Supporting information for

Construction of Tunable Peptide Nucleic Acid Junctions

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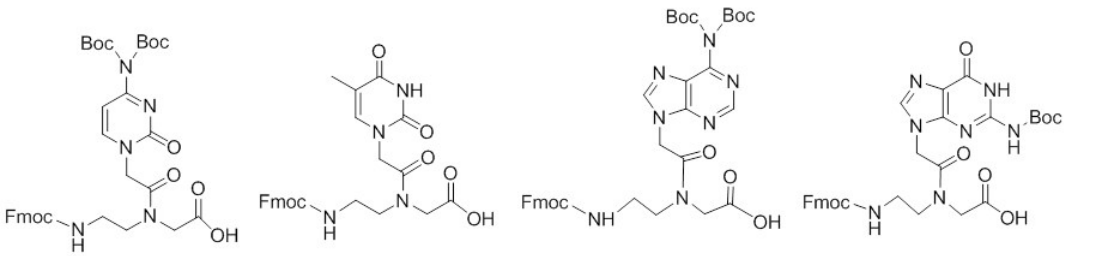
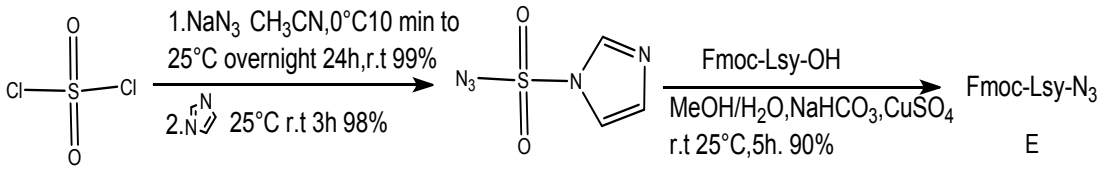
Synthesis of protected PNA monomers and Fmoc-Lys-N_3

Materials. Starting materials, reagents and solvents were purchased from Alfa Aesar and other companies and used without further purification with the exception of the following solvents, which were purified by distillation: THF and CH_3CN. For all reaction oven-dried glasswares were used. ^1H NMR spectra were collected at Bruker AVANCE 400/600 MHz spectrometers at 25 ^oC.

Synthesis of PNA monomers (Fmoc-protected monomers including C, T, A and G; Scheme 1) and Fmoc-Lys-N_3 (Scheme 2) were carried out as previously reported.[1-4]



Scheme S1. PNA monomers (Fmoc-protected monomers).



Scheme S2. Synthesis of Fmoc-Lys-N_3

H[1] NMR spectra of PNA monomers and Fmoc-Lys-N_3

A) Fmoc-protected monomer C:

^{1}H NMR (400 MHz, DMSO; Figure S1, A): 7.99 (m, 1H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.70-7.67 (m, 2H), 7.43-7.26 (m, 5H), 6.81 (m, 1H), 4.86-4.68 (d, 2H), 4.35 (m, 2H), 4.22 (m, 3H), 4.00 (s, 1H), 3.60-3.10 (m, 4H), 1.50 (s, 18H). HRMS (ESI-TOF): Calcd, m/z, 691.2853 found, 714.2735 [M+Na].

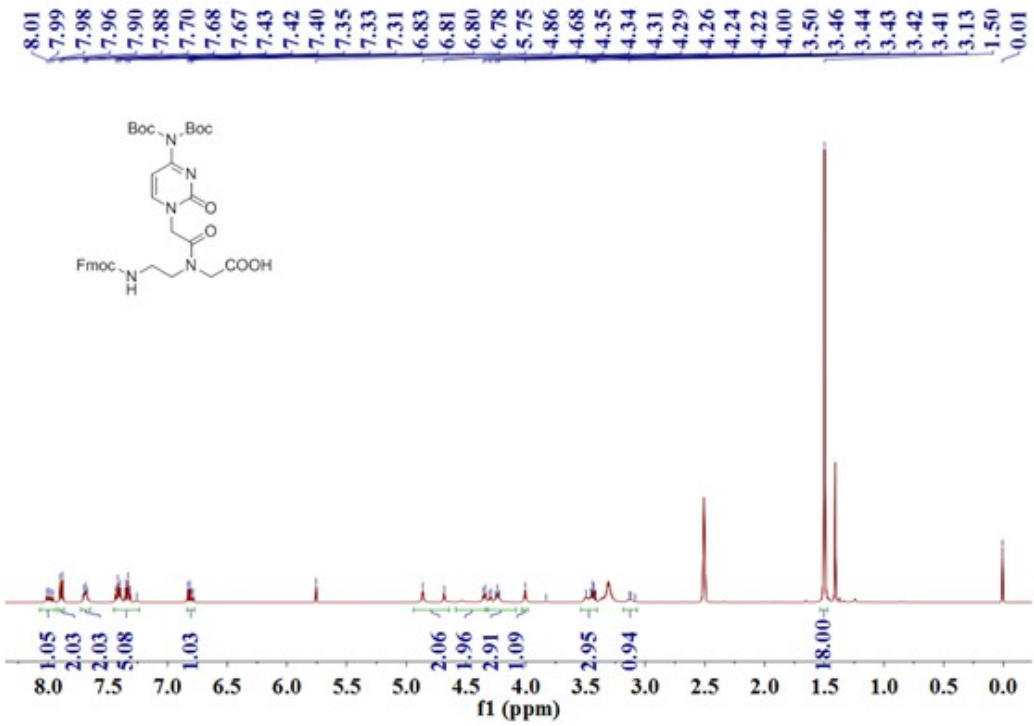


Figure S1, A

B) Fmoc-protected monomer T:

^{1}H NMR (400 MHz, DMSO; Figure S1, B): δ (ppm) 11.28 (d, *J* = 13.8 Hz, 1H), 7.90 (d, *J* = 7.5 Hz, 2H), 7.68 (d, *J* = 7.1 Hz, 2H), 7.32-7.42 (m, 6H), 4.65 (s, 1H), 4.47 (s, 1H), 4.34 (d, *J* = 6.8 Hz, 1H), 4.30-4.20 (m, 2H), 4.14 (s, 1H), 3.98 (s, 1H), 3.42 (m, 1H), 3.10-3.27 (m, 3H), 1.74 (d, *J* = 9.3 Hz, 3H). HRMS (ESI-TOF): Calcd, m/z, 506.1801 found, 529.1665 [M+Na].

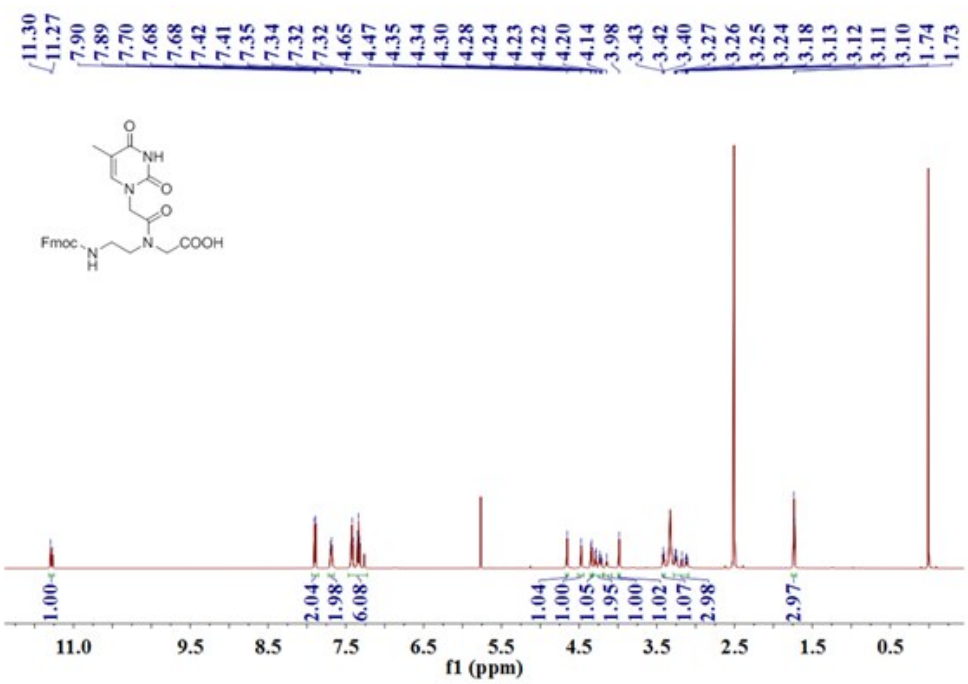


Figure S1, B

C) Fmoc-protected monomer A:

^{1}H NMR (600 MHz, DMSO; Figure S1, C): 8.76 (d, 1H), 8.48 (d, 1H), 7.88 (dd, J = 7.4, 4.4 Hz, 2H), 7.68 (t, J = 7.0 Hz, 2H), 7.53-7.29 (m, 5H), 5.40 (s, 1H), 5.23 (s, 1H), 4.40 (d, J = 6.8 Hz, 1H), 4.27 (m, 3H), 4.05 (s, 1H), 3.57 (m, 1H), 3.36-3.69 (m, 2H), 3.16 (m, 1H), 1.38 (s, 18H). HRMS (ESI-TOF): Calcd, m/z, 715.2966 found, 716.3039 [M+H].

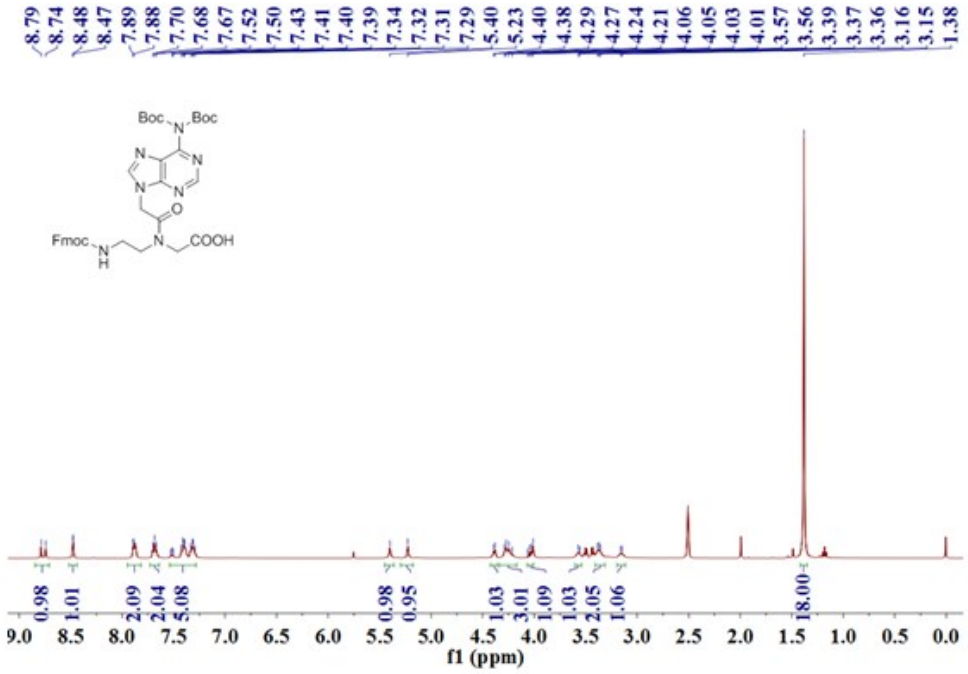


Figure S1, C

D) Fmoc-protected monomer G:

^{1}H NMR (600 MHz, DMSO; Figure S1, D): δ (ppm) 11.37 (d, *J* = 18 Hz, 1H), 11.00-11.17 (d, 1H), 7.88 (dd, *J* = 7.3Hz, 4.8 Hz, 2H), 7.80 (d, 1H) 7.67 (t, *J* = 6.8 Hz, 2H), 7.41-7.31 (m, 5H), 5.08 (s, 1H), 4.91(s, 1H), 4.37 (d, *J* = 6.8 Hz, 1H), 4.29 (d, *J* = 7 Hz, 1 H), 4.23 (m, 2H), 4.01 (s, 1H), 3.50 (t, *J* = 6.7 Hz, 2H), 3.14 (d, *J* = 6.3 Hz, 2H), 1.46 (d, *J* =2.8 Hz, 9H). HRMS (ESI-TOF): Calcd, m/z, 631.2391 found, 654.2278 [M+Na].

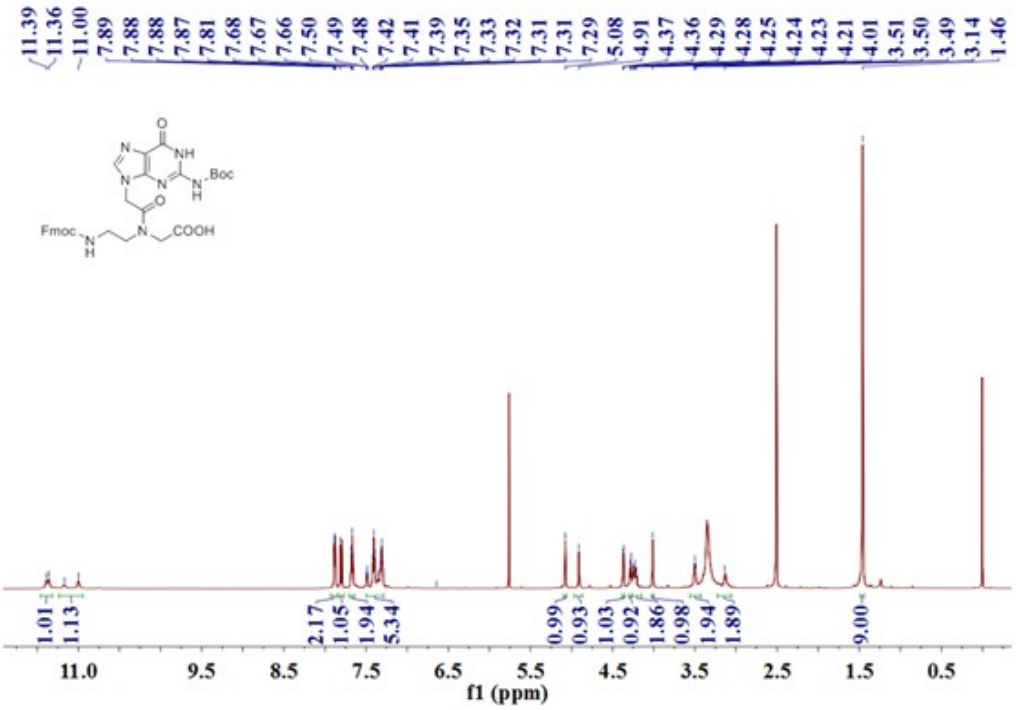


Figure S1, D

E) Fmoc-Lys-N_3:

^{1}H NMR (600 MHz, $CDCl_3$; Figure S1, E) : δ (ppm) 7.80 (d, *J* =8 Hz, 2H), 7.63 (d, J=7.4 Hz, 2H), 7.42 (t, *J* =7.5 Hz, 2H), 7.36 (t, *J* =7.5 Hz, 2H), 5.33 (s, *J* =8, 1H), 4.47-4.30 (m, 3H), 4.26 (t, *J* =7, 1H), 3.32 (t, *J* =6.5 Hz, 2H), 2.01-1.50 (m, 6H).

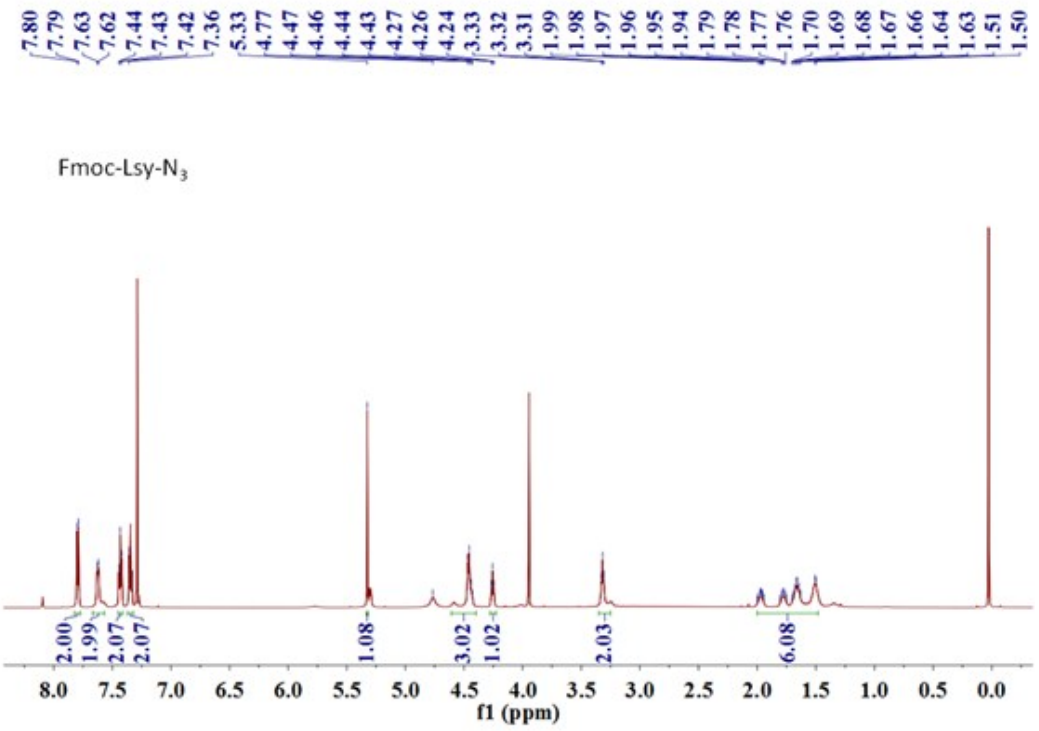


Figure S1, E

Manual synthesis of PNA chains

PNA Oligonucleotides were synthesized using standard solid-phase synthesis protocols.[5,6]

Amino acid coupling on resin (Fmoc-Lys(Boc)-OH, Fmoc-Lys-N_3, 4-Pentynoic acid, Fmoc-Ala-OH)

Weigh 0.1 mmol 0.272 mmol/g Rink-AM Resin (0.368 g) into a reactor, soak the resin in DCM about 2 h to make the polymer swell. Add 20% piperidine into DMF and stay for 15 min. Drain the solvent, then wash the resin five times with DMF. Add 0.1 mmol amino acid, 0.9 ml 0.45 M HOBT and TBTU in DMF, 0.14 ml DIEA onto resin, agitate the resin for 2 h. Drain the solvent, then wash the resin five times with DMF. Use AC_2O and DIEA in DMF to block residual unreacted groups for 30 min, then drain the solvent, and then wash the resin five times with DMF.

PNA coupling on the resin

Removal of the Fmoc from the PNA monomers is achieved by treatment with 20% piperidine in DMF for 30 s, then filter and wash with DMF five times. PNA monomer is prepared for coupling as a 0.2 M solutions in N-methylpyrrlidone. Using HATU together with a base mixture of 0.2 M DIEA and 0.3 M lutidine to activate the

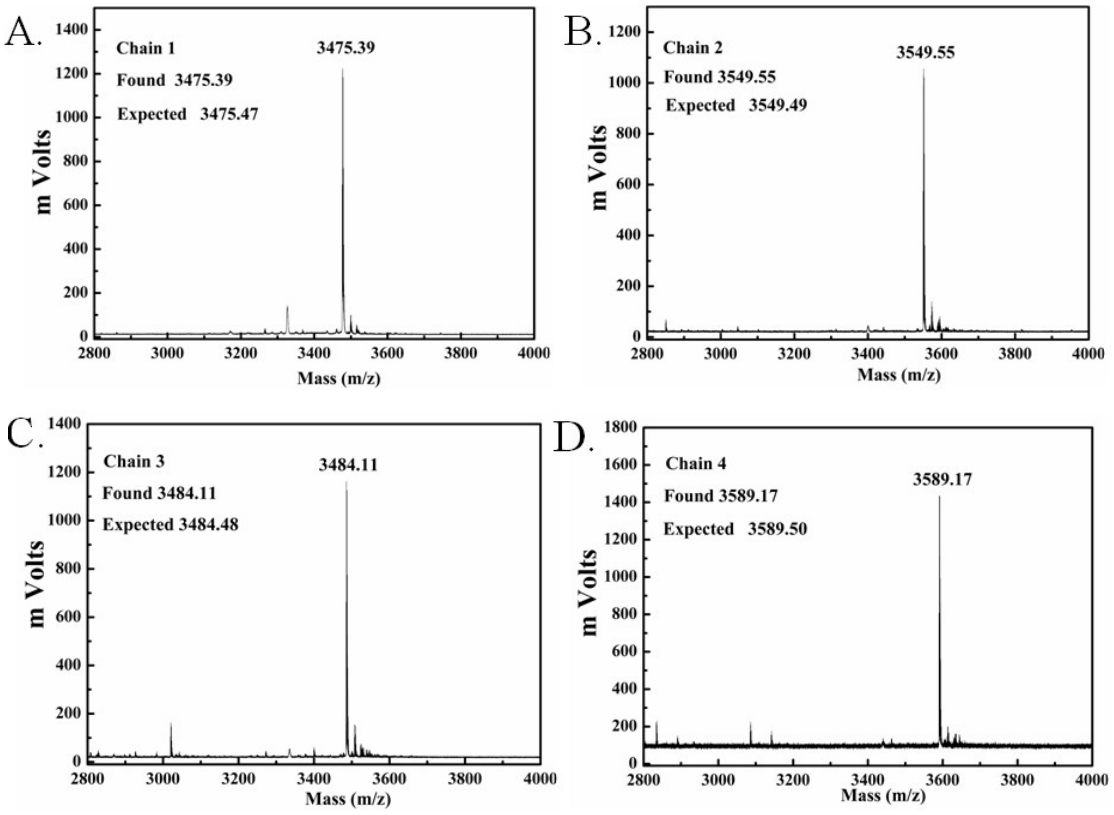
monomer for 2.5 min, Kaiser reagent test is carried out after coupling for 2 h. Coupling the monomer one more time if the test is positive. If the test is negative, the resin is filtered and washed with DMF five times. Capping residual unreacted products by adding AC_2O and DIEA in DMF solution to the resin and agitating for 30 min, then the resin is filtered and washed with DMF five times.

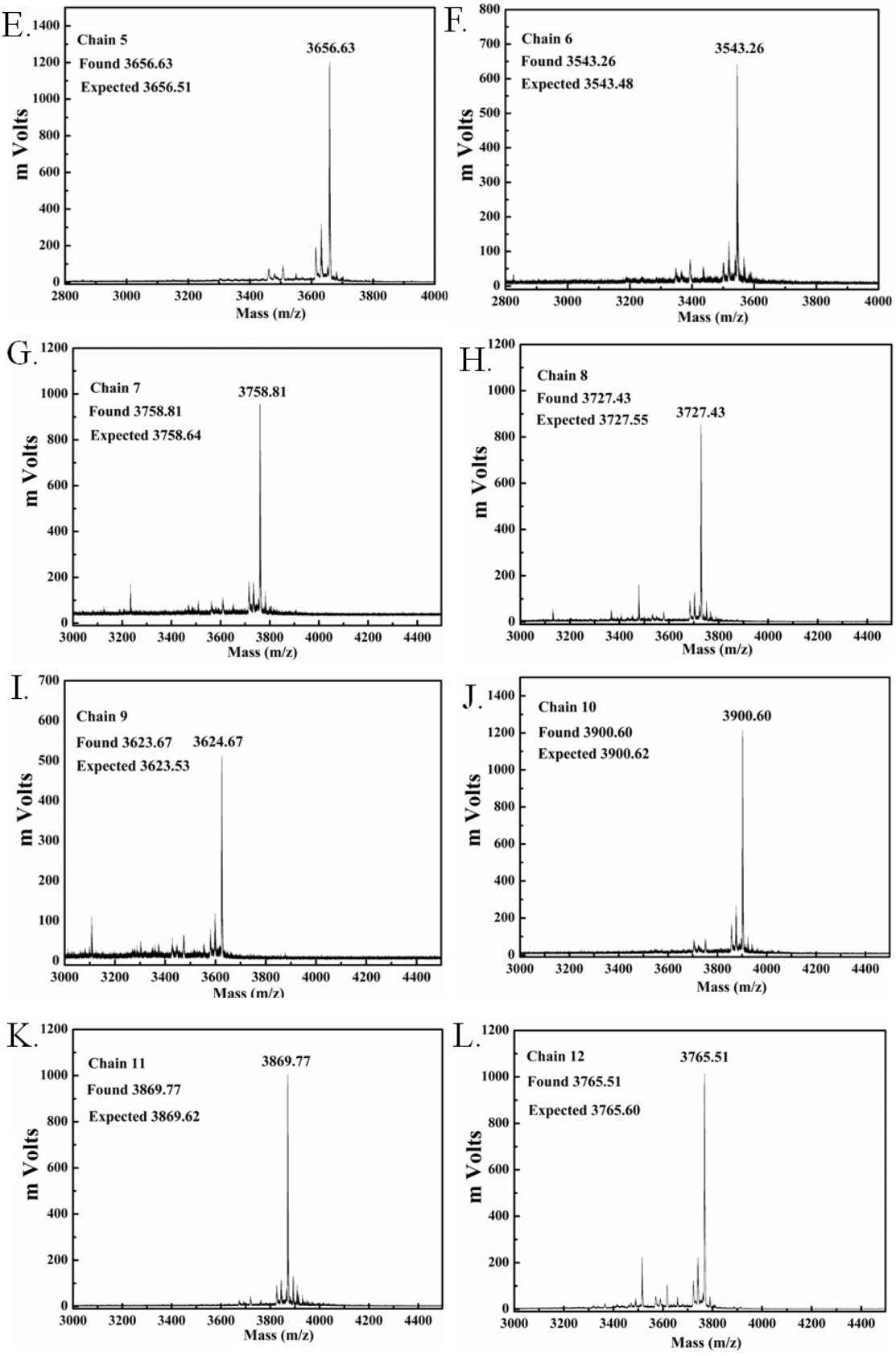
Cleavage of PNA Oligonucleotides from resin.

All PNA products were cleaved from the resin by agitating for 2.5 h in trifluoroacetic acid/1,2-ethanedithiol/thioanisole/phenol/triisopropylsilane/water in the ratios of 90/2.5/2.5/2.5/0.5/2. Products were precipitated in cold ether and lyophilized.

Purification

All PNA Oligonucleotides were purified using reversed-phase high performance liquid chromatography (C_{18} column/50 ^{o}C Shanghai Wufeng) using a H_2O-MeCN (with 0.01%TFA) linear gradient from 100-0% to 65-35% to 5-95%, PNAs were analyzed by MALDI-TOF (AB SCIEX) mass spectrometry using CHCA as the matrix (Figure S2, A-Q). PNA Oligonucleotides used in this study are revealed in table 1. Extinction coefficients for PNA monomers were obtained from NMR spectroscopy (262 nm): A 18409.2; C 3490.2; G 19181.1; T 8734.1. PNA concentrations were determined by UV (UV-6 MAPADA).




E.
Chain 5
Found 3656.63
Expected 3656.51
3656.63
m Volts
Mass (m/z)
F.
Chain 6
Found 3543.26
Expected 3543.48
3543.26
m Volts
Mass (m/z)
G.
Chain 7
Found 3758.81
Expected 3758.64
3758.81
m Volts
Mass (m/z)
H.
Chain 8
Found 3727.43
Expected 3727.55
3727.43
m Volts
Mass (m/z)
I.
Chain 9
Found 3623.67
3624.67
Expected 3623.53
m Volts
Mass (m/z)
J.
Chain 10
Found 3900.60
Expected 3900.62
3900.60
m Volts
Mass (m/z)
K.
Chain 11
Found 3869.77
Expected 3869.62
3869.77
m Volts
Mass (m/z)
L.
Chain 12
Found 3765.51
Expected 3765.60
3765.51
m Volts
Mass (m/z)

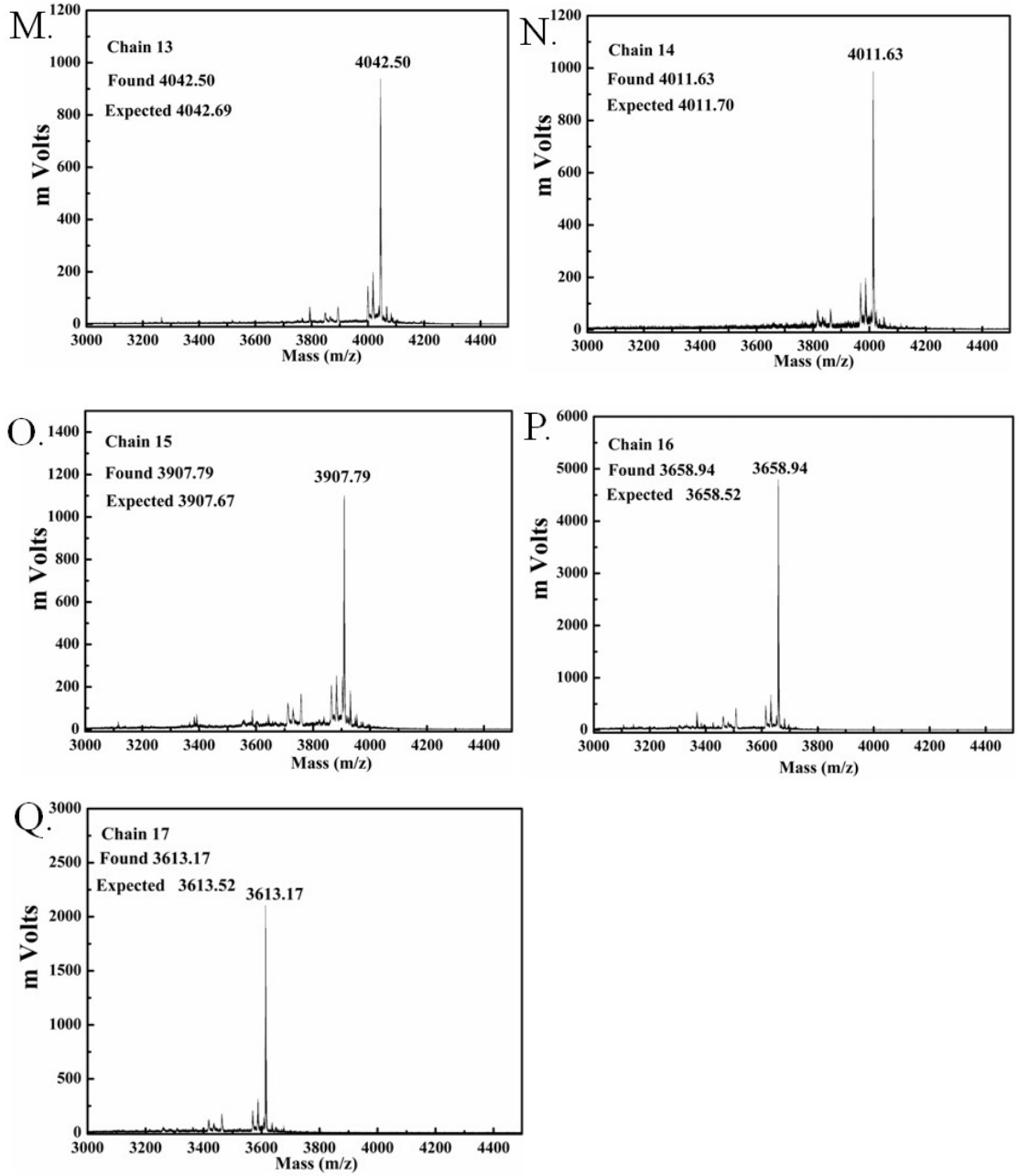


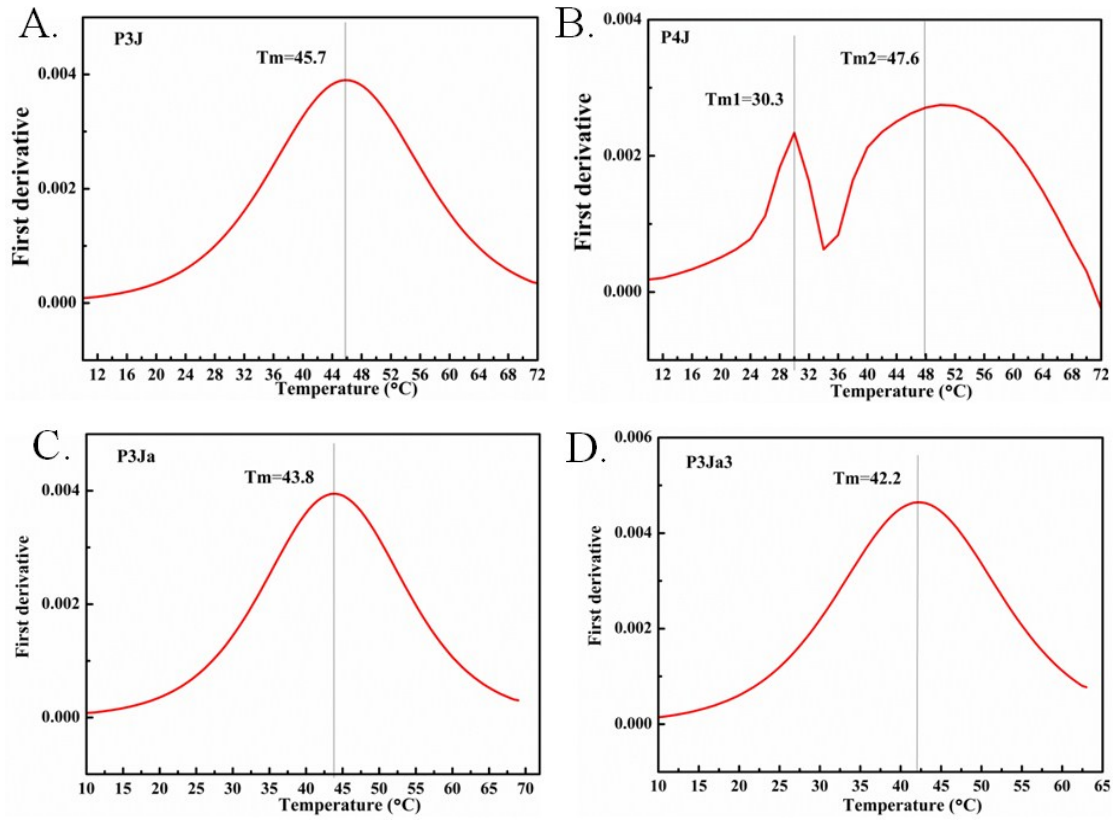
Figure S2. (A-Q) MALDI-TOF mass spectra of 17 single chains

Hybridization, Purification and UV melting of PNA nanostructures

Mixtures for different sets of single chains, that is, chains 1-4; chains 4-6; chains 7-9; chains 10-12; chains 13-15; chains 5 & 6; chains 7 & 8; chains 8 & 9; chains 7 & 9; chains 7, 16 and 17 (the molar ratio of each single chain to the other in any mixture of single chains is 1:1) were prepared in DD water and diluted to 400 μl (6.5 μM). Sample were first mixed at room temperature then heated to 90 ^{o}C for 30 min, and then slowly cooled to 10 ^{o}C at a cooling speed of 0.3 ^{o}C/min, the mixtures were then maintained at 10 ^{o}C for 30 min . (Long Gene L96+)

PNA nanostructures were purified by FPLC (GE. Healthcare) on an cation exchange column (Hitrap^{TM} CM, CMFF), buffer A, 50 mM phosphate, PH=7; buffer B, 50 mM phosphate, 2 M guanidine solution, PH=7. A linear gradient from 100% A to 50% A - 50% B in 50 min was used.

UV measurements were performed on a Perkin Elmer Lambda UV-VIS Spectrometer equipped with a thermoelectrically controlled multicell holder. Samples (purity > 95%) were annealed in pure water prior to measurements, the measurements were repeated at least 3 times. The data from melting experiments were fitted to a standard sigmoidal curve by origin 8, resulting varying Tm values for different samples. The first derivatives from the fitted curves are shown in Figures S3A-G, Tm values are revealed in the derivative curves in a more direct way[7,8].



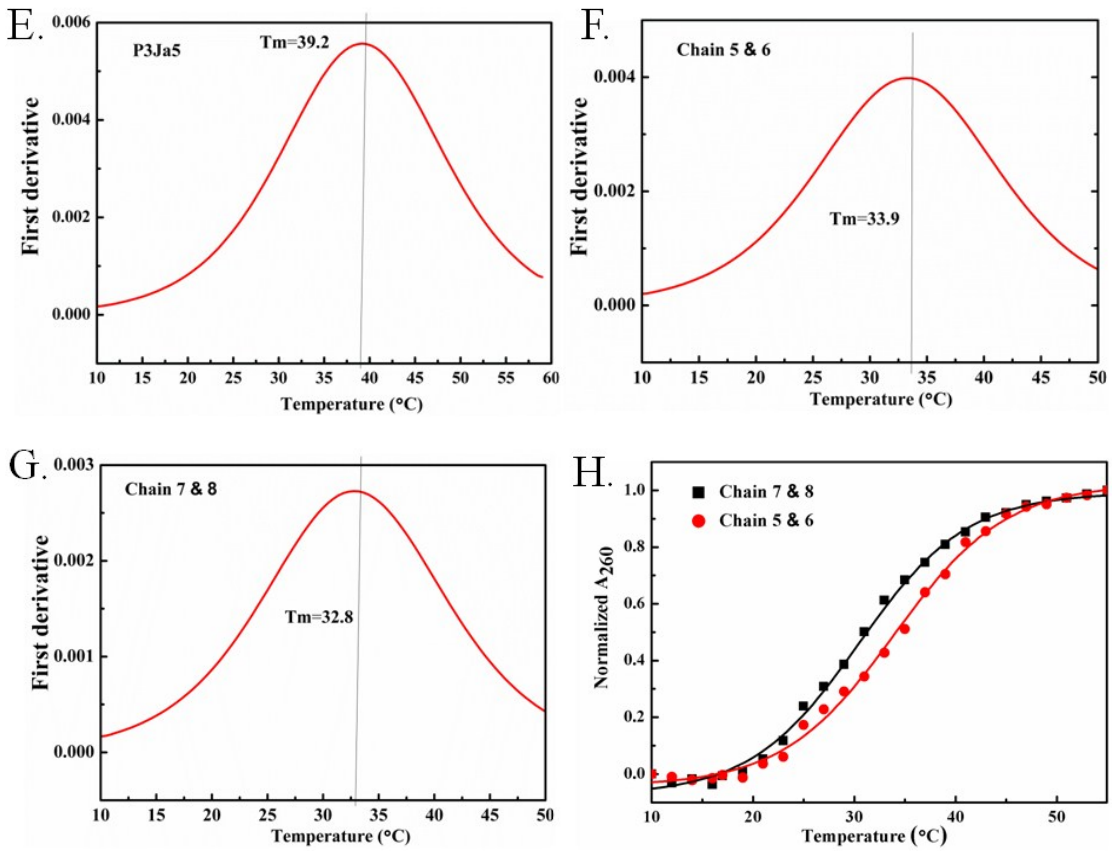


Figure S3. A-G First derivative curves from the fitting curves. A) P3J, B) P4J, C) P3Ja, D) P3Ja3, E) P3Ja5, F) chain 5 & 6, G) chain 7 & 8 H) UV-melting curves for chain 5 & 6, chain 7 & 8.

Control UV-melting curves for two single chains out of three that are required for the assembly of a 3-way junction nanostructure.

Chain 5 & 6, chain 7 & 8 were annealed in pure water. The test conditions of UV-melting curves are the same as described previously. the Tm of chain 5 & 6 and chain 7 & 8 were found to be 33.9±0.2 ºC and 32.8±0.4 ºC (Figures S3F-H).

MS spectra for the major peaks from the FPLC profiles of PNA P4J and the control single chain 1:

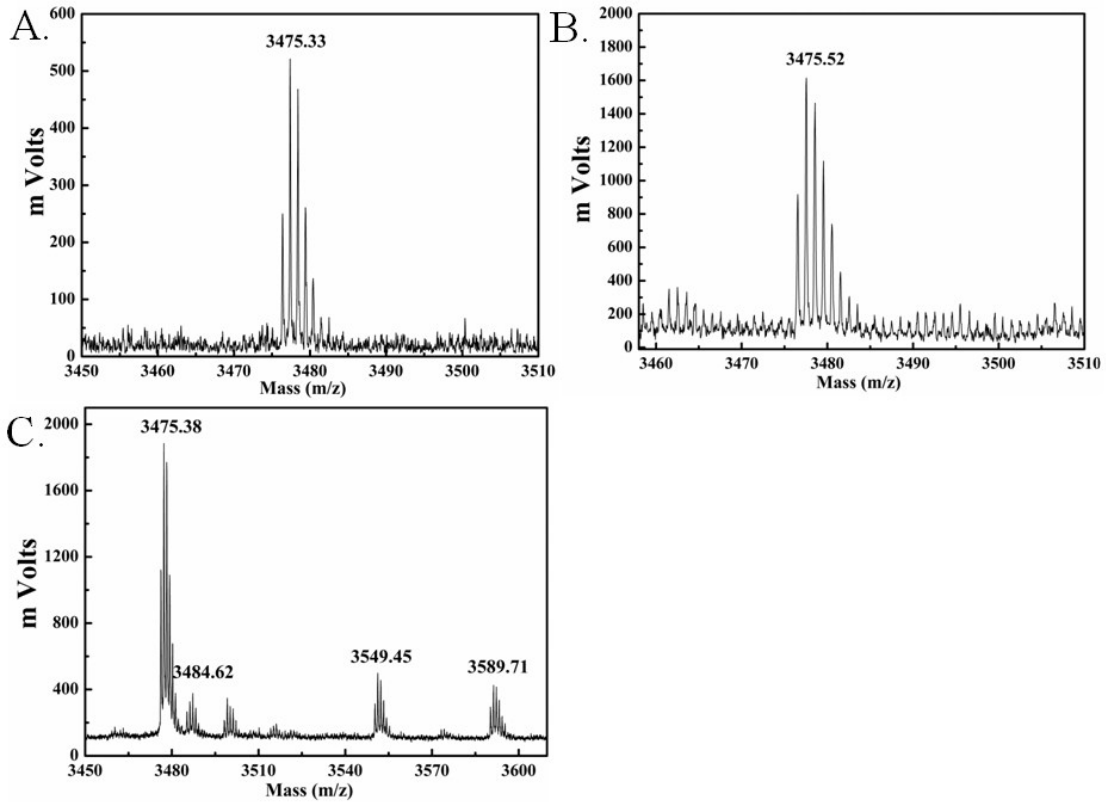
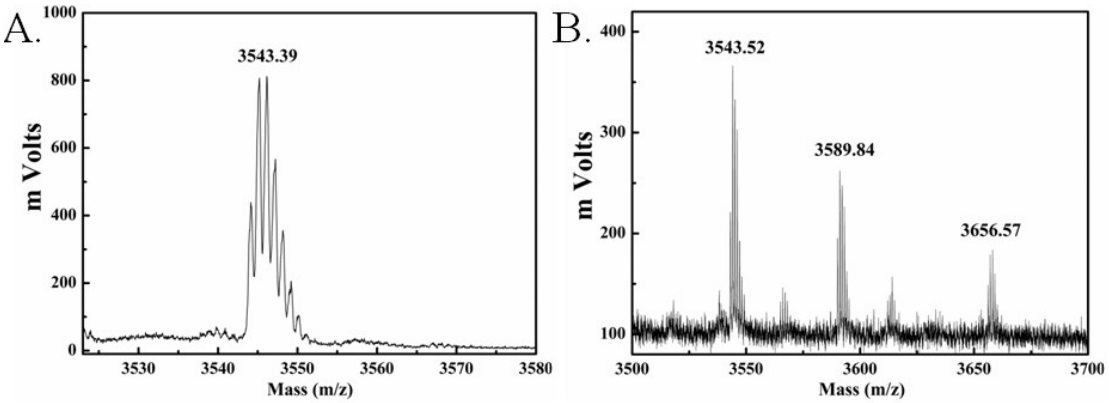


Figure S4. A) MALDI-TOF mass spectrum of chain 1, m/z: Expected 3475.47, Found 3475.33. B) MALDI-TOF mass spectrum for peak 1 of P4J, m/z: chain 1 Expected 3475.47, Found 3475.52 . C) MALDI-TOF mass spectrum for peak 2 of P4J, m/z: chain 1 Expected 3475.47, Found 3475.38 ; chain 2 Expected 3549.49, Found 3549.45; chain 3 Expected 3484.48, Found 3484.62; chain 4 Expected 3589.50, Found 3589.71.

MS spectra for the major peak from the FPLC profiles of PNA P3J, the control single chain 6, chain 5 & 6:



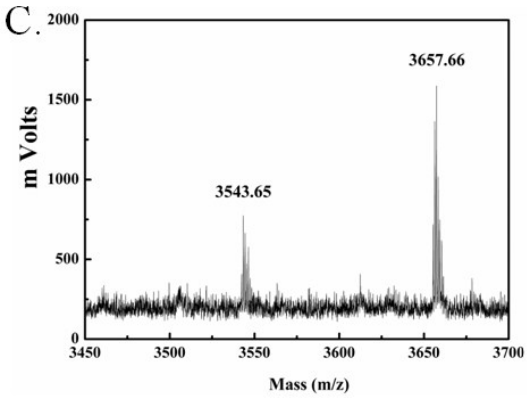
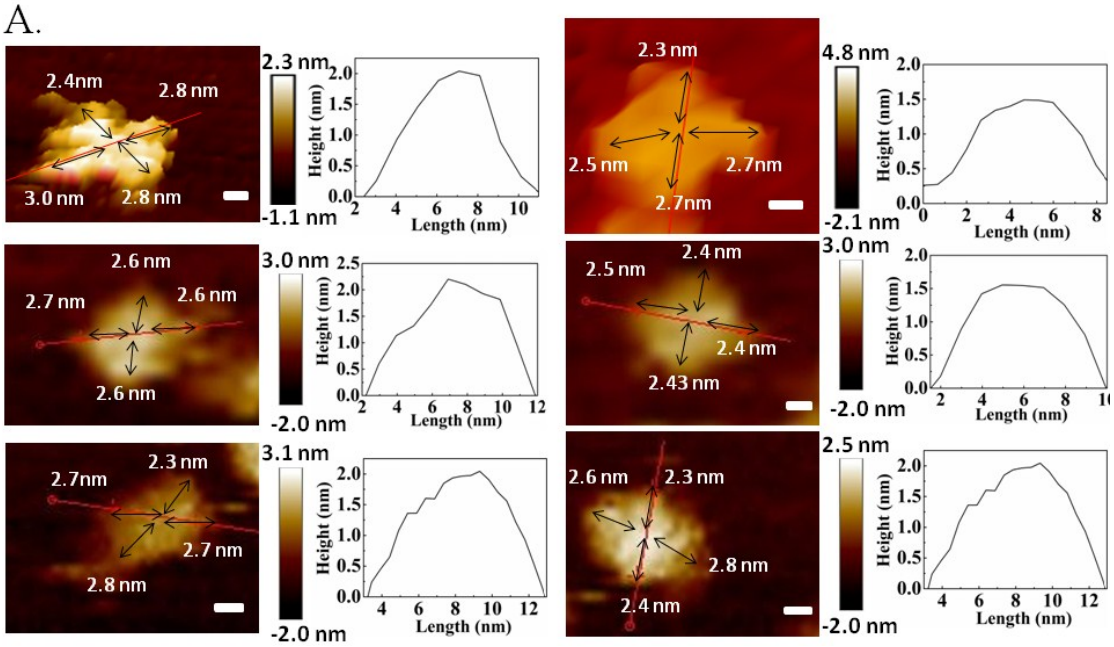


Figure S5. A) MALDI-TOF mass spectrum of chain 6, m/z: Expected 3543.48, Found 3543.39 . B) MALDI-TOF mass spectrum for the major peak of P3J, m/z: chain 4 Expected 3589.80, Found 3589.84; chain 5 Expected 3656.51, Found 3656.57; chain 6 Expected 3543.48, Found 3543.52. C) MALDI-TOF mass spectrum for the major peak of chain 5 & 6, m/z: chain 5 Expected 3656.51, Found 3657.66 ; chain 6 Expected 3543.48, Found 3543.65.

AFM Imaging

Imaging was performed with a Bruker Dimension FastScan Bio AFM equipped with the ScanAsyst mode. The sample solution was deposited onto freshly cleaved mica surface, and left for 5 min at room temperature to allow adsorption of the PNA structures. After addition of 70 μL of water, the sample was scanned with the scan rates between 1 and 3 Hz. Several AFM images were acquired at different areas of the mica surface to ensure the reproducibility of the results. All images were analyzed with NanoScope Analysis 1.50 and Gwyddion 2.38 software. The arm length and pore size are all measured according to the cross section of height profile. The arm length was measured from the first point with 30% of the biggest height to the highest point. The pore size was measured from the highest point to lowest point in the middle.

A.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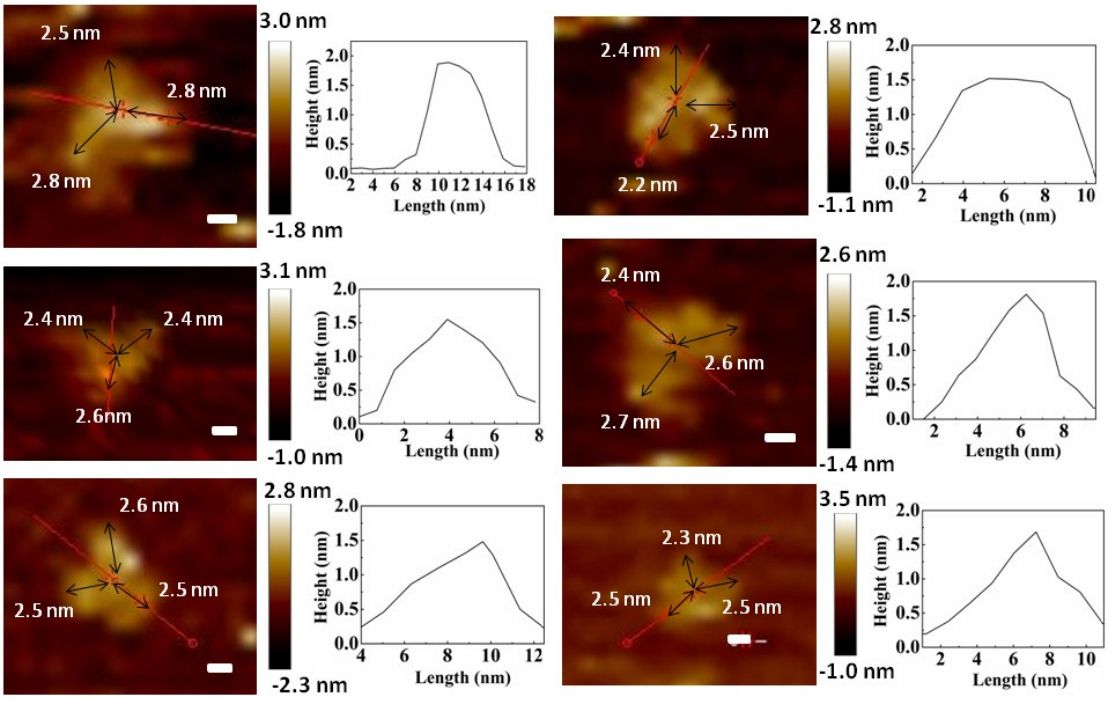
B.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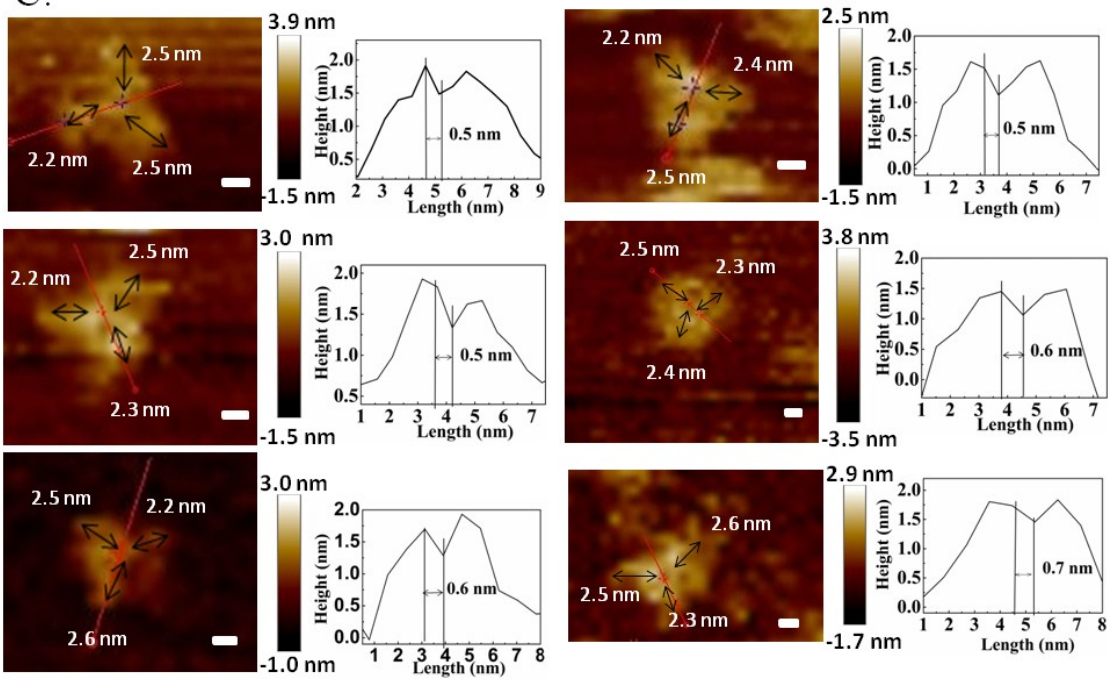
C.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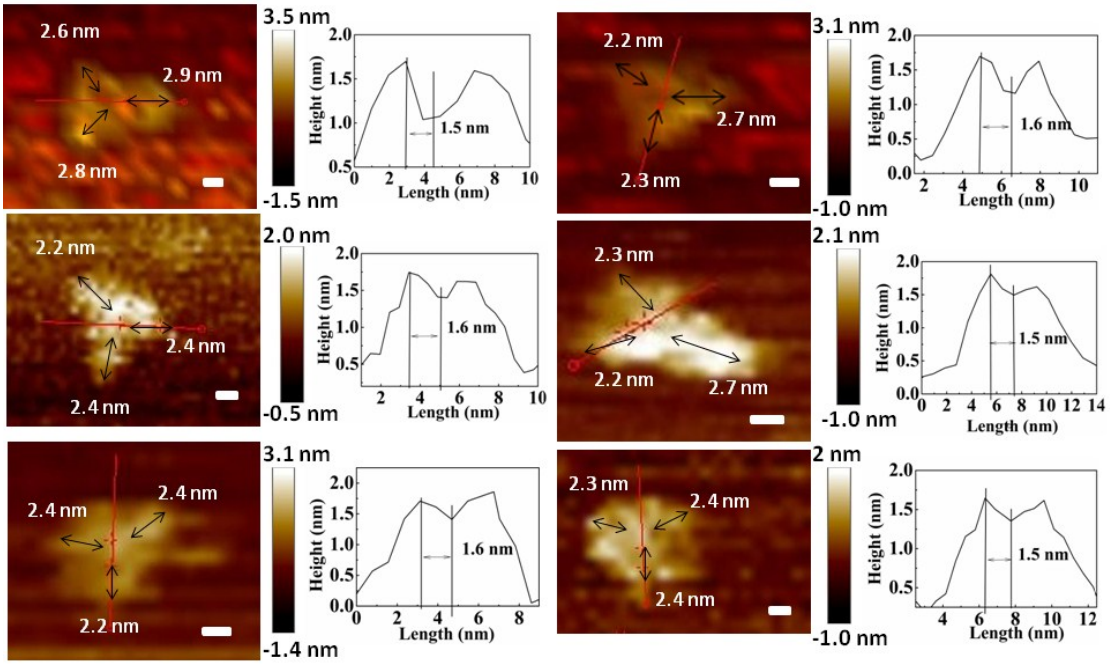
D.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E.

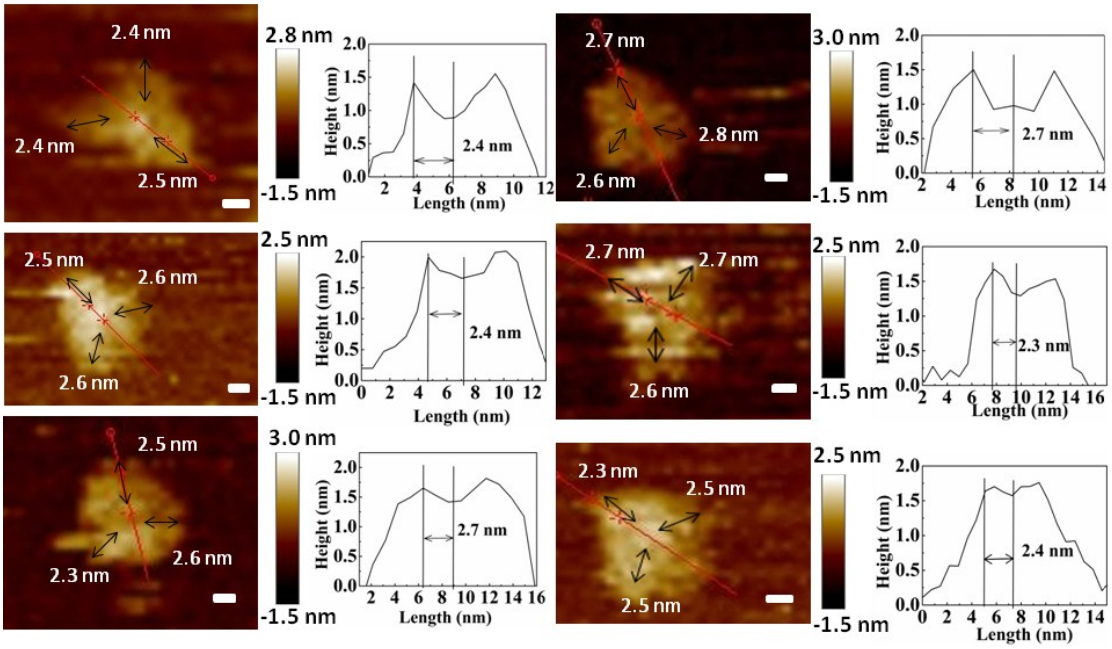


Figure S6 AFM images of the PNA and PNA-peptide nanostructures. A) P4J, B) P3J , C) P3Ja1, D) P3Ja3 and E) P3Ja5. Images are shown with a scale bar at 2 nm. The height cross section corresponding to the red line.

Click Reactions

Click Reactions from P3Ja1, P3Ja3 and P3Ja5. The PNA-Peptide nanostructures (3.25 nmol, 500 μl) were assembled through annealing before the click reactions; sodium ascorbate (0.325 μmol) and $CuSO_4 \cdot 5H_2O$ (0.0325 μmol) were sequentially added under argon gas. The reaction mixtures were incubated at room temperature under argon gas overnight . PNA-Peptide single chains and the click reaction products were purified by C18 column; the column was eluted with A (H_2O)-B (MeCN) with 0.01% TFA, a linear gradient was used: 0-3 min, 0-5% B; 3-7 min, 5-8% B; 7-45 min, 8-22% B; 45-50 min, 22-95% B; 50-55 min, 95-5% B; 55-60 min, 95-5% B (see Figures S7, S8A, S9A and S10A for corresponding HPLC profiles). The final click reaction products were characterized by MALDI-TOF mass spectrometry (see Figures S8B-O, S9B-O, S10B-N and Tables S1-3).

Control click reaction between two single chains out of three that are required for the assembly of a 3-way junction nanostructure.

Chain 7 and chain 8:

Chain 7 and chain 8 were mixed in 1:1 molar ratio and were annealed in pure water before the click reaction. Sodium ascorbate (0.325 μmol) and $CuSO_4 \cdot 5H_2O$ (0.0325 μmol) were sequentially added under argon gas. The reaction mixture was incubated at room temperature under argon gas overnight. The click reaction products were

purified by C18 column and eluted using A (H_2O)-B (MeCN) with 0.01% TFA in a linear gradient: 0-3 min, 0-5% B in A; 3-7 min, 5-8% B in A; 7-45 min, 8-22% B in A; 45-50 min, 22-95% B in A; 50-55 min, 95-5% B in A; 55-60 min, 95-5% B in A (Figure S11A). The final click reaction products were characterized by MALDI-TOF mass spectrometry (Figures S11B-H and Table S4).

Chain 7 and chain 9:
Chain 7 and chain 9 were mixed in 1:1 molar ratio and were annealed in pure water before the click reaction. Sodium ascorbate (0.325 μmol) and $CuSO_4 \bullet 5H_2O$ (0.0325 μmol) were sequentially added under argon gas. The reaction mixture was incubated at room temperature under argon gas overnight. The click reaction products were purified by C18 column and eluted using A (H_2O)-B (MeCN) with 0.01% TFA in a linear gradient: 0-3 min, 0-5% B in A; 3-7 min, 5-8% B in A; 7-45 min, 8-22% B in A; 45-50 min, 22-95% B in A; 50-55 min, 95-5% B in A; 55-60 min, 95-5% B in A (Figure S12A). The final click reaction products were characterized by MALDI-TOF mass spectrometry (Figures S12B-H and Table S5).

Chain 8 and chain 9:
Chain 8 and chain 9 were mixed in 1:1 molar ratio and were annealed in pure water before the click reaction. Sodium ascorbate (0.325 μmol) and $CuSO_4 \bullet 5H_2O$ (0.0325 μmol) were sequentially added under argon gas. The reaction mixture was incubated at room temperature under argon gas overnight. The click reaction products were purified by C18 column and eluted using A (H_2O)-B (MeCN) with 0.01% TFA in a linear gradient: 0-3 min, 0-5% B in A; 3-7 min, 5-8% B in A; 7-45 min, 8-22% B in A; 45-50 min, 22-95% B in A; 50-55 min, 95-5% B in A; 55-60 min, 95-5% B in A (Figure S13A). The final click reaction products were characterized by MALDI-TOF mass spectrometry (Figures S13B-H and Table S6).

Control Click Reactions among chain 7 and two random chains 16 and 17.
Sequences of two random chains are Z-CCTTTGaCGATTTk-k(N_3) and Z-TTCCCGaTGACCCk- k(N_3) (PNA sequences are written from N-terminus to C-terminus), named chain 16 and chain 17, respectively. Chain 7, chain 16 and chain 17 were mixed in 1:1:1 molar ratio, 3.25 nmol, they were dissolved in 500 μl pure water. Sodium ascorbate (0.325 μmol) and $CuSO_4 \bullet 5H_2O$ (0.0325 μmol) were added sequentially under argon gas. The reaction mixture was incubated at room temperature under argon gas overnight. The click reaction products were purified by C18 column and eluted using A (H_2O)-B (MeCN) with 0.01% TFA in a linear gradient: 0-3 min, 0-5% B in A; 3-7 min, 5-8% B in A; 7-45 min, 8-22% B in A; 45-

50 min, 22-95% B in A; 50-55 min, 95-5% B in A; 55-60 min, 95-5% B in A (Figure S14A). The final click reaction products were characterized by MALDI-TOF mass spectrometry (Figures S14B-J and Table S7).

HPLC retention times of different single PNA-Peptide chains.

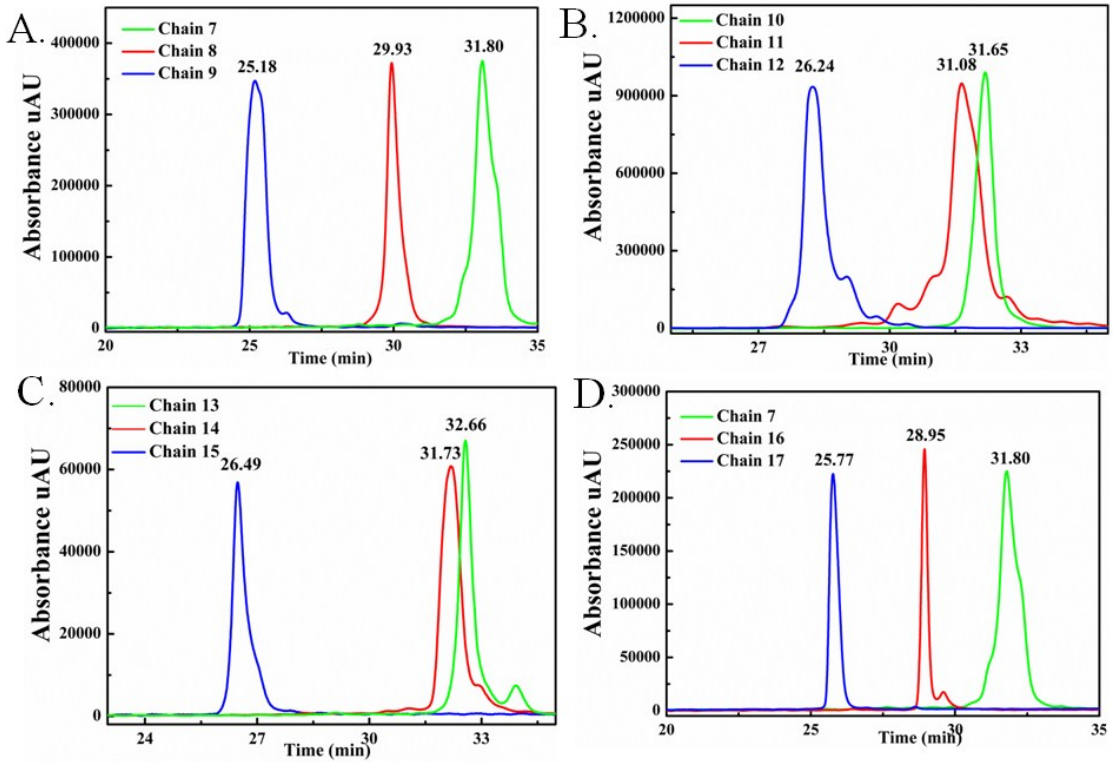
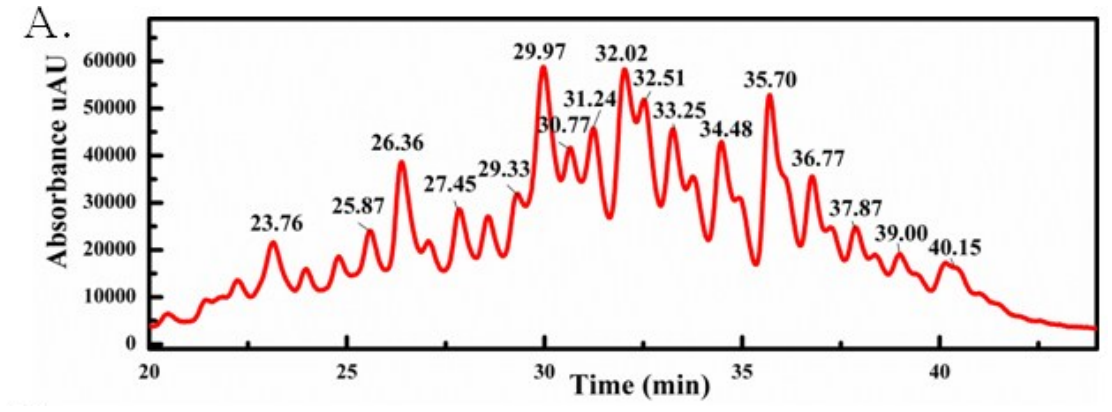
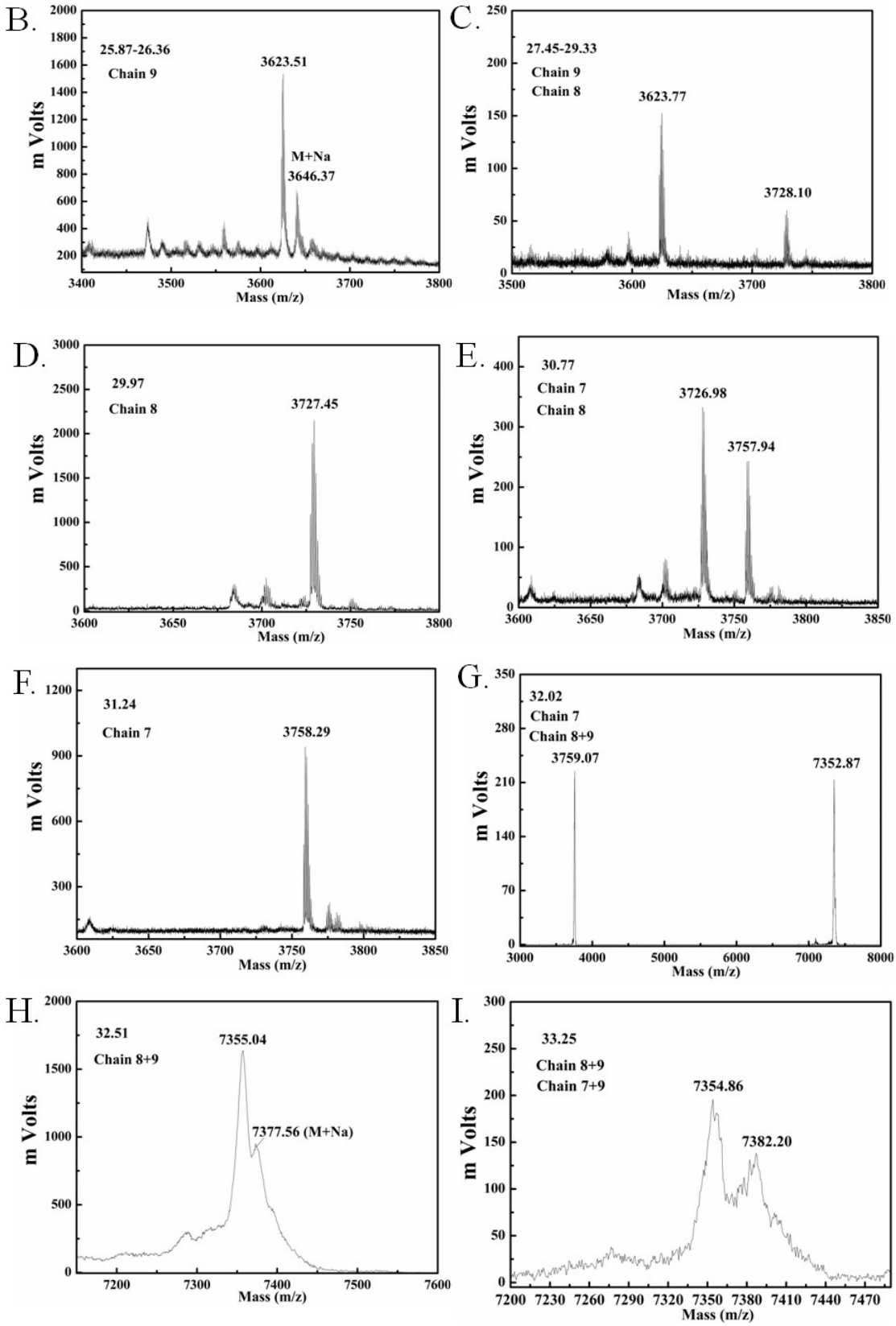


Figure S7. A) HPLC retention times for chains 7-9, B) HPLC retention times for chains 10-12, C) HPLC retention times for chains 13-15, D) HPLC retention times for chains 7, 16-17.

HPLC profiles of click reaction products from P3Ja1 and the product analysis/assignments based on MALDI-TOF mass:




B.
25.87-26.36
Chain 9
3623.51
M+Na
3646.37
m Volts
Mass (m/z)
C.
27.45-29.33
Chain 9
Chain 8
3623.77
3728.10
m Volts
Mass (m/z)
D.
29.97
Chain 8
3727.45
m Volts
Mass (m/z)
E.
30.77
Chain 7
Chain 8
3726.98
3757.94
m Volts
Mass (m/z)
F.
31.24
Chain 7
3758.29
m Volts
Mass (m/z)
G.
32.02
Chain 7
Chain 8+9
3759.07
7352.87
m Volts
Mass (m/z)
H.
32.51
Chain 8+9
7355.04
7377.56 (M+Na)
m Volts
Mass (m/z)
I.
33.25
Chain 8+9
Chain 7+9
7354.86
7382.20
m Volts
Mass (m/z)

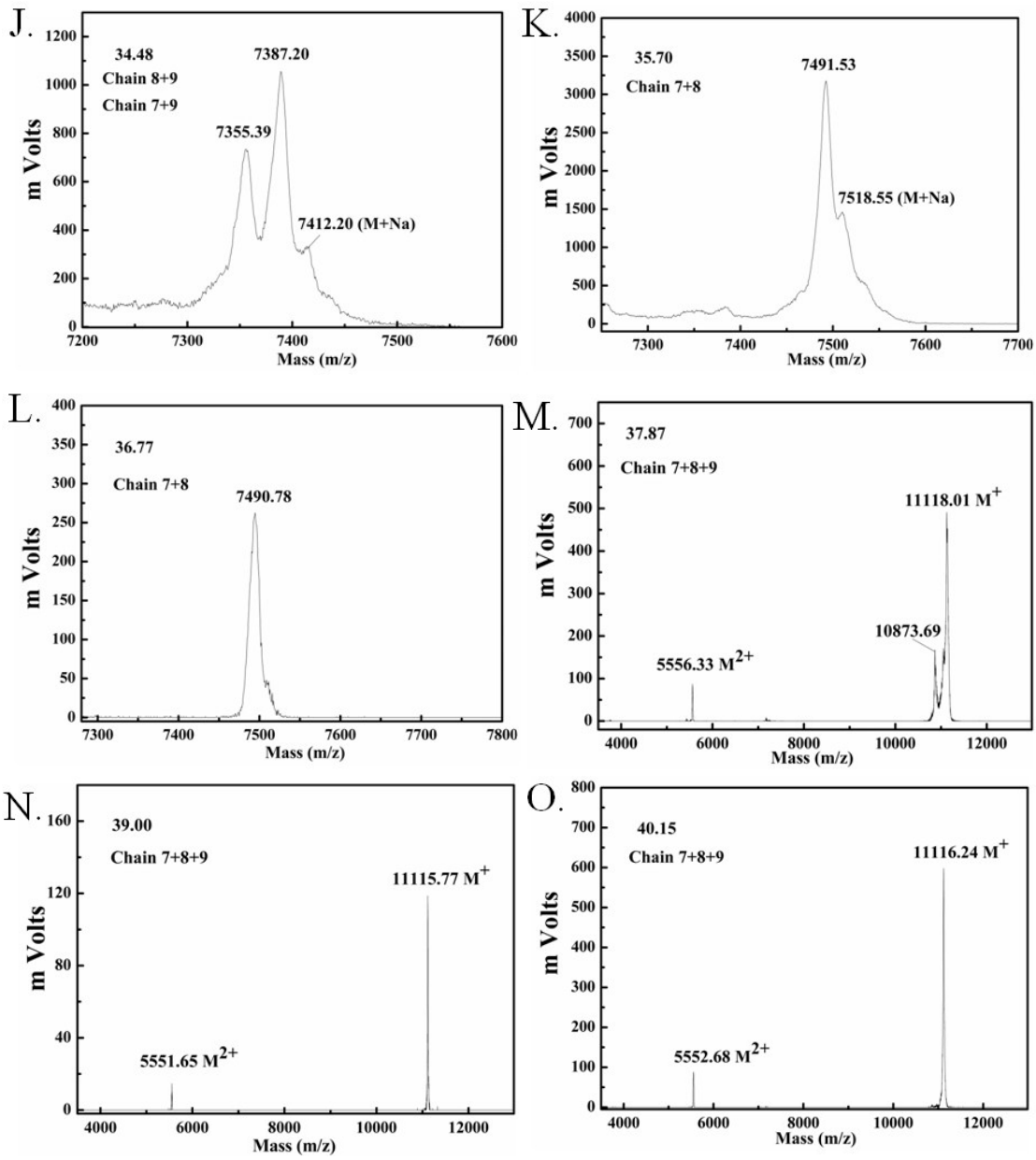


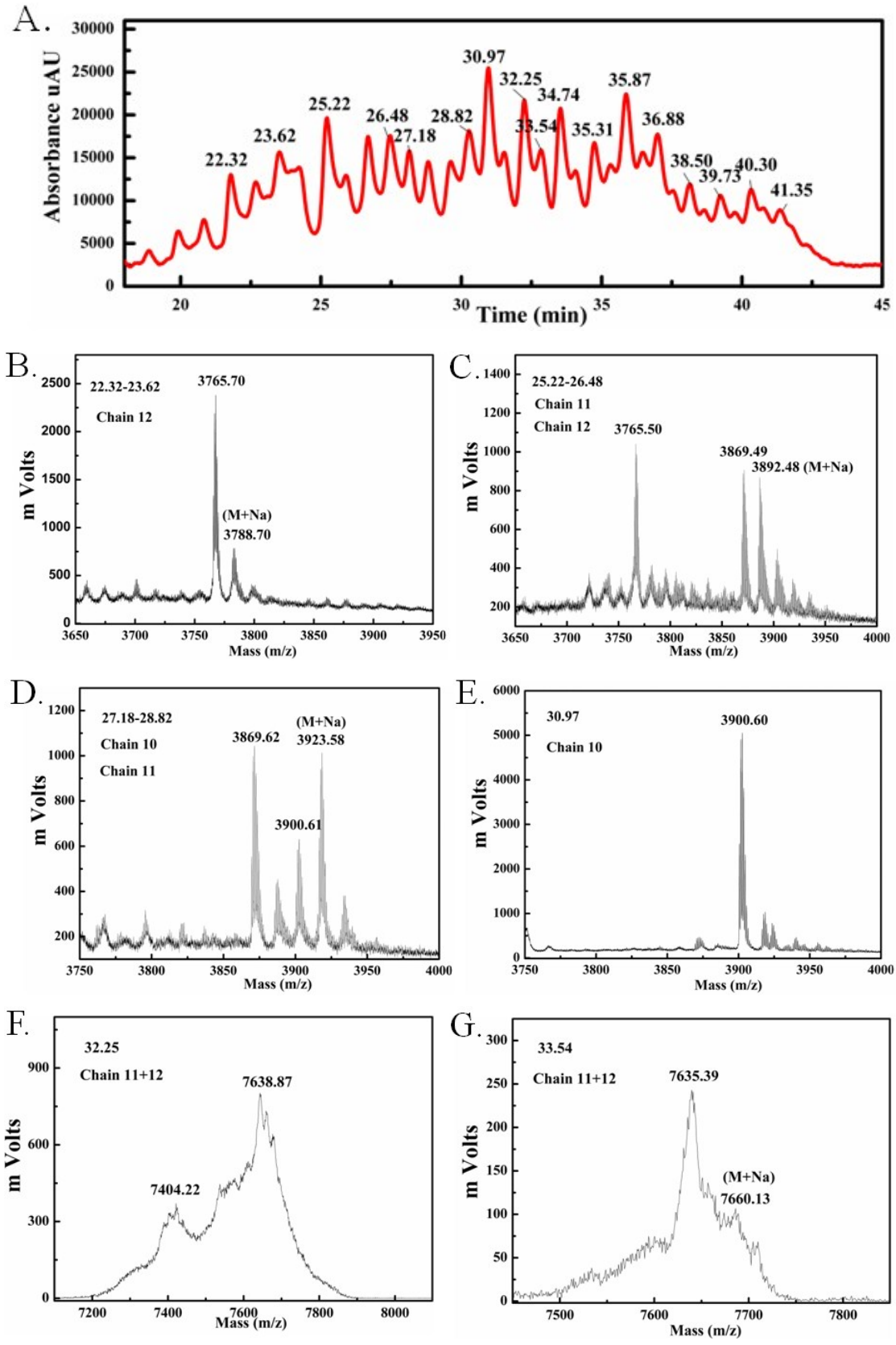
Figure S8. A) HPLC profiles of the click reaction products from P3Ja1, B-O) MALDI-TOF mass analysis of products from P3Ja1 click reaction.

Table S1. MALDI-TOF mass results from P3Ja1 click reaction

peak	Found (m/z)	Name
B) 25.87-26.36	3623.51, 3646.37 (M+Na)	chain 9
C) 27.45-29.33	3623.77; 3728.10	chain 9, chain 8
D) 29.97	3727.45	chain 8
E) 30.77	3726.98; 3757.94	chain 7, chain 8
F) 31.24	3758.29	chain 7
G) 32.02	3759.07; 7352.87	chain 7, chain 8+ chain 9
H) 32.51	7355.04, 7377.56 (M+Na)	chain 8+ chain 9
I) 33.25	7354.86; 7382.20	chain 8+ chain 9, chain 7+ chain 9
J) 34.84	7355.39; 7387.20, 7412.20 (M+Na)	chain 7+ chain 9, chain 7+ chain 8
K) 35.70	7491.53, 7518.55 (M+Na)	chain 7+ chain 8
L) 36.77	7490.78	chain 7+ chain 8
M) 37.87	11118.01 M^+, 5556.33 M^{2+}; 10873.69 (N)	chain 7+ chain 8+ chain 9
N) 39.00	11115.77 M^+, 5551.65 M^{2+}	chain 7+ chain 8+ chain 9
O) 40.15	11116.24 M^+, 5552.68 M^{2+}	chain 7+ chain 8+ chain 9

Theoretically calculated (m/z) for the following potential products: chain 7: 3758.64, chain 8: 3727.55, chain 9: 3623.53, chain 7+ chain 8: 7486.19, chain 7+ chain 9: 7382.17, chain 8+ chain 9: 7351.08, chain 7+ chain 8+ chain 9: 11109.72, chain 7+ chain 7: 7517.28, chain 8+ chain 8: 7455.10, chain 9+ chain 9: 7246.06.
Observed P3Ja1 click reaction products include: chain 7+ chain 8, chain 7+ chain 9, chain 8+ chain 9, chain 7+ chain 8+ chain 9.

HPLC profiles of click reaction products from P3Ja3 and the product analysis/assignments based on MALDI-TOF mass:



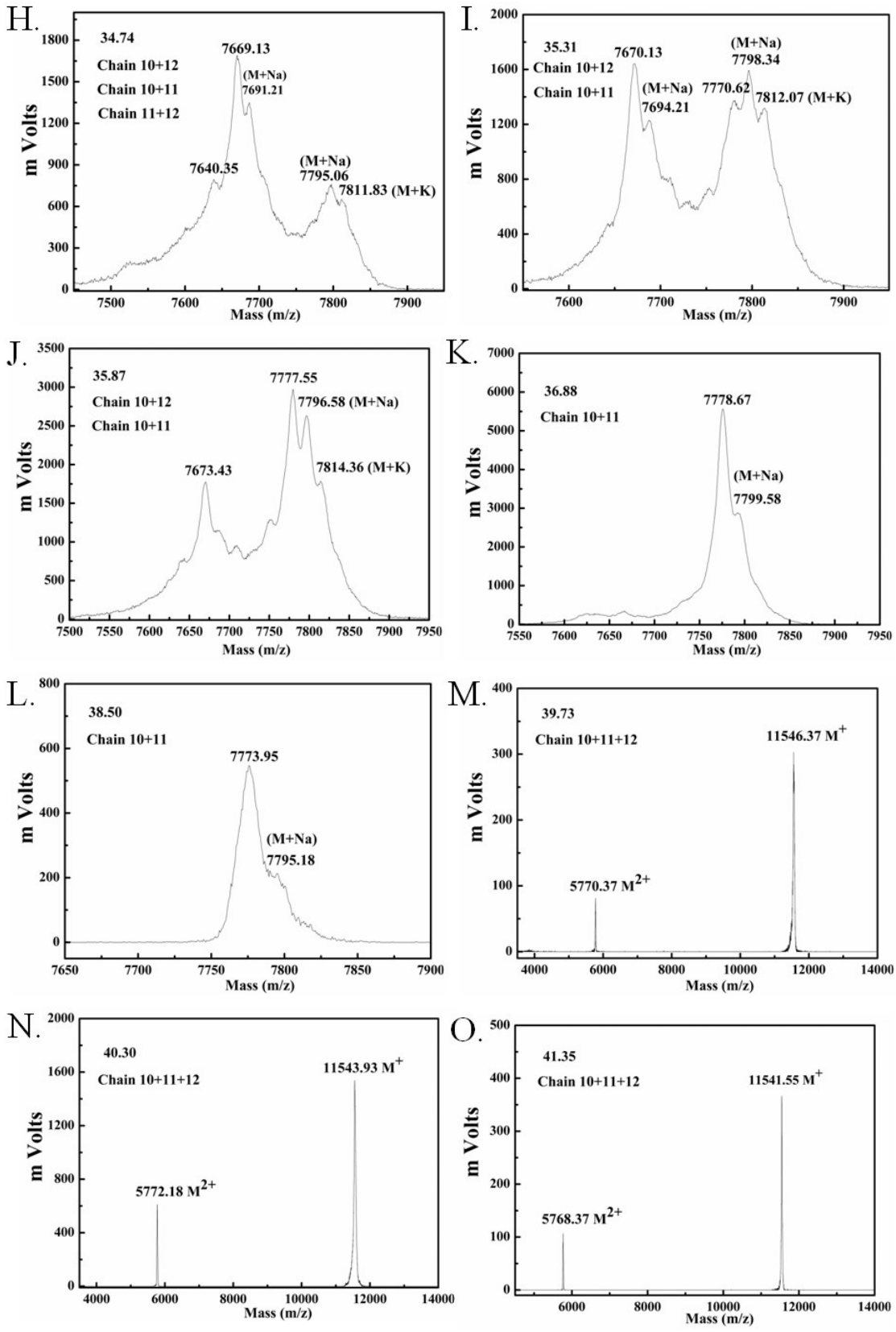


Figure S9. A) HPLC profiles of click reaction products from P3Ja3,
B-O) MALDI-TOF mass analysis of products from P3Ja3 click reaction.

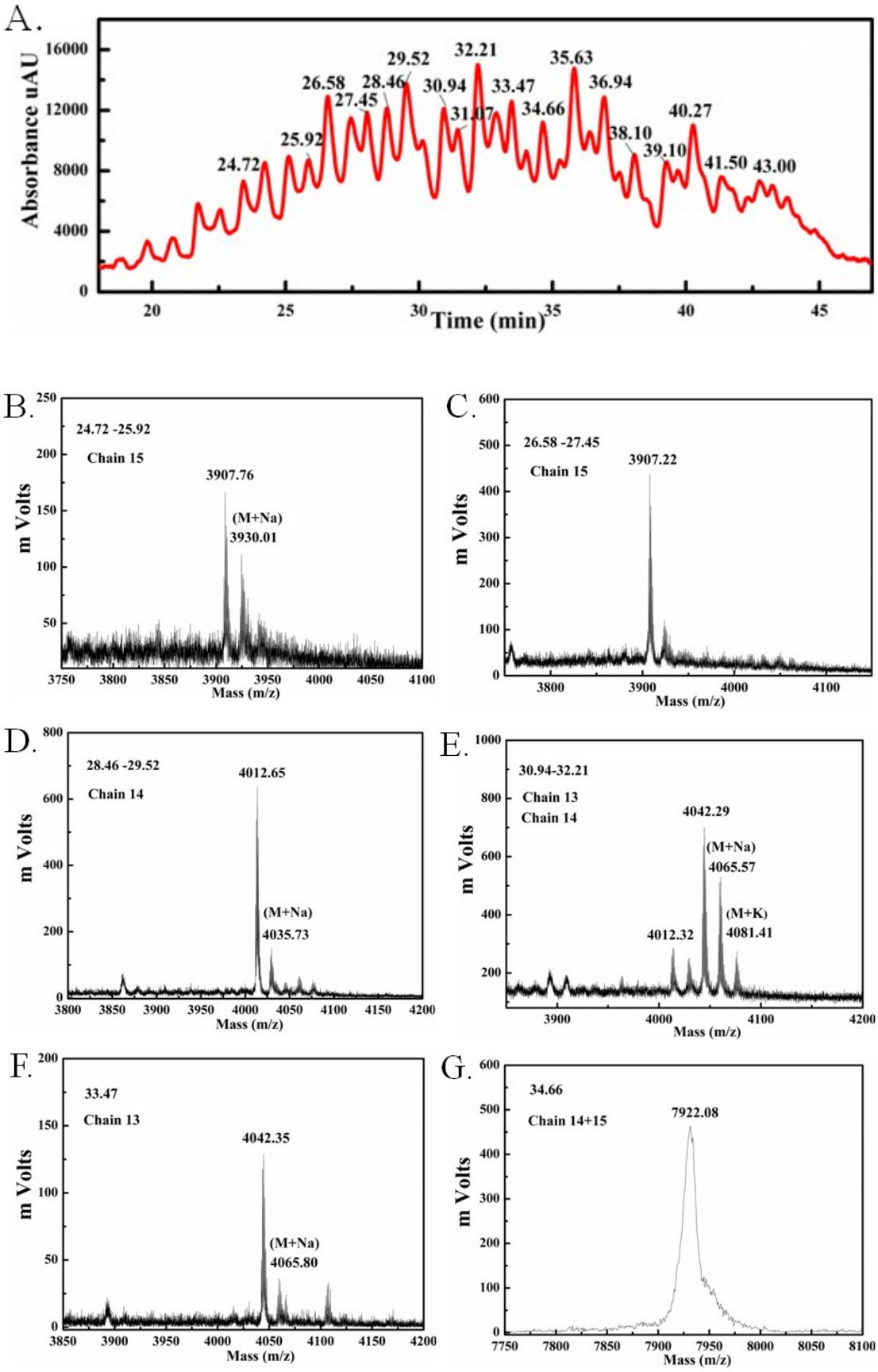
Table S2. MALDI-TOF mass results from P3Ja3 click reaction

peak	Found (m/z)	Name
B) 22.32-23.62	3765.70, 3788.70 (M+Na)	chain 12
C) 25.22-26.48	3765.50; 3864.49, 3892.48 (M+Na)	chain 12, chain 11
D) 27.18-28.82	3869.62; 3900.61, 3923.58 (M+Na)	chain 11, chain 10
E) 30.97	3900.60	chain 10
F) 32.25	7404.22 (N); 7638.87	chain 11+ chain 12
G) 33.54	7635.39, 7660.13 (M+Na)	chain 11+ chain 12
H) 34.74	7640.35; 7669.13, 7691.21 (M+Na); 7795.06 (M+Na)，7811.83 (M+K)	chain 10+ chain 12, chain 10+ chain 11, chain 11+ chain 12
I) 35.31	7670.13, 7694.21 (M+Na); 7770.62，7798.34 (M+Na), 7812.07 (M+K)	chain 10+ chain 12, chain 10+ chain 11
J) 35.87	7673.43, 7777.55, 7796.58 (M+Na), 7814.36 (M+K)	chain 10+ chain 11, chain 10+ chain 12
K) 36.88	7778.67, 7799.58 (M+Na)	chain 10+ chain 11
L) 38.50	7773.95, 7795.18 (M+Na)	chain 10+ chain 11
M) 39.73	11546.37 M^{+}, 5770.37 M^{2+}	chain 10+ chain 11+ chain 12
N) 40.30	11543.93 M^{+}, 5772.18 M^{2+}	chain 10+ chain 11+ chain 12
O) 41.35	11541.55 M^{+}, 5768.37 M^{2+}	chain 10+ chain 11+ chain 12

Theoretically calculated (m/z) for the following potential products: chain 10: 3900.62, chain 11: 3869.62, chain 12: 3765.60, chain 10+ chain 11: 7770.24, chain 10+ chain 12: 7666.22, chain 11+ chain 12: 7635.22, chain 10+ chain 11+ chain 12: 11535.84, chain 10+ chain 10: 7801.24, chain 11 + chain 11: 7739.24, chain 12+ chain 12: 7531.20.

Observed P3Ja3 click reaction products include: chain 10+ chain 11, chain 10+ chain 12, chain 11+ chain 12, chain 10+ chain 11+ chain 12.

HPLC profiles of click reaction products from P3Ja5 and the product analysis/assignments based on MALDI-TOF mass:



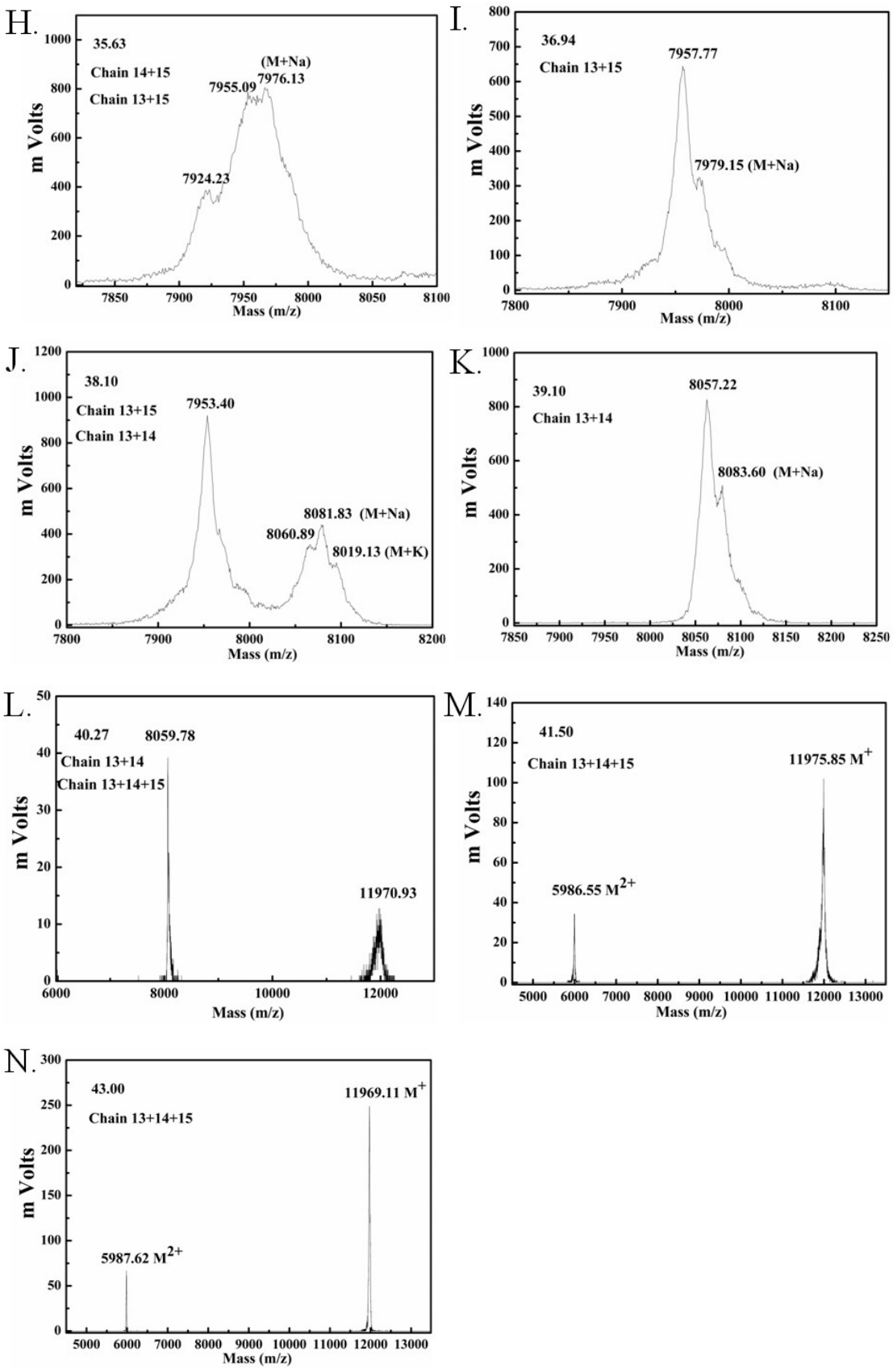


Figure S10. A) HPLC profiles of click reaction products from P3Ja5,
B-N) MALDI-TOF mass analysis of products from P3Ja5 click reaction.

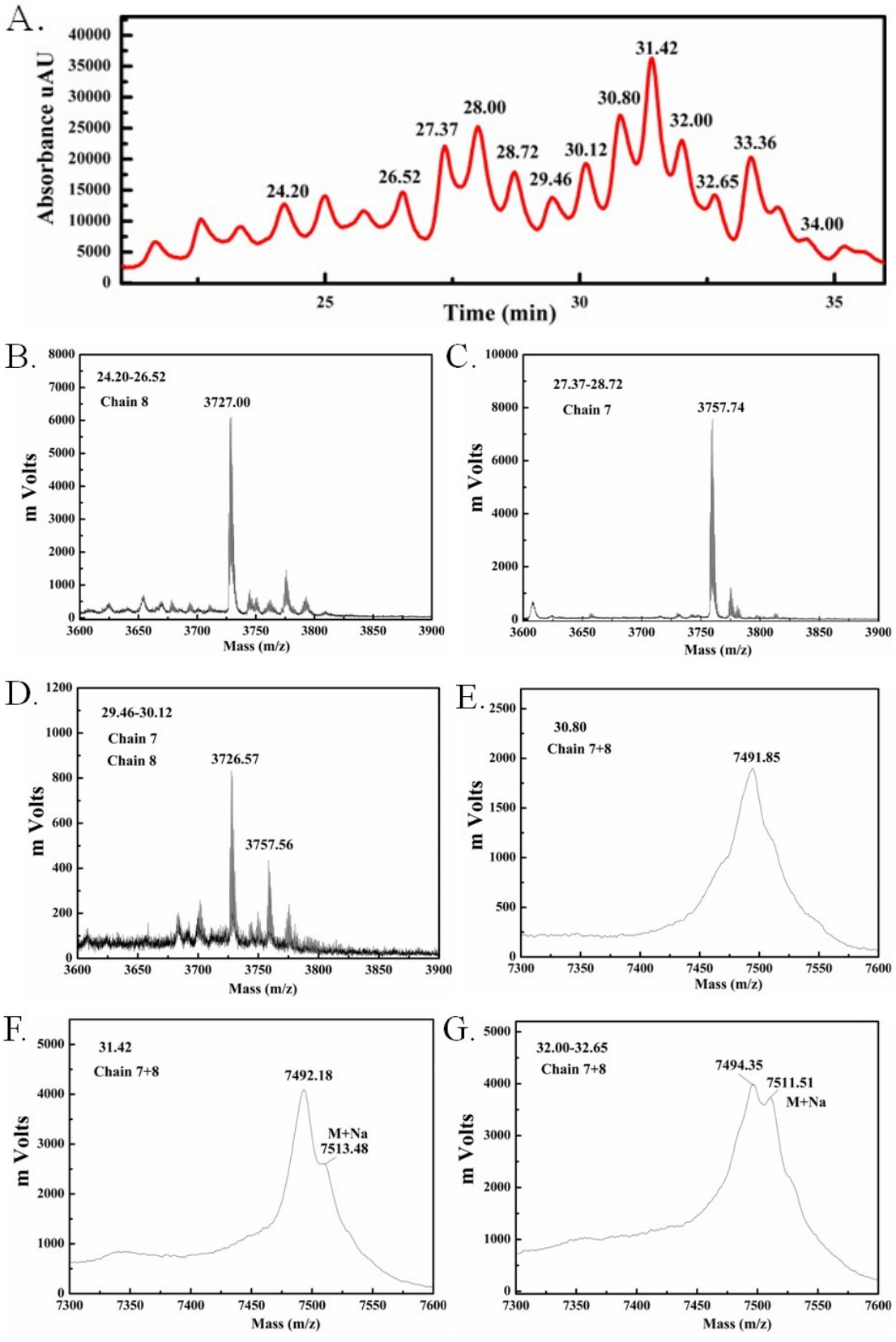
Table S3. MALDI-TOF mass analysis results from P3Ja5 click reaction

peak	Found (m/z)	Name
B) 24.72-25.92	3907.76, 3930.01 (M+Na)	chain 15
C) 26.58-27.45	3907.22	chain 15
D) 28.46-29.52	4012.65, 4035.73 (M+Na)	chain 14
E) 30.94-32.21	4012.32; 4042.29, 4065.57 (M+Na), 4081.41 (M+K)	chain 14, chain 13
F) 33.47	4042.35, 4065.80 (M+Na)	chain 12
G) 34.66	7922.08	chain 14+ chain 15
H) 35.63	7924.23; 7955.09, 7976.13 (M+Na)	chain 14+ chain 15，chain 13+ chain 15
I) 36.94	7957.77, 7979.15 (M+Na)	chain 13+ chain 15
J) 38.10	7953.40; 8060.89, 8081.83 (M+Na), 8019.13 (M+K)	chain 13+ chain 14，chain 13+ chain 15
K) 39.10	8057.22, 8083.60 (M+Na)	chain 13+ chain 14
L) 40.27	8059.78, 11970.93 (M+Na)	chain 13+ chain 14+ chain 15
M) 41.50	11975.85 M^{+}, 5986.55 M^{2+}	chain 13+ chain 14+ chain 15
N) 43.00	11969.11 M^{+}, 5987.62 M^{2+}	chain 13+ chain 14+ chain 15

Theoretically calculated (m/z) for the following potential products: chain 13: 4042.69, chain 14: 4011.70, chain 15: 3907.67, chain 13+ chain 14: 8054.39, chain 13+ chain 15: 7950.36, chain 14+ chain 15: 7919.37, chain 13+ chain 14+ chain 15: 11962.06. chain 13+ chain 13: 8085.38, chain 14+ chain 14: 8023.4, chain 15+ chain 15: 7815.34.

Observed P3Ja5 click reaction product include: chain 13+ chain 14, chain 14+ chain 15, chain 13+ chain 15, chain 13+ chain 14+ chain 15.

HPLC profiles of the control click reaction (between chain 7 and chain 8) products and the product analysis/assignments based on MALDI-TOF mass:



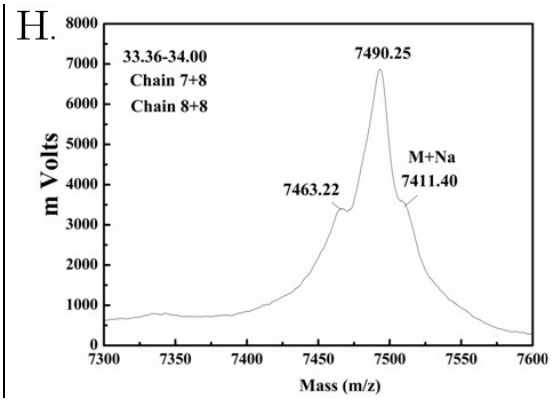


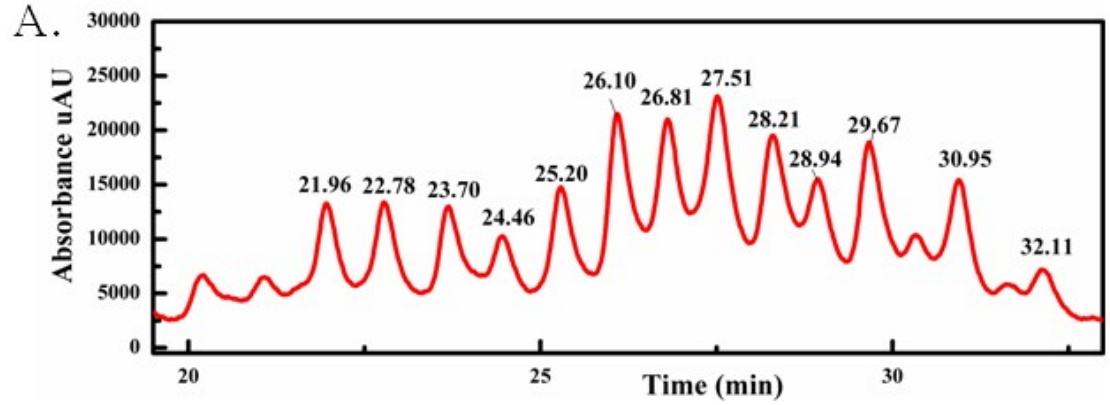
Figure S11. A) HPLC profiles of click reaction products from chain 7 and chain 8; B-H) MALDI-TOF mass analysis of products from the click reaction between chain 7 and chain 8.

Table S4. MALDI-TOF mass analysis results from the click reaction between chain 7 and chain 8

peak	Found (m/z)	Name
B) 24.20-26.52	3727.00	chain 8
C) 27.37-28.72	3757.74	chain 7
D) 29.46-30.12	3726.57, 3757.56	chain 7, chain 8
E) 30.80	7491.85	chain 7+ chain 8
F) 31.42	7492.18, 7513.48 (M+Na)	chain 7+ chain 8
G) 32.00-32.65	7494.35, 7511.51 (M+Na)	chain 7+ chain 8
H) 33.36-34.00	7463.22; 7490.25, 7411.40 (M+Na)	chain 7+ chain 8, Chain 8+ chain 8

Observed click reaction products include: chain 7+ chain 8 (major) and chain 8+ chain 8 (minor).

HPLC profiles of the control click reaction (between chain 7 and chain 9) products and the product analysis/assignments based on MALDI-TOF mass:



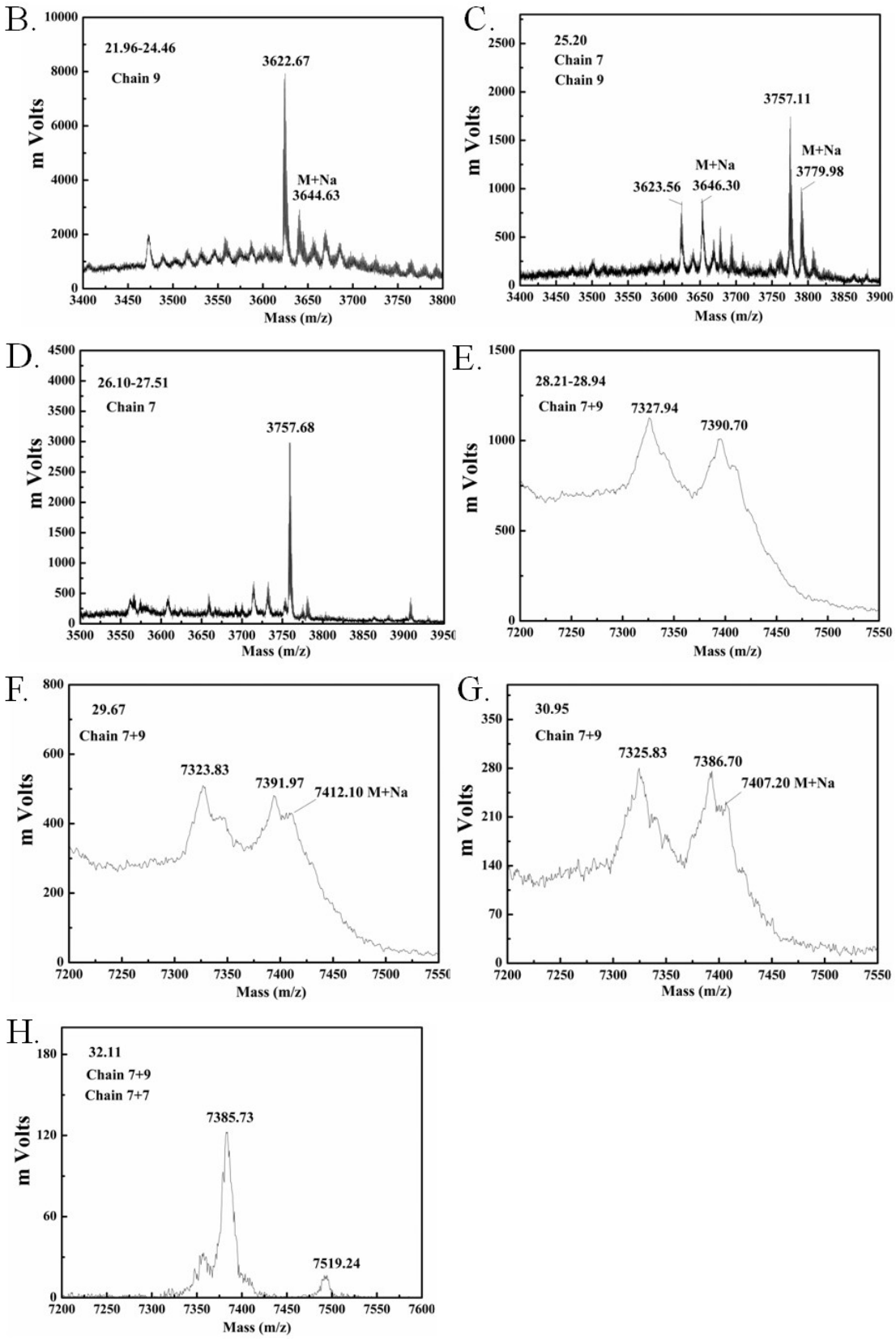


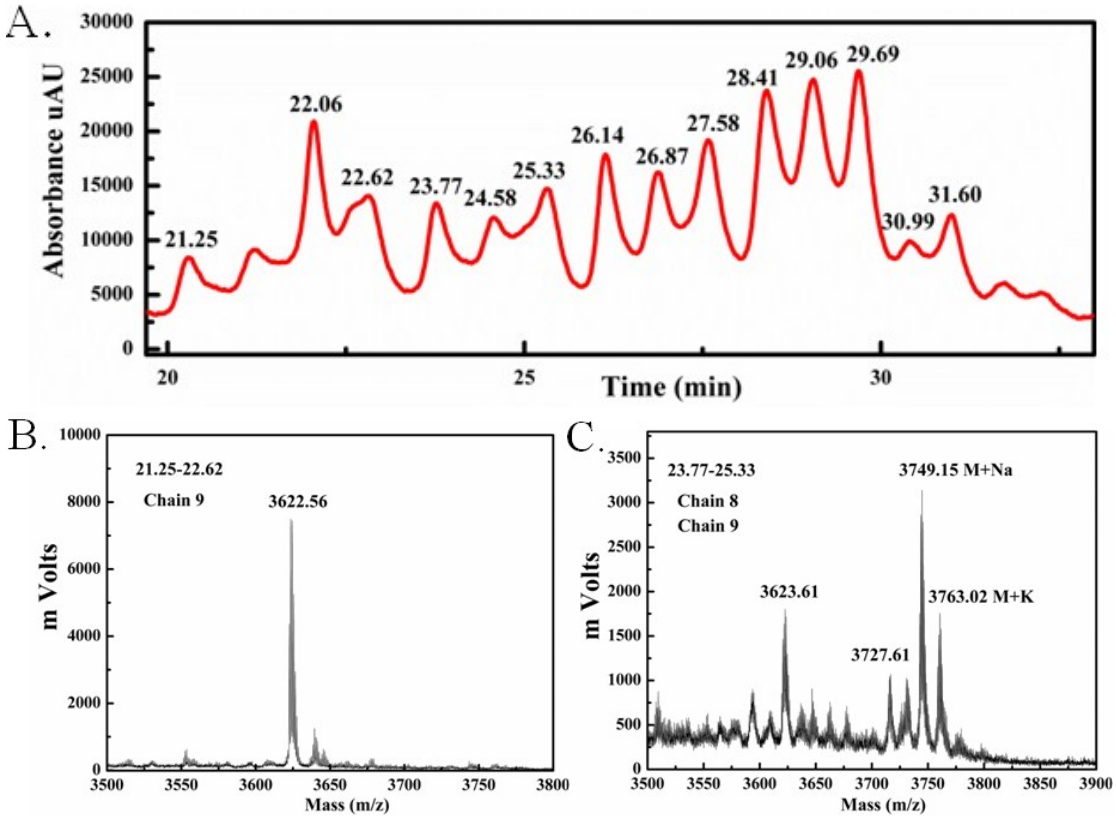
Figure S12. A) HPLC profiles of click reaction products from chain 7 and chain 9; B-H) MALDI-TOF mass analysis of products from the click reaction between chain 7 and chain 9.

Table S5. MALDI-TOF mass analysis results from the click reaction between chain 7 and chain 9

peak	Found (m/z)	Name
B) 21.96-24.46	3622.67, 3644.63 (M+Na)	chain 9
C) 25.20	3623.56, 3646.30 (M+Na), 3757.11, 3779.98 (M+Na)	chain 7, chain 9
D) 26.10-27.51	3757.68	chain 7
E) 28.21-28.94	7327.94(N), 7390.70	chain 7+ chain 9
F) 29.67	7323.83 (N), 7391.97, 7412.10 (M+Na)	chain 7+ chain 9
G) 30.95	7325.83(N), 7386.70, 7407.20 (M+Na)	chain 7+ chain 9
H) 32.11	7385.73, 7519.24	chain 7+ chain 9, chain 7+ chain 7

Observed click reaction products include: chain 7+ chain 9 (major) and chain 7+ chain 7 (minor).

HPLC profiles of the control click reaction (between chain 8 and chain 9) products and the product analysis/assignments based on MALDI-TOF mass:



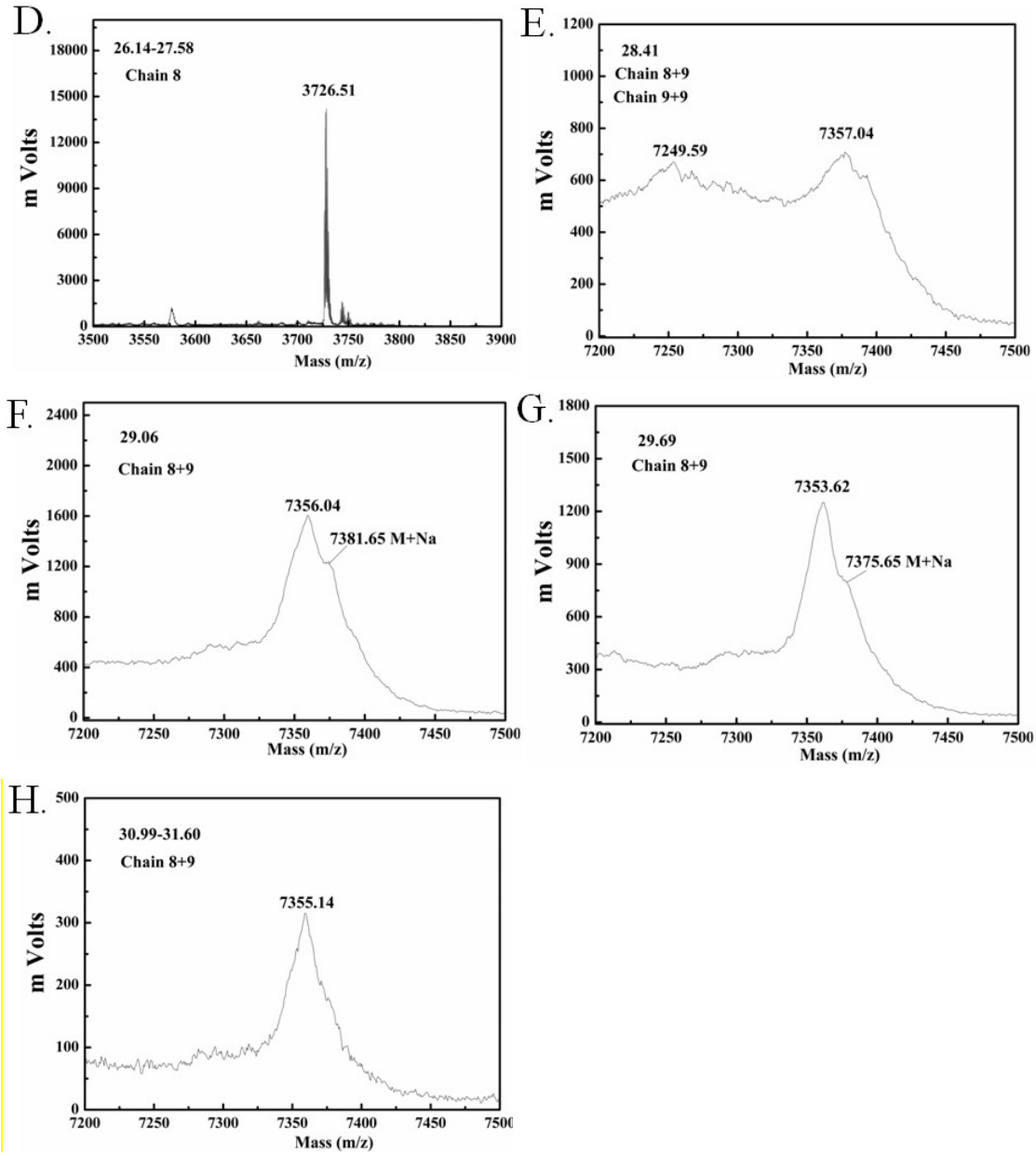


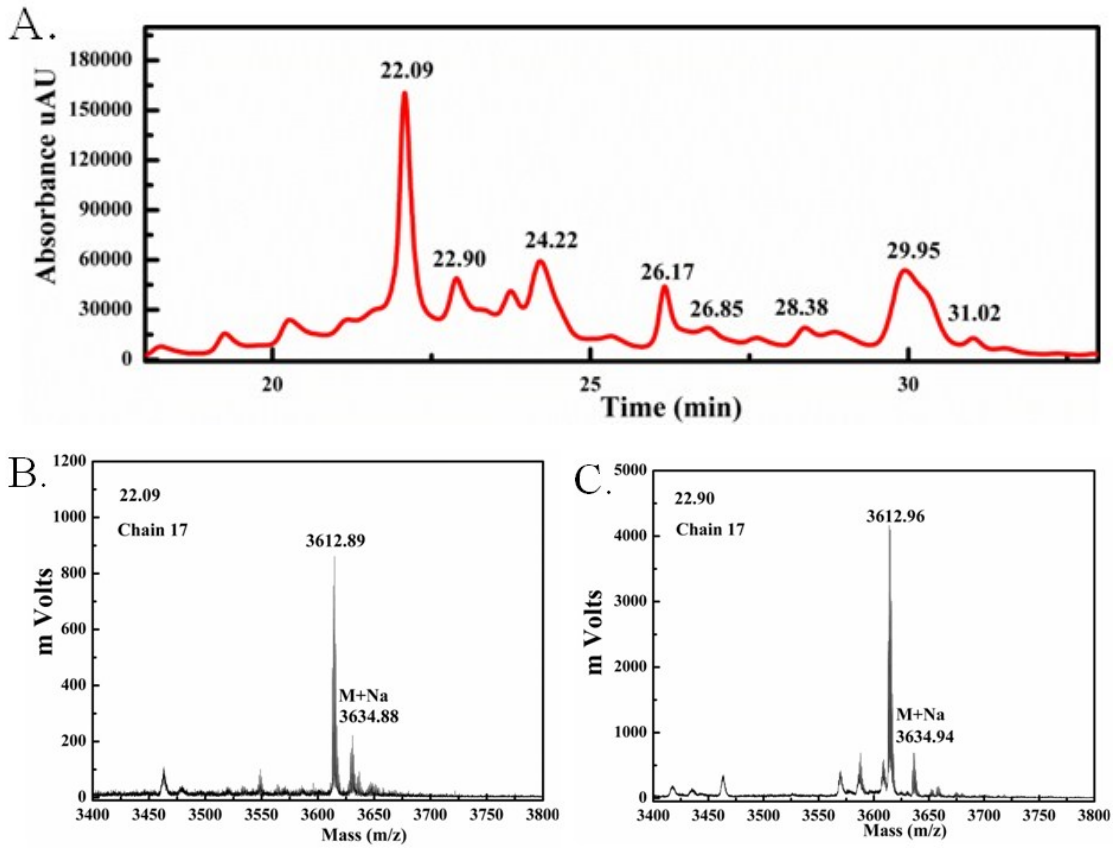
Figure S13. A) HPLC profiles of click reaction products from chain 8 and chain 9; B-H) MALDI-TOF mass analysis of products from the click reaction between chain 8 and chain 9.

Table S6. MALDI-TOF mass analysis results from the click reaction between chain 8 and chain 9

peak	Found (m/z)	Name
B) 21.25-22.62	3622.56	chain 9
C) 23.77-25.33	3623.61; 3727.61, 3749.15 (M+Na), 3763.02 (M+K)	chain 8, chain 9
D) 26.14-27.58	3726.51	chain 8
E) 28.41	7249.59, 7357.04	chain 8+ chain 9, chain 9+ chain 9
F) 29.06	7356.04, 7381.65 (M+Na)	chain 8+ chain 9
G) 29.69	7353.62, 7357.65 (M+Na)	chain 8+ chain 9
H) 30.99-31.60	7355.14	chain 8+ chain 9

Observed click reaction products include: chain 8+ chain 9 (major) and chain 8+ chain 9 (minor).

HPLC profiles of the control click reaction (among chain 7 and random chains 16 & 17) products and the product analysis/assignments based on MALDI-TOF mass:



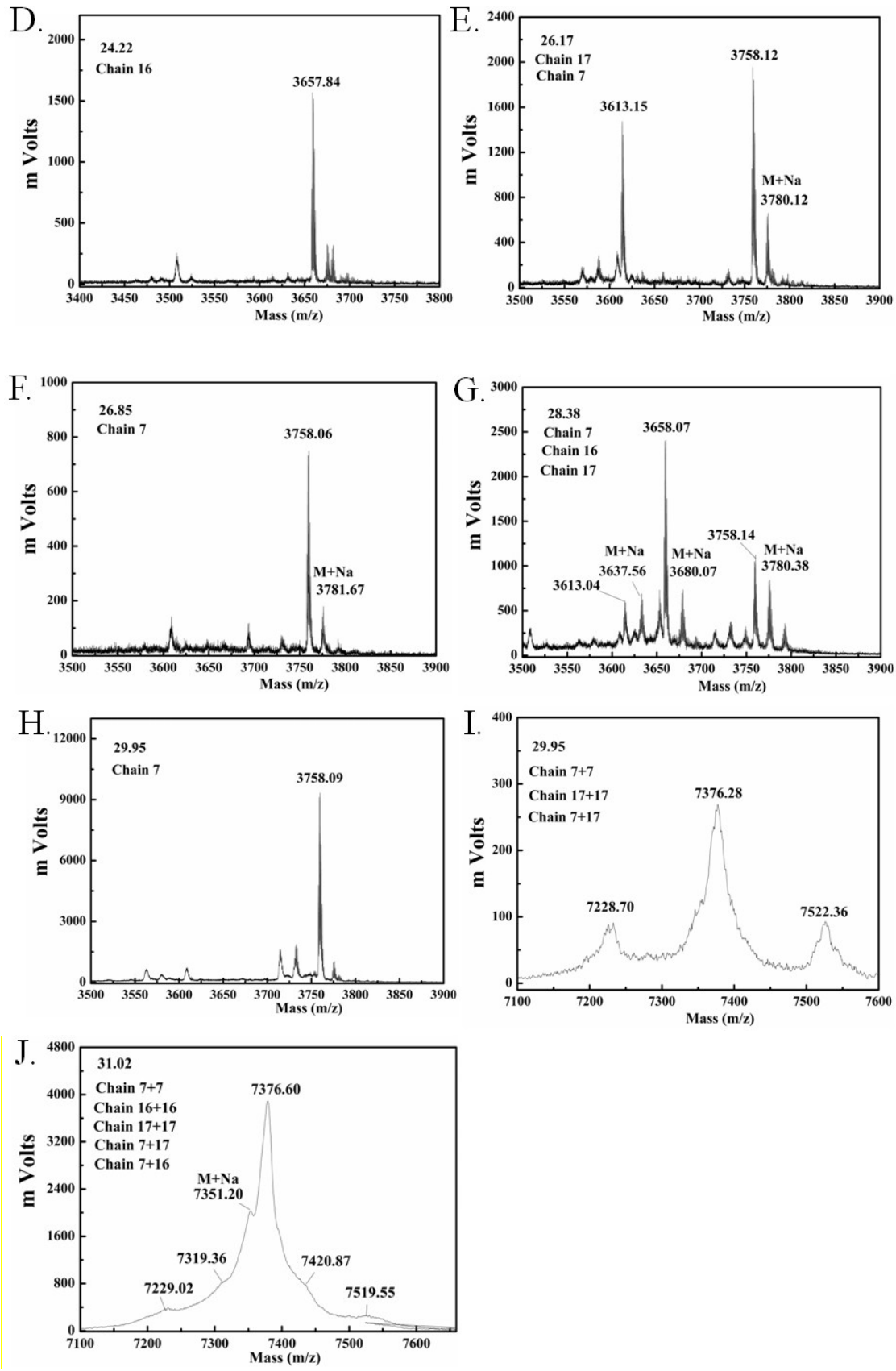


Figure S14. A) HPLC profiles of click reaction products from Chain 7, chain 16 and chain 17; B-J) MALDI-TOF mass analysis of products from the click reaction

among chain 7, chain 16 and chain 17.

Table S7. MALDI-TOF mass analysis results from the click reaction among chain 7, chain 16 and chain 17

peak	Found (m/z)	Name
B) 22.09	3612.89, 3634.88(M+Na)	chain 17
C) 22.90	3612.96, 3634.94(M+Na)	chain 17
D) 24.22	3657.84	chain 16
E) 26.17	3613.15; 3758.12, 3780.12(M+Na)	chain 7, chain 17
F) 26.85	3758.06, 3781.67(M+Na)	chain 7
G) 28.38	3613.04; 3637.56(M+Na); 3658.07, 3680.07(M+Na); 3758.14, 3780.38(M+Na)	chain 7, chain 16, chain 17
H/I) 29.95	3758.09; 7228.70; 7376.28; 7522.36	chain 7, chain 7+ chain 7, chain 7+ chain 17, chain 17+ chain 17
J) 31.02	7229.02; 7319.36; 7351.20; 7376.60; 7420.87; 7519.55	chain 7+ chain 7, chain 16+ chain 16, chain 17+ chain 17, chain 7+ chain 17, chain 7+ chain 16

Theoretically calculated (m/z) for the following potential products: chain 7: 3758.64, chain 16: 3658.52, chain 17: 3613.52, chain 7+16: 7417.16, chain 7+ chain 17: 7372.16, chain 16+ chain 17: 7272.04, chain 7+ chain 7: 7517.28, chain 16+ chain 16: 7317.14, chain 17+ chain 17:7226.04.
Observed click products include: chain 7+ chain 7, chain 16+ chain 16, chain 17+ chain 17, chain 7+ chain 17, chain 7+ chain 16.

Control experiment results:

Click reactions were carried out using any two from the set of three single chains including chains 7-9 as control experiments, the observed major click products are those between two different single chains, only minor click products are those among the same single chains, higher order click reaction products were not observed. Control click reactions were also carried using chain 7 together with two random sequences including chains 16 and 17, the observed click products include chain 7+ chain 7, chain 16+ chain 16, chain 17+ chain 17, chain 7+ chain 16, chain 7+ chain 17. For the click reaction using random sequences, the efficiency is very low and the yields of all click products are very low as compared to other click reactions carried

out in this study. A possible explanation is that formations of base-paired or partially base-paired complementary structures would bring the alkyne and azido reaction partners into close proximity and make the click reactions easier to proceed.

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