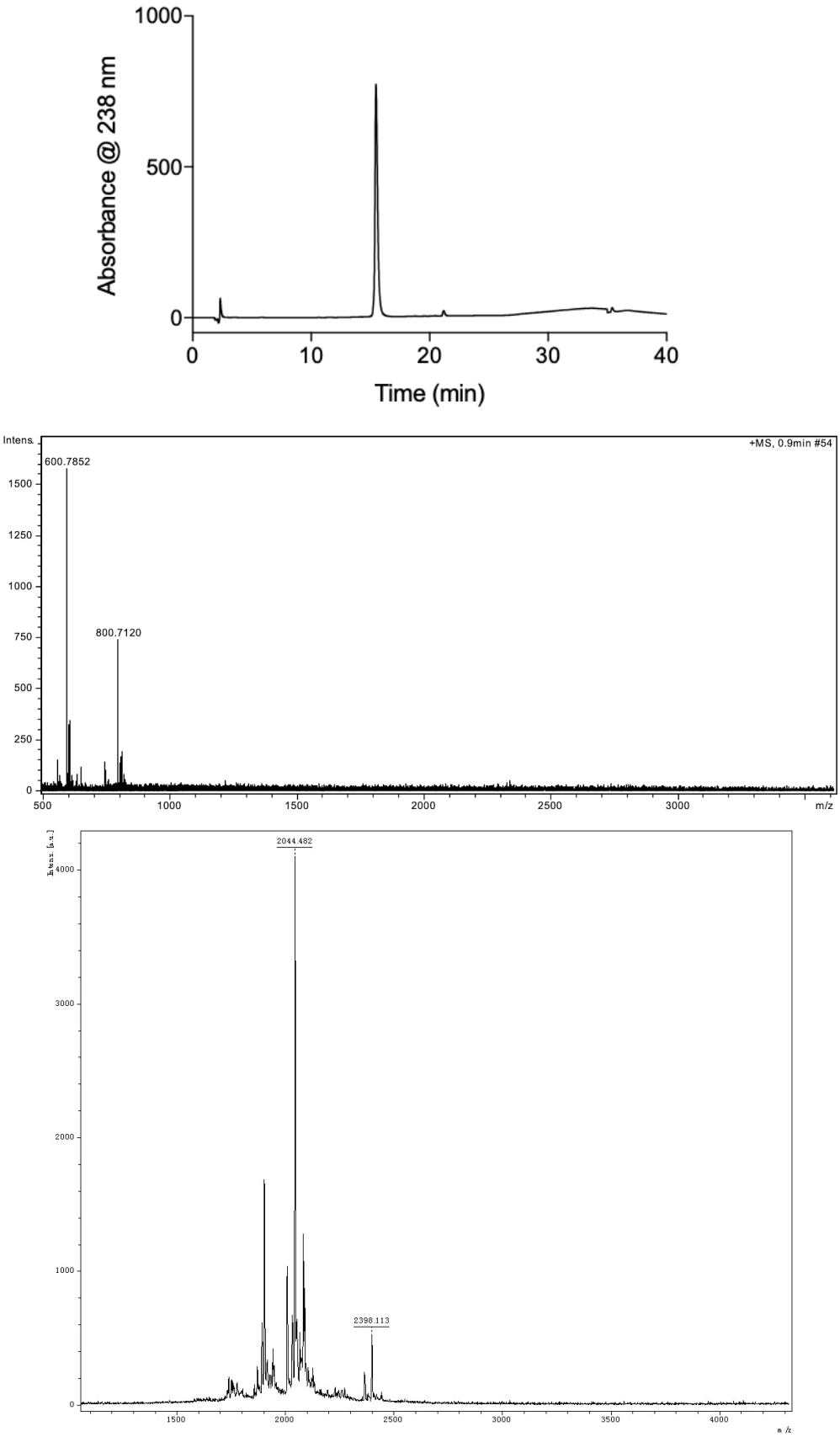


Figure S9.39. HPLC, ESI and MALDI of FITC-βAla-$(EK^{2M})_3$-G. ESI: Mass found: [M+4H] 600.7852, [M+3H] 800.7120, mass calculated: [M+4H] 600.7884, [M+3H] 800.7154. MALDI: Mass found: 2044.482 (fragmentation), 2398.113, mass calculated 2398.121.

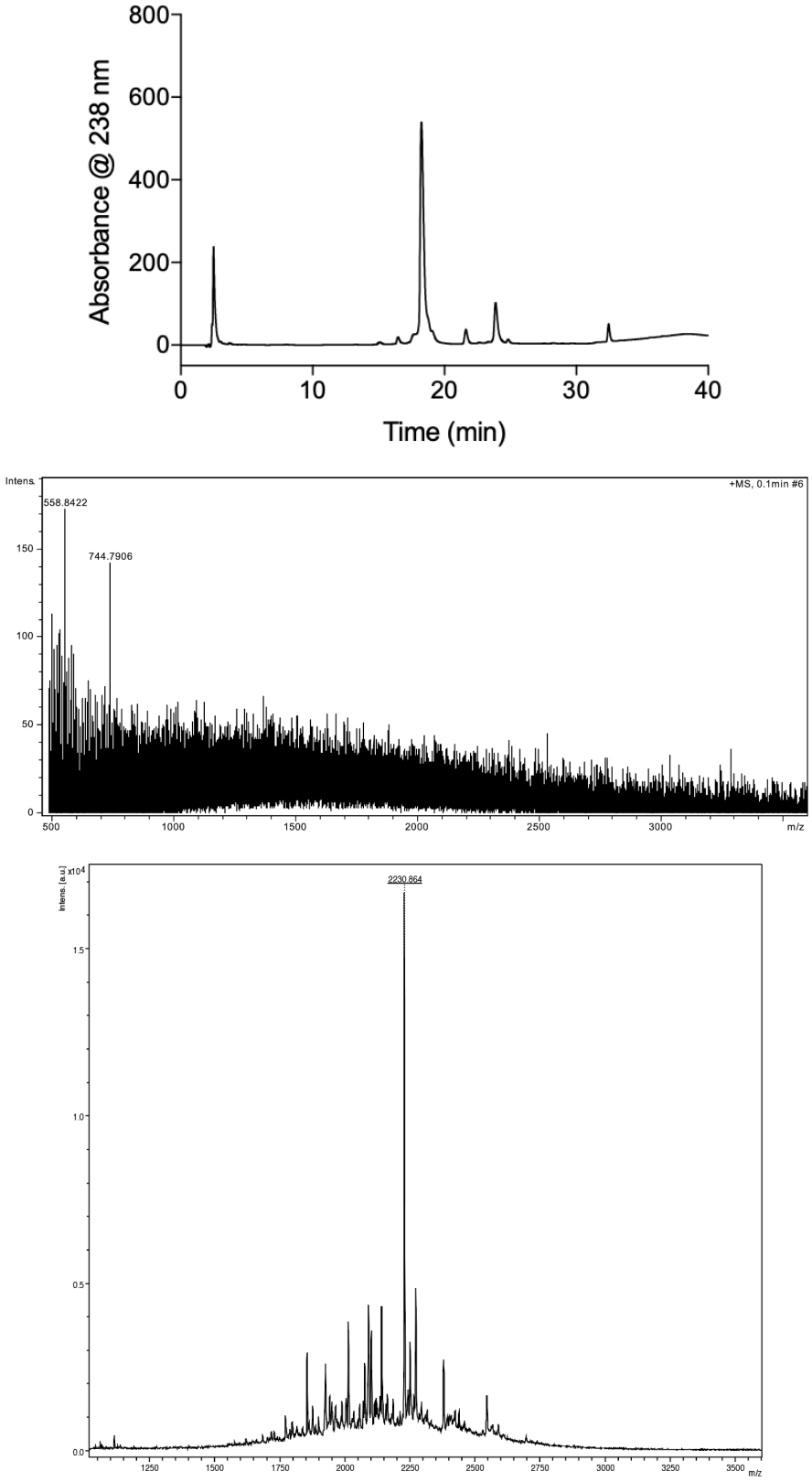
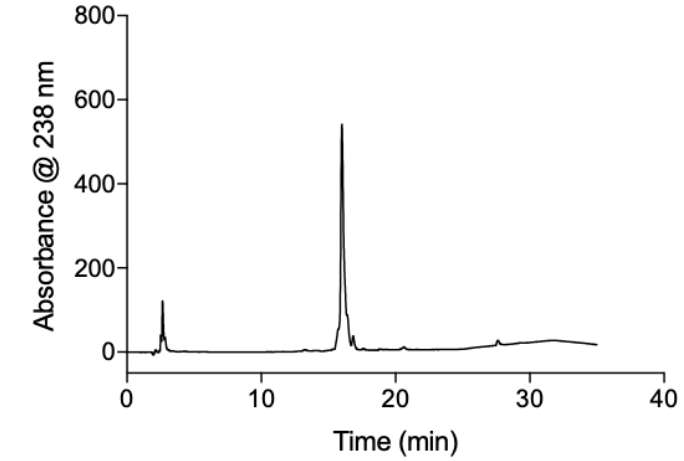
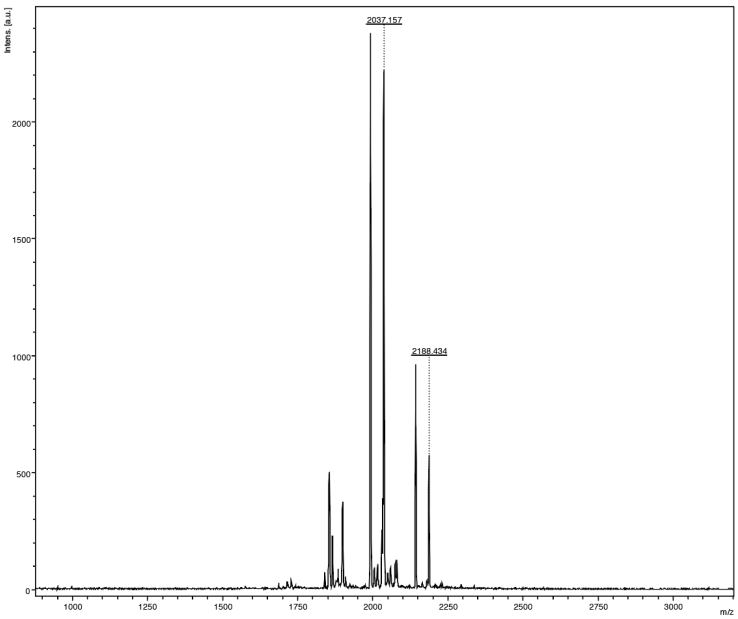


Figure S9.40. HPLC, ESI of FITC-βAla-$(SO^{2M})_3$-G and MALDI of alkyne-Ala-$(SO^{2M})_3$-G. ESI: Mass found: [M+4H] 558.8422, [M+3H] 744.7906, mass calculated: [M+4H] 558.7687, [M+3H] 744.6892. MALDI: Mass found: 2230.854, mass calculated 2230.043.





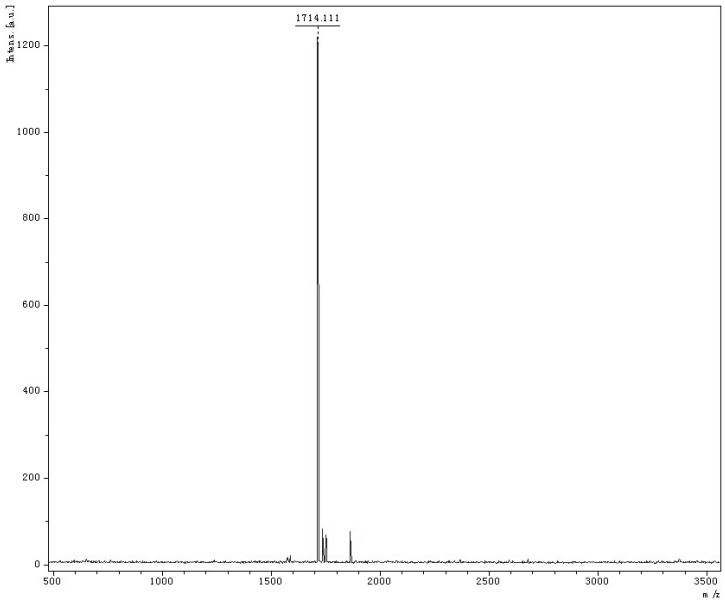


Figure S9.41. HPLC and MALDI of FITC-βAla-$(SB^{2M})_3$-G and MALDI of alkyne-βAla-$(SB^{2M})_3$-G. MALDI of FITC-βAla-$(SB^{2M})_3$-G: Mass found: 2188.434, 2037.157 (fragmentation), mass calculated 2187.995. MALDI of alkyne-βAla-$(SB^{2M})_3$-G: Mass found: 1714.111, mass calculated 1712.900.

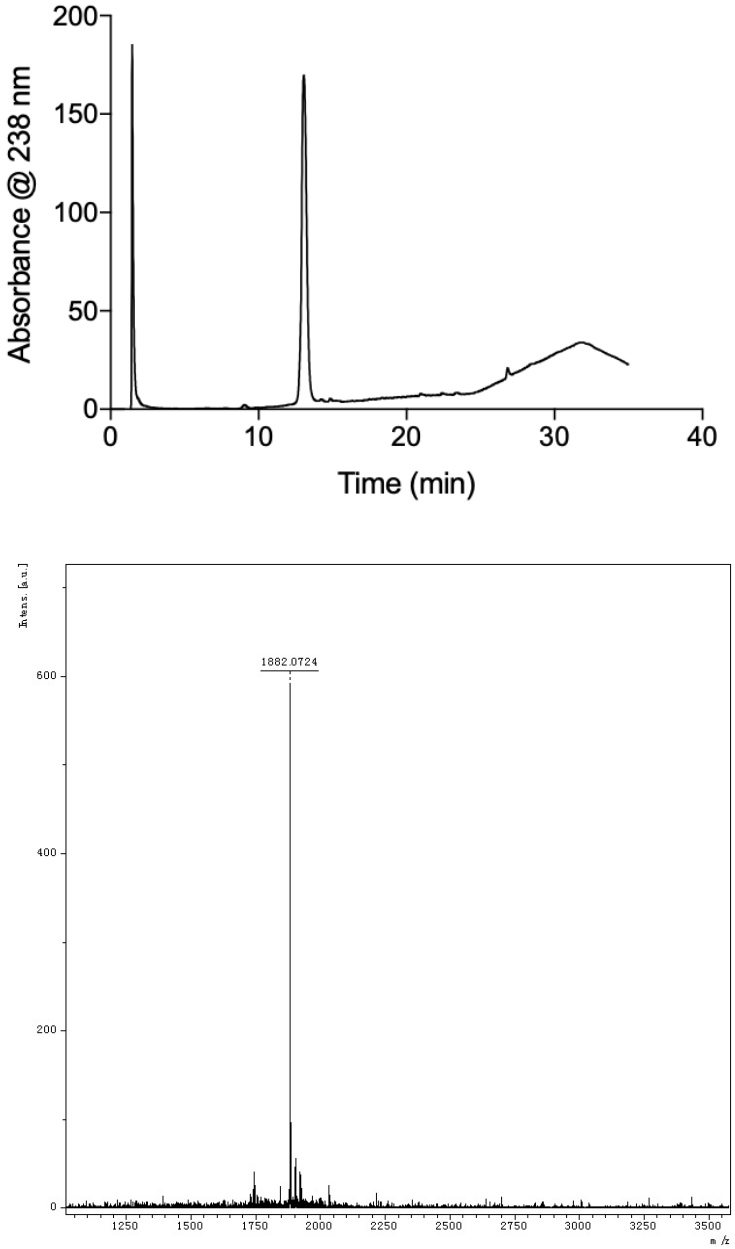


Figure S9.42. HPLC of FITC-βAla-$(EO^{2M})_3$-G and MALDI of alkyne-βAla-$(EO^{2M})_3$-G. MALDI: Mass found: 1882.072, mass calculated 1881.982.

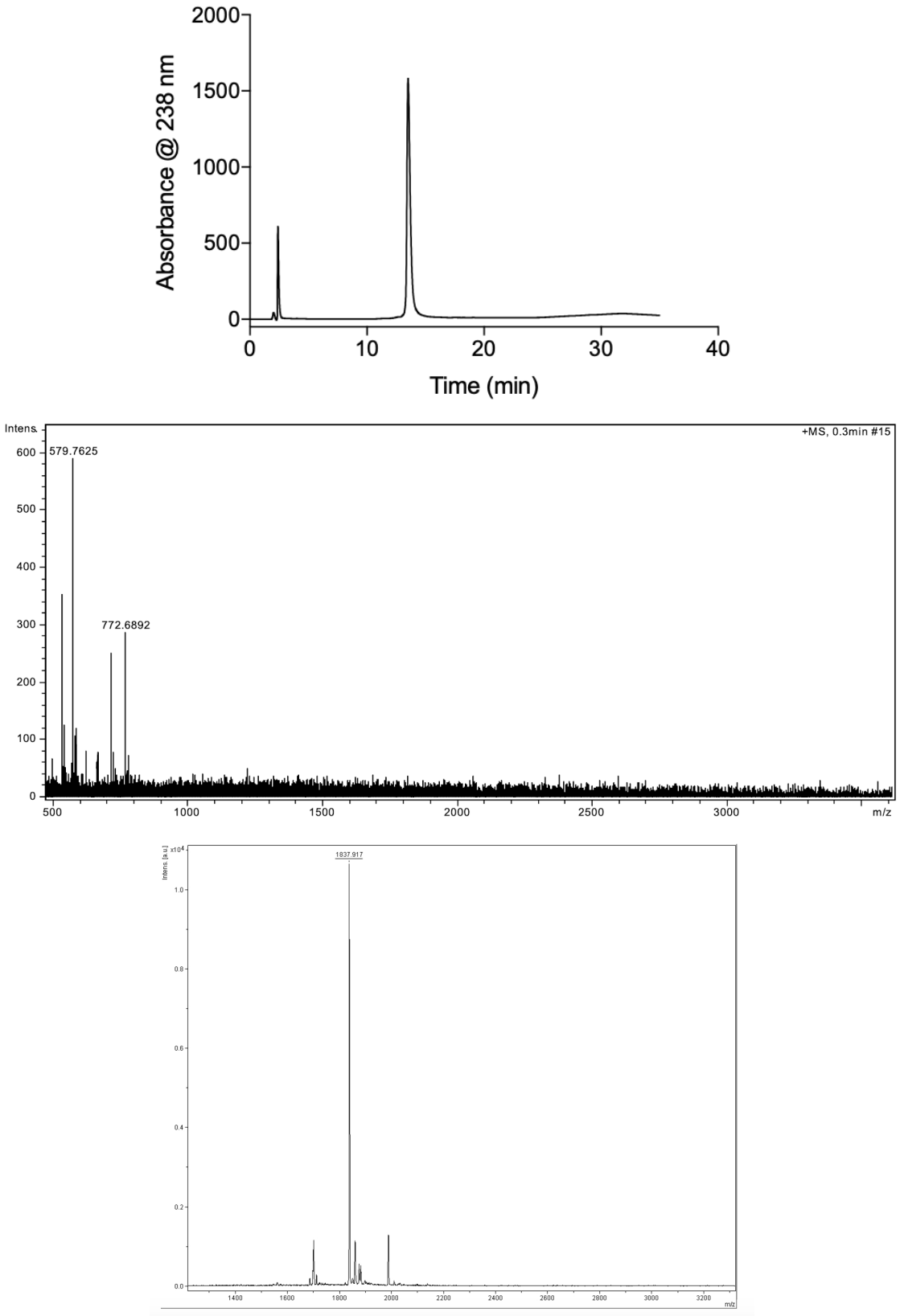


Figure S9.43. HPLC, ESI of FITC-βAla-$(EB^{2M})_3$-G and MALDI of alkyne-Ala-$(EB^{2M})_3$-G. ESI: Mass found: [M+4H] 579.7625, [M+3H] 772.6892, mass calculated: [M+4H] 579.7649, [M+3H] 772.6841. MALDI: Mass found: 1837.917, mass calculated 1838.932.

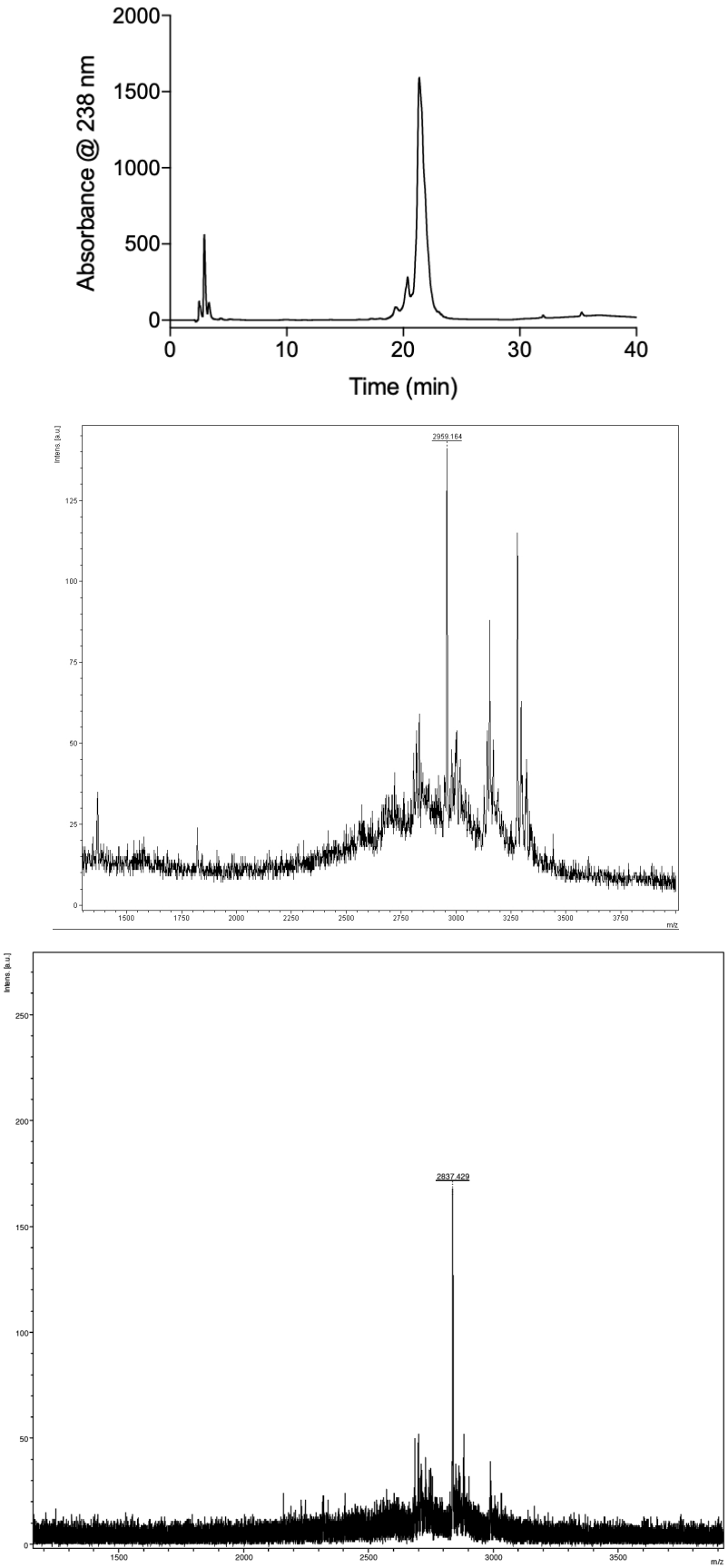


Figure S9.44. HPLC, ESI of FITC-βAla-$(SK^{2M})_5$-G and MALDI of alkyne-βAla-$(SK^{2M})_5$-G. MALDI of alkyne-βAla-$(EK^{2M})_5$-G: Mass found: 2837.429, mass calculated 2835.572. MALDI of FITC-βAla-$(SK^{2M})_5$-G (fragmentation): Mass found: 2959.164.

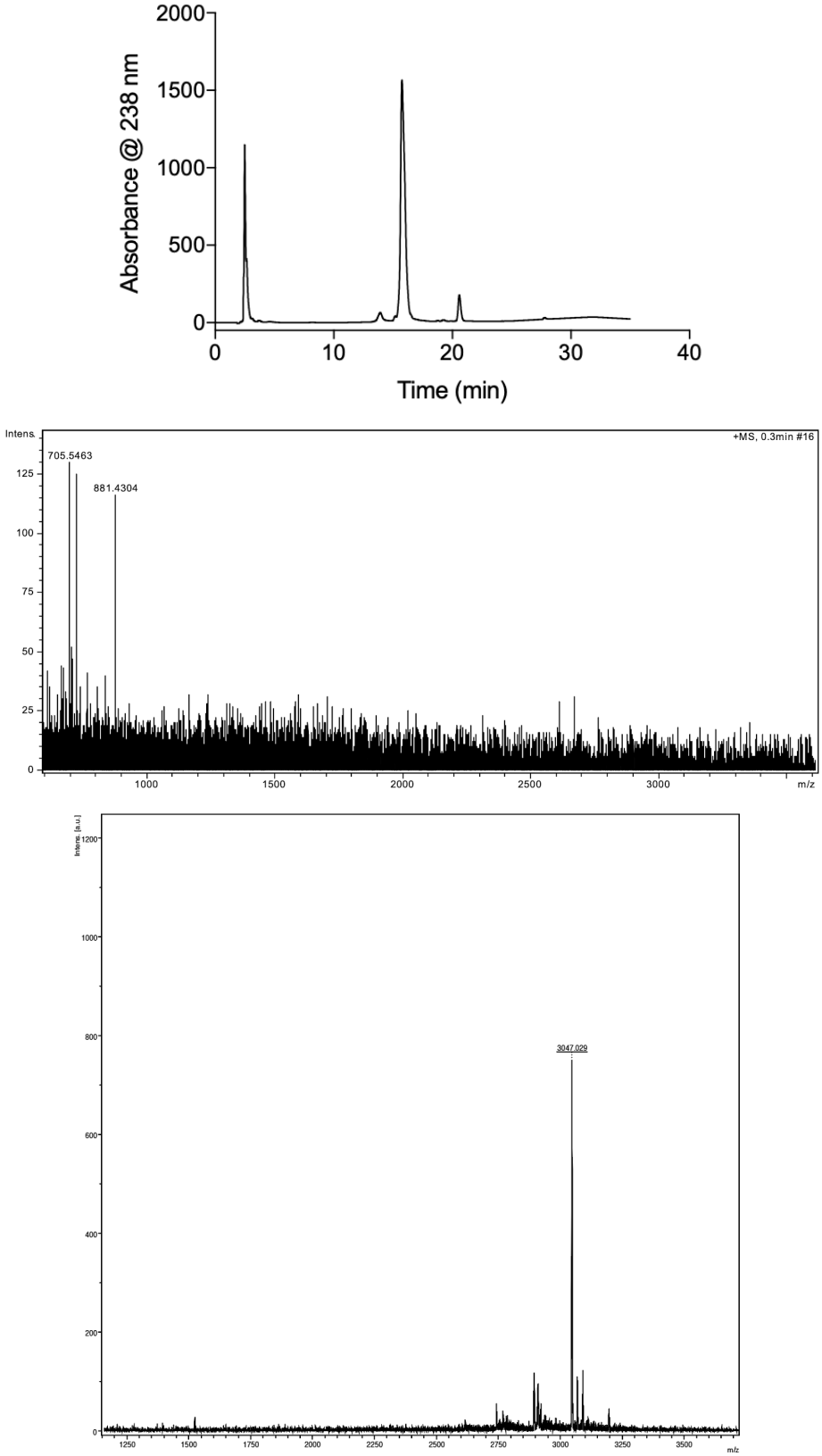


Figure S9.45. HPLC, ESI of FITC-βAla-$(EK^{2M})_5$-G and MALDI of alkyne-βAla-$(EK^{2M})_5$-G. ESI: Mass found: [M+5H] 705.5463, [M+4H] 881.4304, mass calculated: [M+5H] 705.3520, [M+4H] 881.4382. MALDI: Mass found: 3047.029, mass calculated 3045.625.

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(2) Ikawa, Y.; Fukada, K.; Watanabe, S.-I.; Shiraishi, H.; Inoue, T. Design, Construction, and Analysis of a Novel Class of Self-Folding RNA. *Structure* **2002**, *10* (4), 527–534.
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