Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Self-assembled peptide nucleic acid-microRNA nanocomplex for dual modulation of cancer-related microRNAs

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Contents

Materials and Methods

1. General materials and methods

HeLa and KB cells were purchased from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All chemicals and solvents were purchased from J&K chemicals (Shanghai, China), TCI chemicals (Shanghai, China) or Energy Chemical (Shanghai, China) and used as received. PNA monomers were purchased from Leon biological Technology (Nanjing China). Amino acids and resins were purchased from GL Biochem (Shanghai, China). miR-34a, Cy3-labeled miR-34a and NC were purchased from RiboBio (Guangzhou, China). miR-34a: 5'-UGGCAGUGU-CUUAGCUGGUUGU-3' (guide strand), 5'-CAAUCAGCAAGUAUACUGCCCU-3' (passenger strand). NC: 5'-UUCUCCGAACGUGUCACGU-3' (guide strand), 5'-ACGUGACACGUUCGG-AGAA-3' (passenger strand). High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin(P/S), Trypsin-EDTA, Lipofectamine 2000, Lyso tracker green, DRAQ5, and TRIZOL reagents were purchased from Life Technologies (Shanghai, China). Antibodies were purchased from Abcam (Shanghai, China). Protease inhibitor cocktail was purchased from Cell Signaling (Shanghai, China). Luciferase assay kits were purchased from Promega (Beijing, China). MTT, RIPA buffer and 5*SDS loading buffer were purchased from Solarbio (Beijing, China). Annexin V-FITC/PI staining kit was purchased from BD Biosciences (Shanghai, China). All aqueous solutions were treated by diethyl pyrocarbonate (DEPC) before use.

MALDI-TOF MS were done on ABSCIEX MALDI-TOF-TOF 4800 PLUS. HPLC was carried out on Agilent 1200 LC with CH_3CN/H_2O (1‰ CF_3COOH) as eluents. The sizes of nanocomplexes were measured by using Brookhaven 90Plus Analyzer. Zeta potential of nanocomplexes were measured by using Malvern Nano-Z Analyzer. Transmission electron microscopy (TEM) images were taken on JEM-1011 transmission electron microscope. Confocal images were taken on TCS SP5 (Leica) two-photon laser scanning focusing microscope. Apoptosis assay were taken on FACS calibur (BD).

2. Synthesis and characterization of chemical compounds

Synthesis of PA-R9

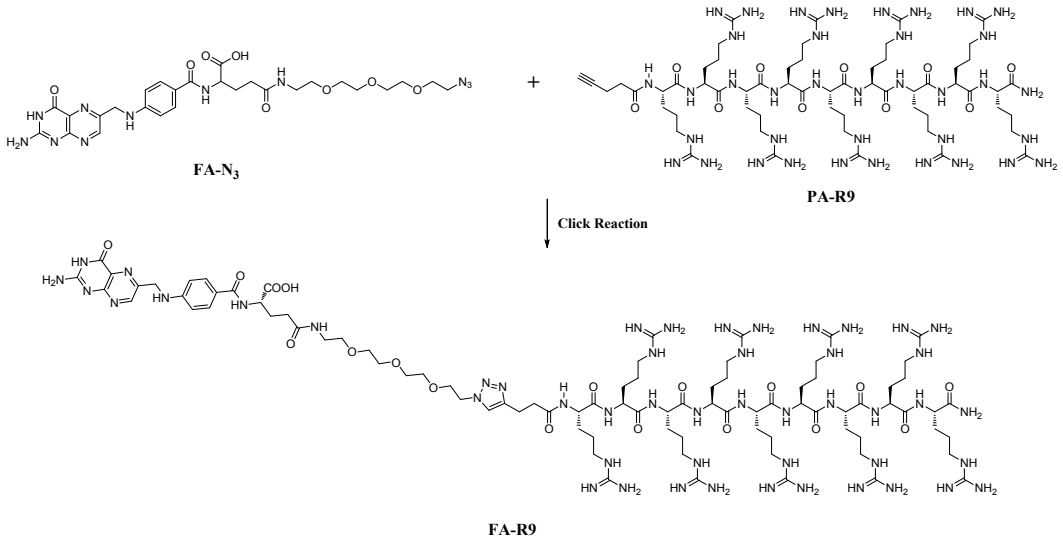
PA-R9 was synthesized according to previous report.[1] **PA-R9** was synthesized using a standard solid phase peptide synthesis (SPPS) protocol by Rink Amide-MBHA Resin and N-Fmoc-protected amino acids. The resins were swelled in dry N,N-Dimethylformamide (DMF) for 15 minutes and then treated with 20% piperidine (in DMF) for 15 minutes to remove the protecting group. After wash by DMF, Fmoc-protected amino acid (1.2 equiv.) was coupled to the free amino group on the resin using HBTU as the coupling reagent. These two steps were repeated to elongate the peptide chain, which were carried out by the standard Fmoc SPPS protocol. The resins were washed with DMF for 3~5 times after each step. At the final step, 4-pentynoic acid (3 equiv.) was loaded by using HBTU (3 equiv.) and DIPEA (6 equiv.). Then, the peptide was cleaved with 95% TFA (2.5% ddH_2O and 2.5% Triisopropylsilane) to get crude product. The crude product was then purified by reverse phase HPLC and lyophilized to give a white powder. MALDI-TOF MS calculated: 1502.9661 $[M+H]^+$, found 1502.9865.

Synthesis of Maleimide-PNA

Maleimide-PNA was synthesized using a standard SPPS protocol by 2-chlorotrityl chloride resin and PNA monomer. 2-chlorotrityl chloride resin was swelled in dry 1-Methyl-2-pyrrolidone (NMP) for 15 min, and then the first PNA monomer was loaded onto the resin at its C-terminal with PNA monomer (5 equiv.) and N, N-diisopropylethylamine (DIPEA) in NMP for 3 h. After wash with NMP (3×5 mL), the resin was treated with the blocking solution (NMP/MeOH/DIPEA, 16/3/1) for 15 min to deactivate the unreacted sites. Then, the resins were treated with 20% piperidine (in NMP) for 15 min to remove the protecting group, followed by coupling Fmoc/Boc protected PNA monomer (3 equiv.) to the free amino group on the resin using HBTU as the coupling reagent. These two steps were repeated to elongate the PNA chain, which were carried out by the standard Fmoc SPPS protocol. 3-Maleimidopropionic acid was also loaded using same protocol. The resin was washed with NMP for 3 ~ 5 times after each step. At the final step, the PNA was cleaved with 95% TFA (2.5% ddH_2O and 2.5% Triisopropylsilane) for 2 h and then the resulted crude product was purified by reverse phase HPLC and lyophilized to give a white powder. MALDI-TOF MS

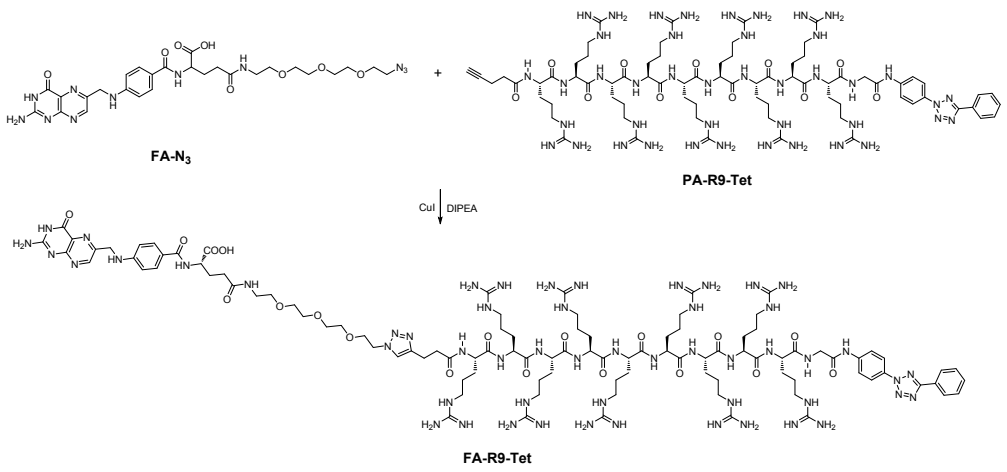
calculated: 2360.9009 $[M+H]^+$, found 2360.6841.

Synthesis of FA-R9



FA-N_3 was synthesized according to previous report.[1] **FA-R9** was synthesized by dissolving **PA-R9** (0.01 mmol, 15 mg) and **FA-N_3** (0.015 mmol, 9.6 mg) in a DMSO/ddH_2O (2/1) solution. Then, sodium ascorbate (200 mg dissolved in ddH_2O), $CuSO_4$ (3.2 mg dissolved in ddH_2O), THPTA (50 mg dissolved in DMSO) were added to the mixture, and the solution was stirred at 30 °C. The reaction was monitored with HPLC until it completed. The resulted mixture was purified by reverse phase HPLC and lyophilized to give a yellow powder (8.7 mg, 40.6%). MALDI-TOF MS calculated: 2144.4220 $[M+H]^+$, found 2144.8159.

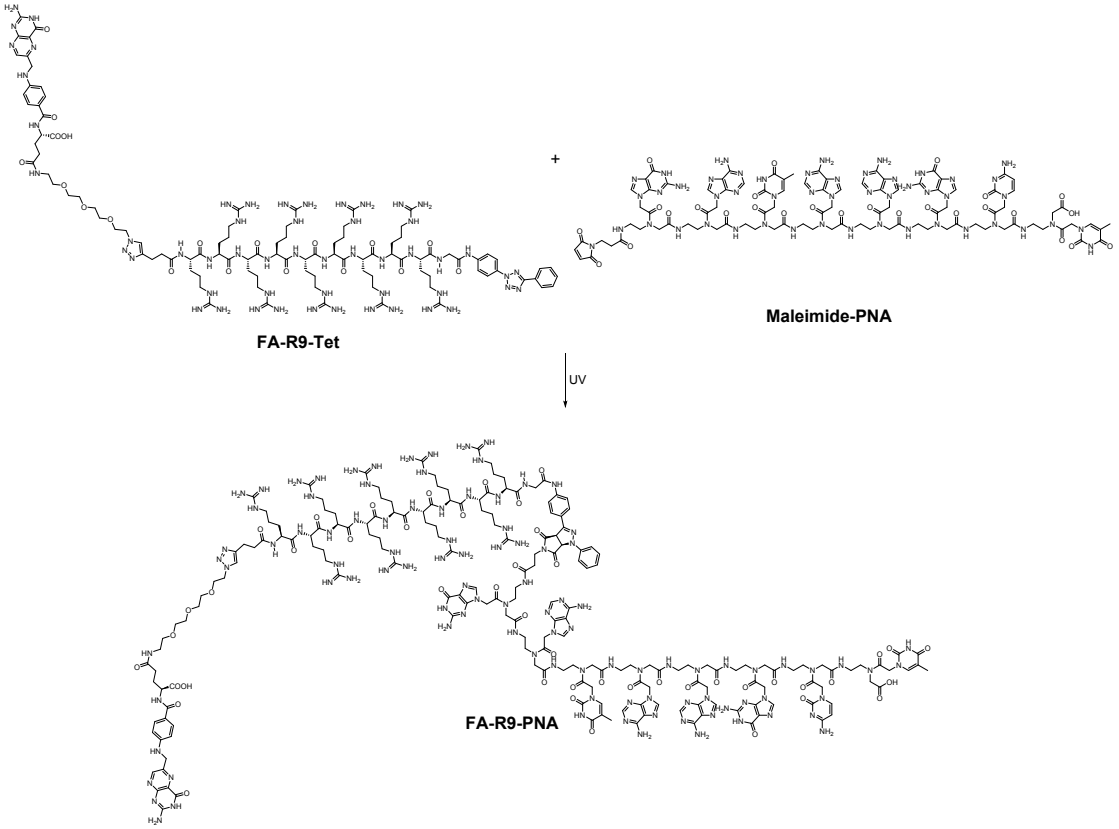
Synthesis of FA-R9-Tet



PA-R9-Tet was synthesized according to previous report.[2] **FA-R9-Tet** was synthesized by

dissolving **FA-N_3** (0.1 mmol, 64.1 mg) and **PA-R9-Tet** (0.05 mmol, 89 mg) in dry DMSO. Then Cuprous iodide (0.05 mmol, 10 mg dissolved in ddH_2O with 17.5 μL DIPEA) were added to the mixture and the solution was stirred at room temperature. The reaction was monitored with HPLC until it completed. The resulted mixture was purified by reverse phase HPLC and lyophilized to give a yellow powder (52.8 mg, 43.6%). MALDI-TOF MS: calculated: 2393.3261 $[M+H-N_2]^+$; found: 2394.5759.

Synthesis of FA-R9-PNA



FA-R9-PNA was synthesized by dissolving **FA-R9-Tet** (0.01 mmol, 24.2 mg) and **Maleimide-PNA** (0.01 mmol, 23.6 mg) in a CH_3CN/PBS (1/1) solution. Then, the mixture was irradiated with a hand-held 302 nm UV lamp for 30 minutes. The reaction was monitored with HPLC until it completed. The resulted mixture was purified by reverse phase HPLC and lyophilized to give a yellow powder (42.1 mg, 88.1%). High Resolution MS: calculated: 4752.2175 $[M+H]^+$; found: 4752.0000.

3. Cell culture

HeLa, RAW264.7 and HUVEC cells were cultured in high glucose DMEM containing 10% FBS and 1% P/S, and maintained in 5% CO_2 at 37 ^oC.

KB cells were cultured in RMPI 1640 containing 10% FBS and 1% P/S and maintained in 5% CO_2 at 37 ^oC.

4. Gel-shift assay

FA-R9-PNA and miR-34a (100 pmol) were mixed at different molar ratios of 10:1, 20:1, 30:1, 40:1 and 50:1. After standing at room temperature for 30 minutes, they were analyzed by 2% agarose gels stained with ethidium bromide. Naked miRNA served as a control.

5. Dynamic Light Scattering and Zeta potential analysis

FA-R9-PNA and miR-34a (100 pmol) were mixed at different molar ratios of 10:1, 20:1, 30:1, 40:1 and 50:1, then they were allowed to stand at room temperature for 30 minutes. The samples were diluted to 500 μL with ddH_2O and were used for dynamic light scattering (DLS) and Zeta potential analysis.

6. Serum Stability Test

100 pmol of miR-34a was incubated with FA-R9-PNA at a 1/40 molar ratio for 30 min at room temperature. Then, the nanocomplex was added into DMEM supplemented with 10% FBS, and the mixture was further incubated for 24 h, during which the diameters of nanoparticles were measured by DLS at 0 h, 2 h, 6 h, 12 h and 24 h time points.

7. RT-qPCR Assay

Analysis of miR-34a and miR-21 levels in HeLa cells after treatment with nanocomplexes formed by FA-R9-PNA/miR-34a at different molar ratios: HeLa cells were seeded in 12 well plates and transfected with 30 pmol miRNA mimics by using FA-R9-PNA at different molar ratios. The final concentration of miR-34a was 100 nM. After 24 h, total RNA was isolated by using TRIZOL reagent according to manufacturer's instruction. miR-34a and miR-21 in cells were quantified using Taqman miRNA detection assay. The reactions were carried out using

0.5 μg of RNA extracted from cells and U6 was used as internal control.

Analysis of miR-34a and miR-21 levels in different cells after treatment with PMN-21^{anti}, PMN-34a or PMN-34a/21^{anti}: HeLa, RAW264.7, KB and HUVEC cells were seeded in 12 well plates. When the cell density reached 80%, these cells were treated with PMN-21^{anti}, PMN-34a and PMN-34a/21^{anti}. The final concentrations of NC, miR-34a, FA-R9 and FA-R9-PNA were 100 nM, 100 nM, 4 μM and 4 μM, respectively. After 24 h, total RNA was isolated using TRIZOL reagent according to manufacturer's instruction. miR-34a and miR-21 in cells were quantified using Taqman miRNA detection assay. The reactions were carried out using 0.5 μg of RNA extracted from cells and U6 was used as internal control.

8. Confocal fluorescence imaging

HeLa cells were seeded on 35 mm glass-bottom tissue culture dishes. When the cell density reached 80%, miR-34a^{Cy3} was transfected into Hela cells using FA-R9-PNA at a 40/1 peptide/miRNA ratio. The final concentration of miR-34a^{Cy3} was 100 nM. At different time points, the cells were washed three times with PBS, then stained with DRAQ5 and Lyso tracker-green, and then fixed with 4% paraformaldehyde. Finally, the cells were washed with PBS for three times and immediately imaged by laser confocal microscopy.

9. Luciferase Assay

HeLa cells were seeded in 24 well plates and transfected the following day with 0.4 μg Luciferase reporter plasmids and 0.4 μg β-galactosidase expressing plasmids by using Lipofectamine 2000 according to manufacturer's protocol. β-galactosidase was used as an internal control. After 4 h, cells were further transfected with PMN-21^{anti}, PMN-34a or PMN-34a/21^{anti}. The final concentrations of NC, miR-34a, FA-R9 and FA-R9-PNA were 400 nM, 400 nM, 16 μM and 16 μM, respectively. Luciferase signals were measured after 72 h by using Luciferase assay kits according to manufacturer's instruction.

10. Western Blotting Analysis

HeLa cells were seeded in 60 mm dish, after transfection of PMN-21^{anti}, PMN-34a and PMN-34a/21^{anti} for 72 h, protein was extracted. For purification of protein, 1× protease

inhibitor cocktail was added to RIPA buffer to prevent protein degradation. Protein extracted from HeLa cell samples was diluted in 1 × SDS loading buffer, and then pre-denatured and resolved in 12% SDS-PAGE, transferred to PVDF membrane, blocked in 5% non-fat milk in TBST, and blotted with primary antibodies for GAPDH, PDCD4 and SIRT1. After wash, the membrane was further incubated with the appropriate secondary antibody and finally visualized using ECL reagents.

11. MTT Assay

HeLa cells were seeded in 96-well plates with a concentration of 5,000 cells per well. 24 hours later, they were transfected by using PMN-21^{anti}, PMN-34a or PMN-34a/21^{anti} at different concentrations of miRNA mimics or NC. MTT assay was carried out after 72 h. For each well, 50 μL MTT solution (final concentration: 1 mg/mL) was added. 100 μL DMSO was added after 4 hours' incubation and absorbance at 560 nm was measured to indicate the cell viability.

12. Flow Cytometry Analysis

Hela cells were seeded in 12 well plates. When reaching ~80% confluence, cells were transfected with PMN-21^{anti}, PMN-34a and PMN-34a/21^{anti}. The final concentrations of NC, miR-34a, FA-R9 and FA-R9-PNA were 400 nM, 400 nM, 16 μM and 16 μM, respectively. Untreated cells served as blank controls. The cells were cultured for 72 hours and harvested respectively for apoptosis analysis. The apoptotic cells were identified through flow cytometry by using an Annexin V-FITC/PI staining kit according to manufacturer's protocol.

References

1. Xiao Xiao, Xingxing Wang, Haidong Gao, Xi Chen, Jinbo Li, Yan Zhang. *Chem. Asian J.* **2018**, *13,* 3845-3849.
2. Xiao Xiao, Xingxing Wang, Yuqi Wang, Tianren Yu, Lei Huang, Lei Chen, Jinbo Li, Chenyu Zhang, Yan Zhang. *Chem. Eur. J.* **2018**, *24*, 2277-2285.

Supporting Figures

Figure S1. Zeta potentials of nanocomplexes formed by FA-R9-PNA with miR-34a.

FA-R9-PNA and miR-34a were mixed in PBS buffer with molar ratios ranging from 10/1 to 50/1. Data are shown as mean ± SEM (n=3).

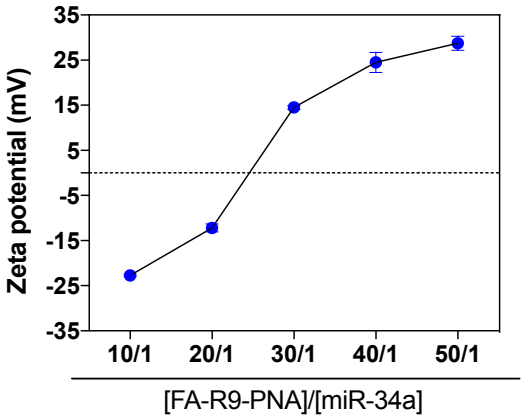


Figure S2. TEM image of PMN-34a/21^{anti}.

TEM image of nanocomplex formed by FA-R9-PNA/miR-34a 40/1. Scale bar: 200 nm.

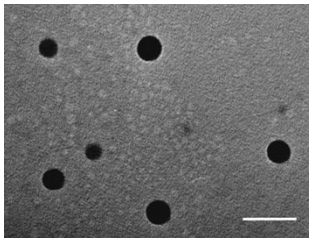


Figure S3. Stability of PMN-34a/21^{anti} in cell culture medium.

PMN-34a/21^{anti} formed by FA-R9-PNA with miR-34a (40/1) were added into DMEM supplemented with 10% FBS. The final concentrations of miR-34a and FA-R9-PNA were 100 nM and 4 μM, respectively. Hydrodynamic sizes of PMN were directly analyzed by DLS after incubation for 0, 2, 6, 12, or 24 h. Data are shown as mean ± SEM (n=3).

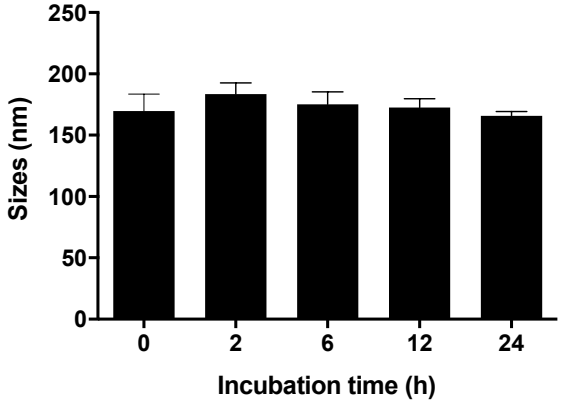


Figure S4. Comparison of the delivery efficiency by PMN-34a/21^{anti} and Lipofectamine 2000.

Relative expression levels of (a) miR-34a and (b) miR-21 in HeLa cells that were treated with Lipo-miR-34a, Lipo-PNA or PMN-34a/21^{anti} for 24 h. The final concentrations of miR-34a and PNA anti-miR-21 were 100 nM and 4 μM, respectively. Data are shown as mean ± SEM (n=3).

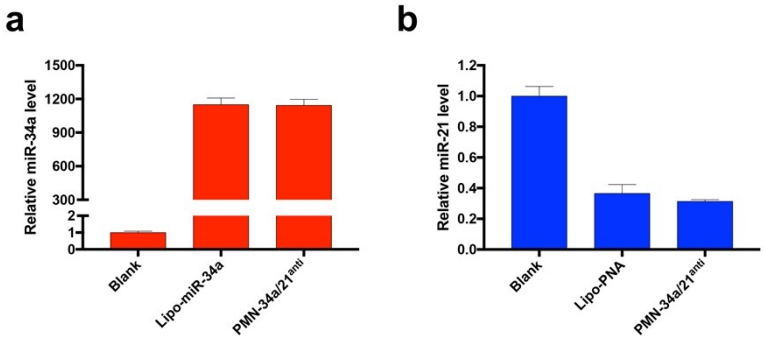


Figure S5. Hydrodynamic sizes of PMN-21^{anti}, PMN-34a and PMN-34a^{Cy3}/21^{anti}.

PMN-21^{anti}: FA-R9-PNA/NC, 40/1; PMN-34a: FA-R9/miR-34a, 40/1; PMN-34a^{Cy3}/21^{anti}: FA-R9-PNA/ miR-34a^{Cy3}, 40/1. Data are shown as mean ± SEM (n=3).

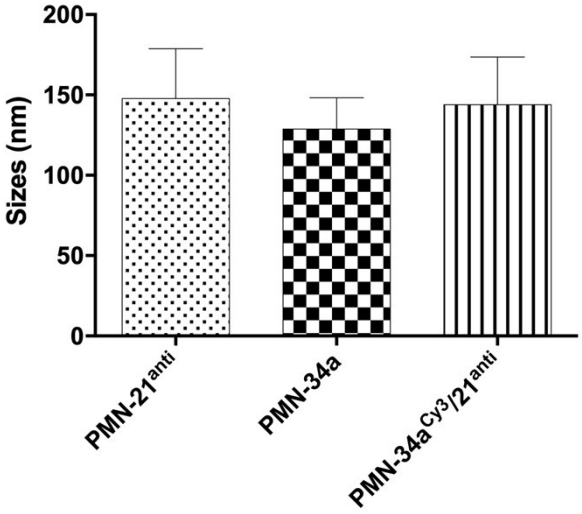


Figure S6. Targeted delivery of miR-34a and PNA anti-miR-21 into KB cells by PMN-34a/21^{anti}.

RT-qPCR analysis of miR-34a and miR-21 levels in KB cells after treatment with PMN-21^{anti}, PMN-34a or PMN-34a/21^{anti}. The final concentrations of NC, miR-34a, FA-R9 and FA-R9-PNA were 100 nM, 100 nM, 4 μM and 4 μM, respectively. Data are shown as mean ± SEM (n=3).

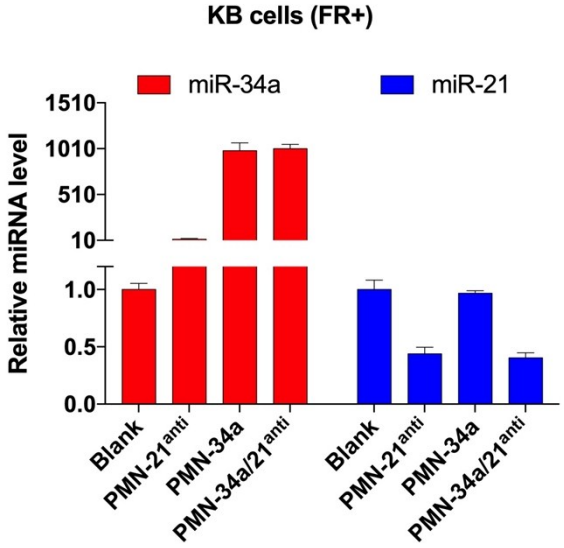


Figure S7. Reduction in cell viability induced by FA-R9/NC, PMN-34a, PMN-21^{anti}, or PMN-34a/21^{anti}.

Relative cell viability of HeLa cells after treatment with FA-R9/NC, PMN-34a, PMN-21^{anti}, or PMN-34a/21^{anti} for 72 h. FA-R9/NC (40/1) was used as negative control. The concentrations of NC or miR-34a ranged from 0 to 500 nM. Data are shown as mean ± SEM (n=3).

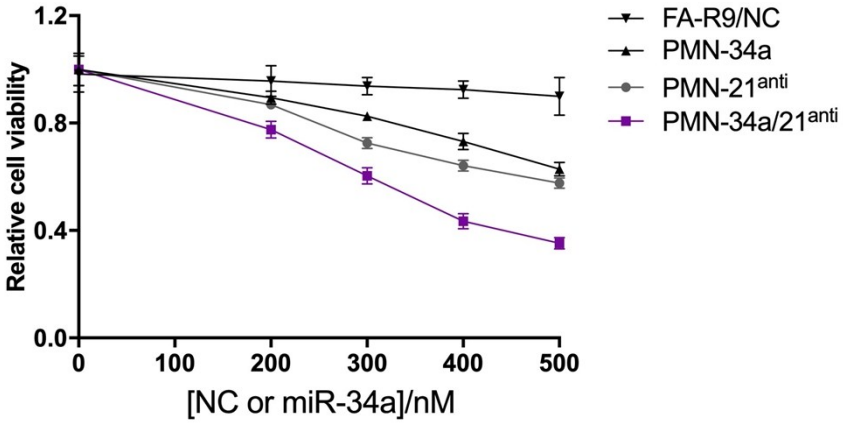
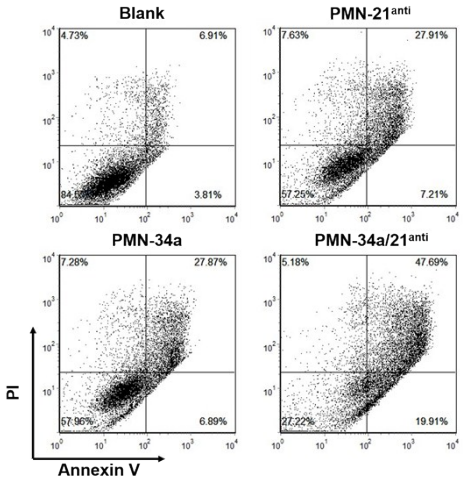


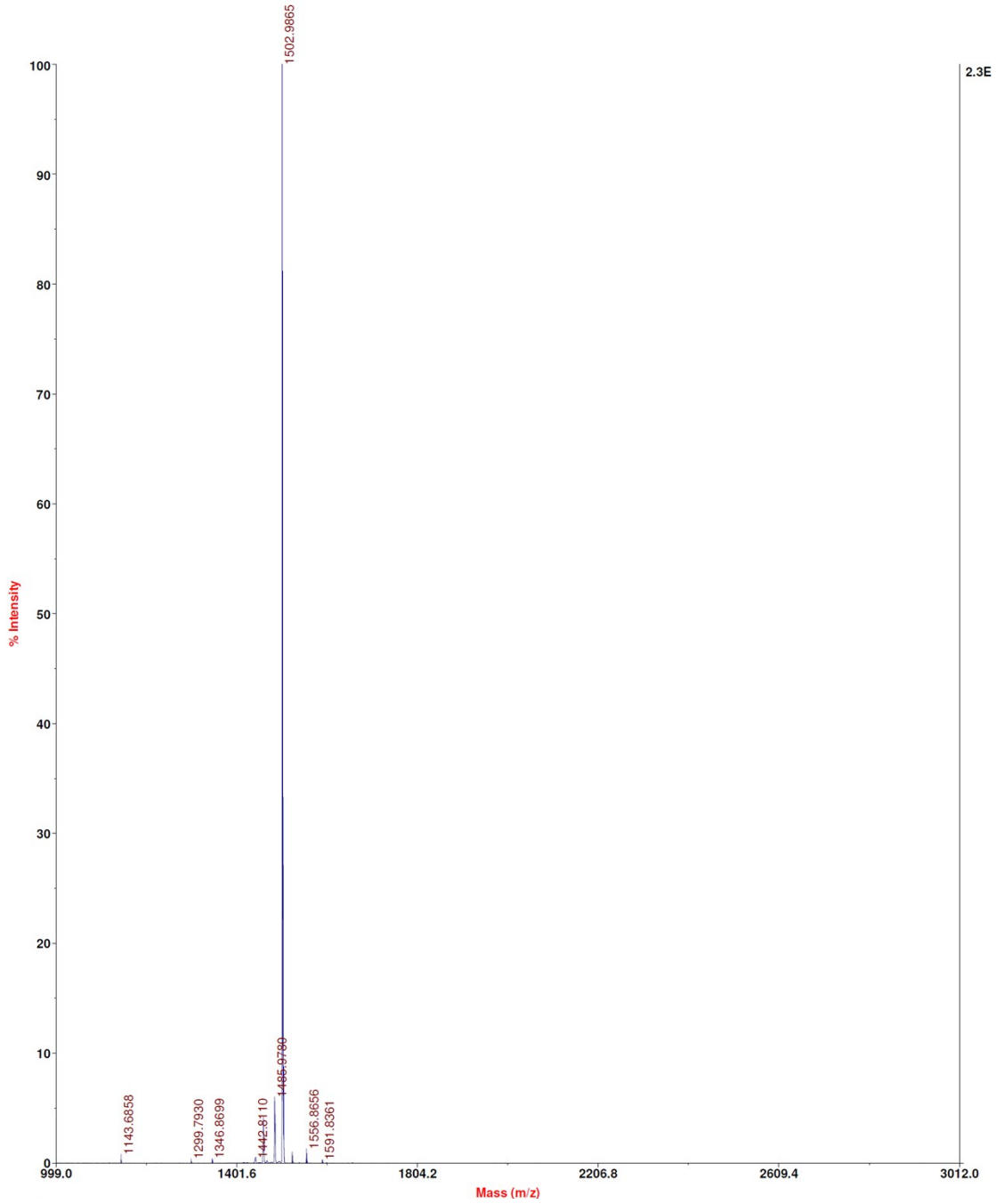
Figure S8. Flow cytometry analysis of apoptosis in HeLa cells after treatment with PMN-21^{anti}, PMN-34a, or PMN-34a/21^{anti}.

The final concentrations of NC, miR-34a, FA-R9 and FA-R9-PNA were 400 nM, 400 nM, 16 μM and 16 μM, respectively.

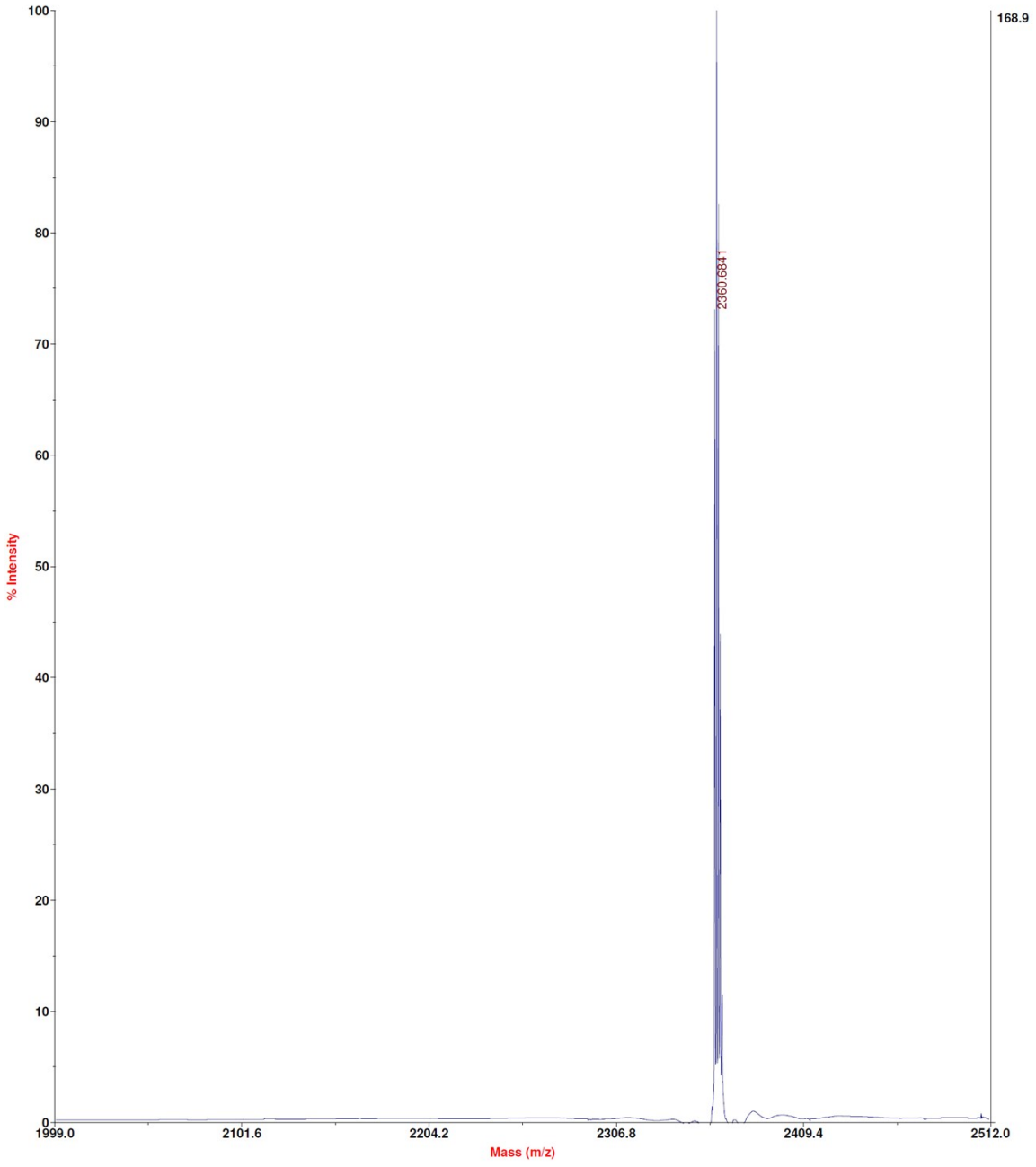


MS Spectra

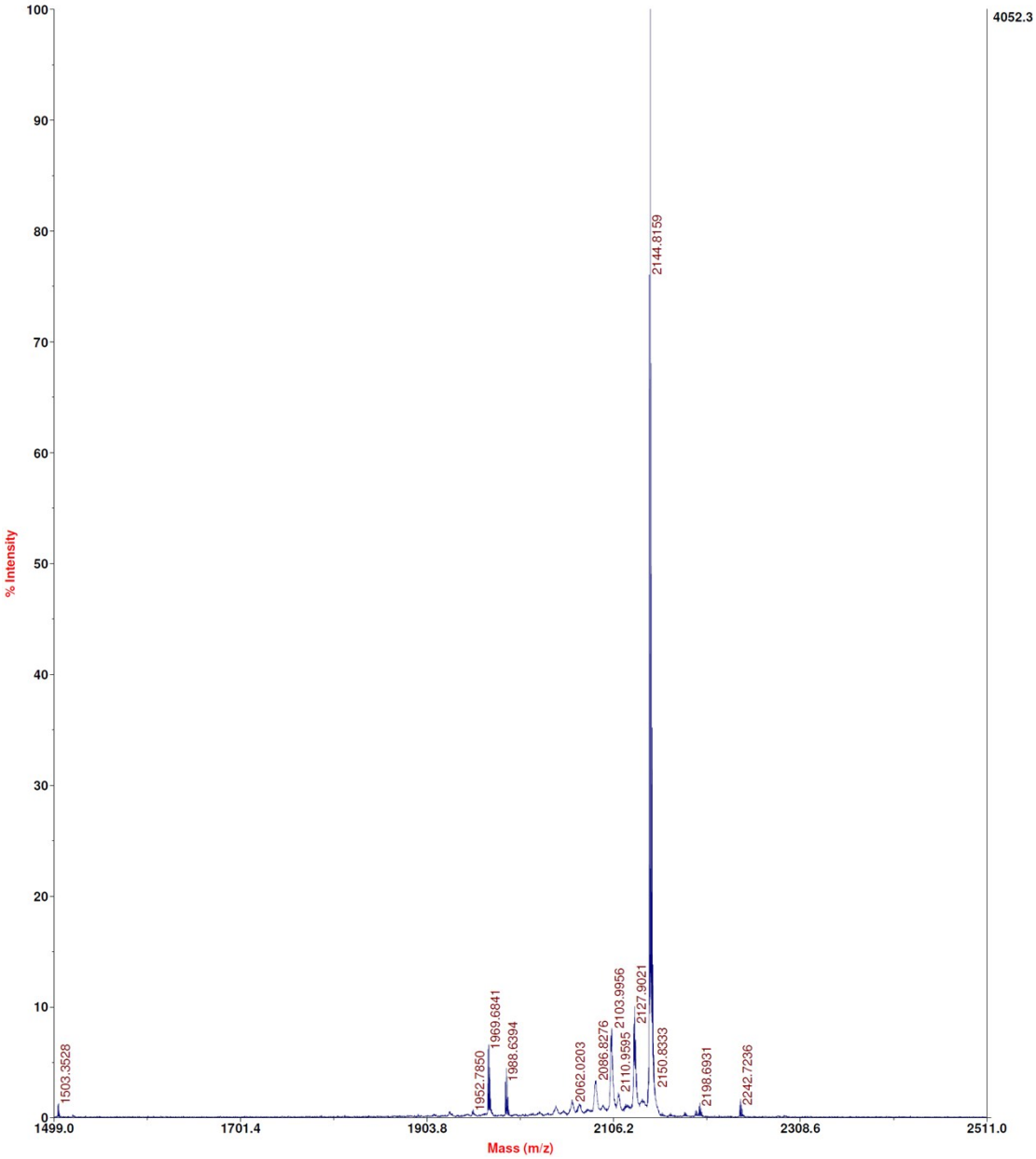
PA-R9



Maleimide-PNA



FA-R9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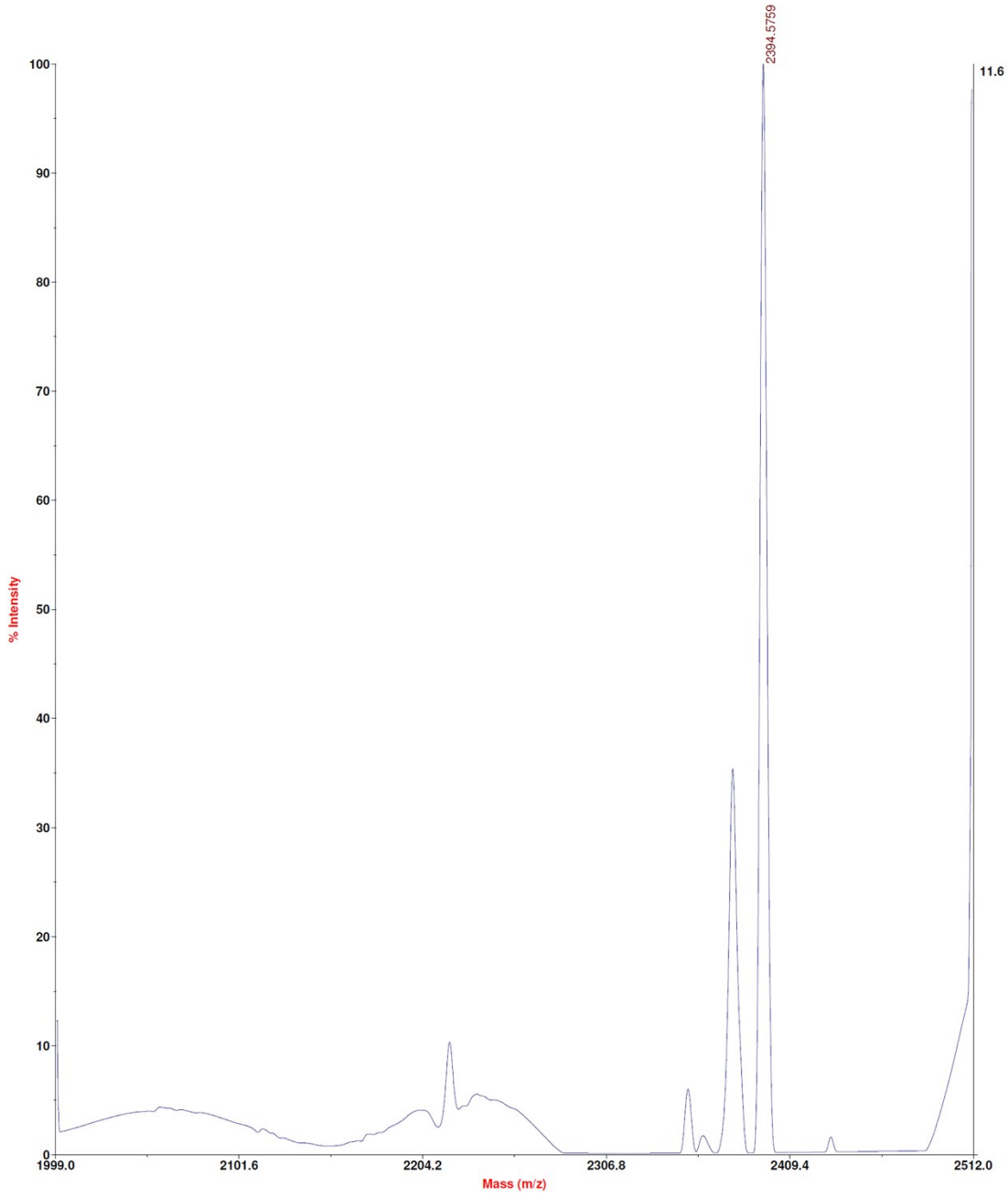
FA-R9-Tet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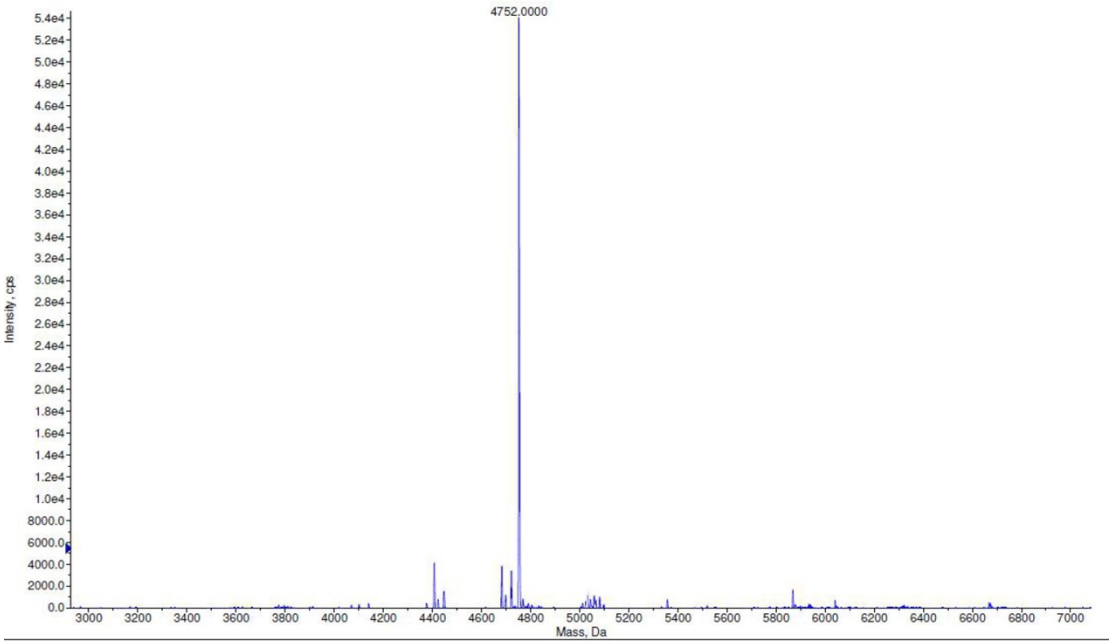
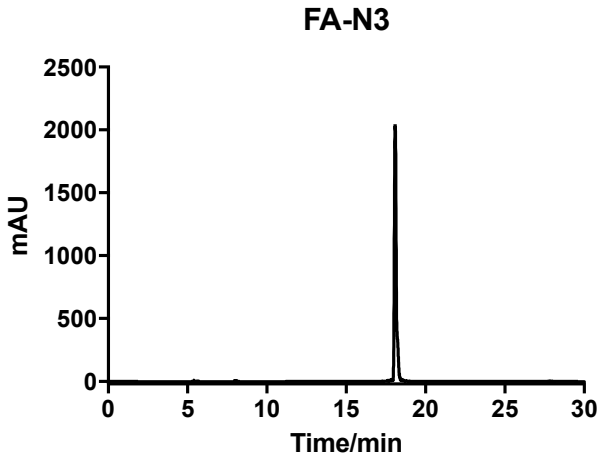
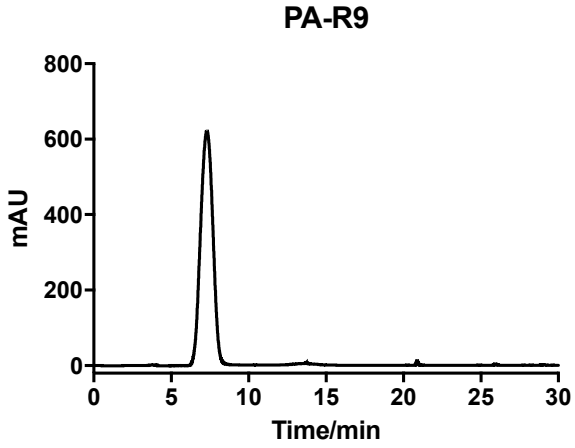
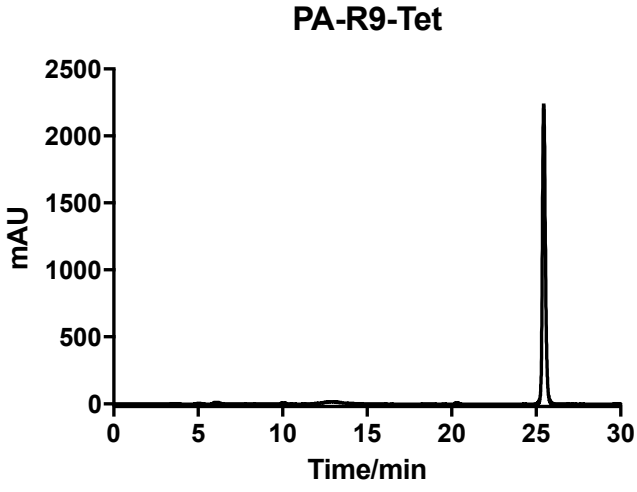
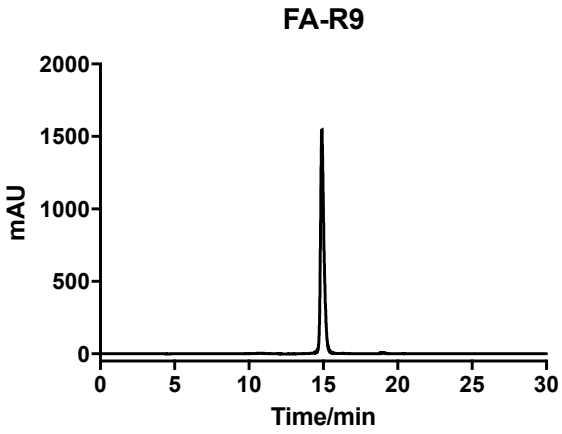


HPLC analysis of peptide and PNA conjugates





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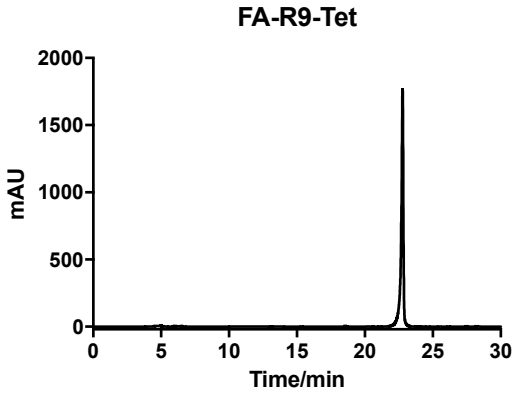
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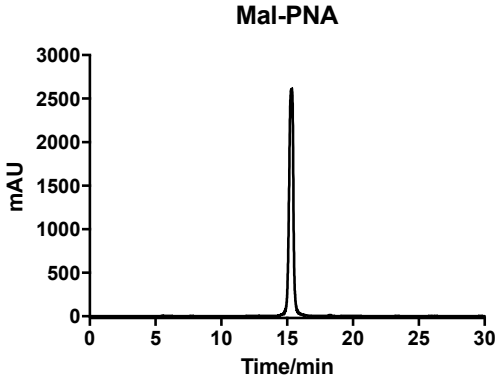
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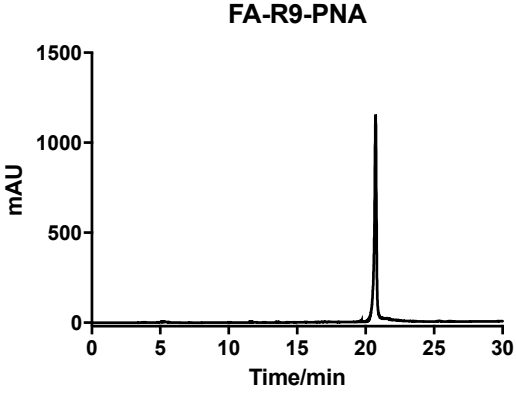
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