Supplementary Information

General Informaion

Materials

All solvents and reagents used for solid phase peptide (SPPS) synthesis were purchased from commercial suppliers including GL Biochem (Shanghai, China), Energy Chemical (Shanghai, China), Hanhong Chemical (Shanghai, China) and Tenglong Logistics (Shenzhen, China) and were used without further purification unless otherwise stated.

All air and moisture sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. Reactive liquid compounds were measured and transferred by gas-tight syringes and were added in the reaction flask through rubber septa. Tetrahydrofuran (THF) were freshly distilled from sodium benzophenoneketyl. Toluene was distilled from CaH_2. Unless otherwise noted, all reagents were obtained commercially and used without further purification.

NMR spectrum:

^1H and ^{13}C NMR spectra were collected on 500 MHz NMR spectrometers (Bruker AVANCE) using $CDCl_3$. Chemical shifts are reported in parts per million (ppm). Chemical shifts for protons are reported in parts per million downfield and are referenced to residual protium in the NMR solvent ($CDCl_3$ = δ 7.26). Chemical shifts for carbon are reported in parts per million downfield and are referenced to the carbon resonances of the solvent ($CDCl_3$ = δ 77.0). Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz (Hz), integration.

Mass spectroscopy:

Mass spectra were in general recorded on a QSTAR Elite (ABI).

Chromatography:

Column chromatography was performed with silica gel (200-300 mesh ASTM).

Experimental Section

Peptide synthesis

Peptides were synthesized on Rink-amide-MBHA resin using manual Fmoc/tBu solid phase peptide synthesis. Coupling was performed using HCTU/DIPEA for 3 h with N_2 bubbling. Coupling between PEG2000 and peptide was conducted using EDC/DIPEA for 5 h with N_2 bubbling in DCM. Cy5 labelling was performed in solution with the solution of Cy5-NHS ester in $NaHCO_3$ (10 mM, pH = 8.0) overnight. Final resins were treated with 95% (v/v) TFA/TIS/H_2O (95 : 2.5 : 2.5) for 2 h. After air removal of most of the TFA, products were triturated with hexane/Et_2O (1: 2), dissolved in CH_3CN/H_2O (1 : 1). Crude peptides were purified on RP-HPLC (Shimadzu (Kyoto, Japan), Agilent (Santa Clara, CA, USA) Zorbax SB-Aq: 4.6 × 250 mm, 220 and 254 nm) and confirmed by Shimadzu LC-MS 2020 mass spectrometer equipped with Agilent Zorbax SB-Aq column.

Intramolecular disulfide bond formation

Air oxidation: The solution containing the deprotected peptide at a dilute solution (lower than 0.2 mg/mL)

was adjusted to PH 8.0 by NH_4HCO_3. The reaction was slowly stirred for 24 h, and completely building of intramolecular disulfide bond was verified by HPLC and mass spectrometry.

Cell Viability

100 μL of 4 × 10^4/mL cell suspension was seeded in each well of the 96 well plate and allowed to grow in DMEM supplemented with 10% FBS supplied with 5% CO_2 overnight. Then, the cells were treated with serial dilution of compounds at 310K in 5% FBS containing media for 24 h. At the end of the compounds exposure, 20 μL of MTT reagent was added and incubated at 310K for 4 h. The absorbance of formazan product was measured at 490 nm by a microplate reader (Perkin Elmer, Envision, 2104 Multilabel Reader). Cells without peptide treatment were regarded as control.

Confocal microscopy imaging

MDA-MB-231 cells or HEK293T cells were seeded on coverslip at 310K in the presence of 5% CO_2. Then cells were then incubated with 22 μM FEB-2000, FEB-2000-iRGD or IR-783 for 6 h. After that, FEB derivatives and IR-783 containing media was removed followed by washing with phosphate buffered saline (PBS) twice. Then nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min then washed with PBS. The coverslips were mounted onto slides and visualized by confocal laser scanning microscope.

Xenograft tumour model

Four-week-old nude mice were purchased from the Vital River (Beijing, China). Ambient temperature was controlled at 293 to 295 K with 12 h light/12 h dark cycles. Approximately 1 × 10^6 MDA-MB-231 cells were injected into the mammary fat pads of female mice in a vehicle of 4 mg/mL Matrigel (BD). One week later, mice bearing tumours around 50 mm^3 in volume were randomly divided into groups. FEB-2000 and FEB2000-iRGD were injected intravenously at a dose of 270 μM, 100 μL. Cy-iRGD were injected intravenously at a dose of 50 μM, 100 μL. Mice were anesthetized with isoflurane and imaged using IVIS Spectrum Pre-clinical *In Vivo* Imaging System (535 nm/640 nm for FEB derivatives and 640 nm/680 nm for Cy5 derivatives). Fluorescence was calculated with Living Image. The values for each mouse were averaged.

The animal experiments complied to the Regulations of Guangdong Province on the Administration of Laboratory Animals, with the approval number of SIAT-IRB-160128-YYS-LZG-A0164.

Spectral characterizations of FEB derivatives.

UV-Vis-NIR spectrophotometer (Cary 6000i) with background correction was employed to measure the optical absorption spectrum of FEB derivatives in the range of 300-1,200 nm. A home build setup was used to measure the fluorescence spectrum in the region of 500-900 nm using an array detector (Princeton OMA-V) and a spectrometer (Acton SP2300i) under a 535-nm diode laser (RMPC lasers) excitation. The obtained emission spectra were further corrected by the detector sensitivity profile and the absorbance features of the filter.

Quantum yield measurement

Determination of fluorescence quantum yield of FEB2000 and FEB2000-iRGD. Fluorescence quantum yield of these dyes was measured in water according to a previous reported method. The commercial

fluorescent dye RHB was used as the reference sample with the quantum yield of 31%. RHB was dissolved in water, and diluted to different concentration with absorbance value at 500 nm of below 0.1, using a ultraviolet-visible- near-infrared absorbance spectrometer (Cary 6000i). The fluorescence spectra in the range of 500- to 800-nm were collected under the 500 nm diode laser (RMPC lasers) excitation. The absorption and emission of FEB-2000 and FEB2000-iRGD in water were measured using the same method. The integrated fluorescence intensity was plotted against absorbance at the excitation wavelength of 500 nm and fitted into a linear function. The slope of FEB derivatives were compared with dye RHB, and the quantum yields were determined by following equation.

$$QY_{FEB} = QY_{RHB} \cdot \frac{slope_{FEB}}{slope_{RHB}} \cdot \left(\frac{n_{FEB}}{n_{RHB}}\right)^2$$

***In vivo* toxicity**

FEB2000 and FEB2000-iRGD (270 μM, 100 μL) was injected intravenously into nude mice. All collected tissue sections (4 μm) were stained with hematoxilin and eosin (H&E) and subsequently processed for histopathological examination under light microscope.

Schemes and Figures

Fig. S1 Absorption coefficiency: FEB-2000= 28530 M/cm, FEB-2000-iRGD= 27300 M/cm.

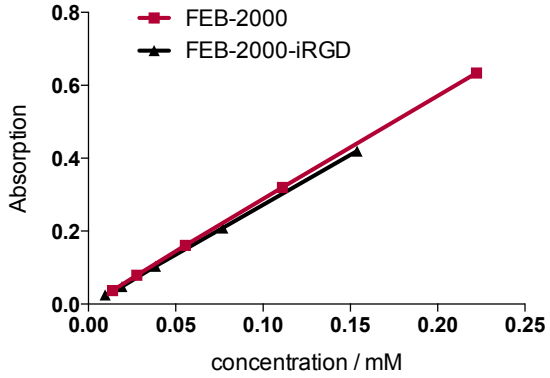


Fig. S2 Fluorescence intensity of FEB-2000 (Left) and FEB-2000-iRGD (Right) in water, PBS, and FBS measured over 1 week.

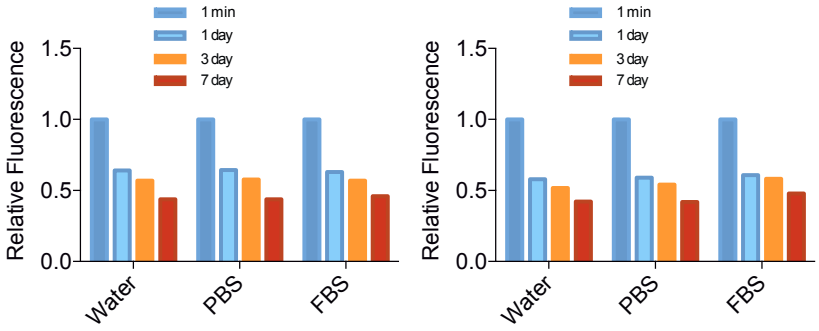


Fig. S3 Quantum yield measurement of molecular fluorophore FEB2000，FEB2000-iRGD，RHB. Integrated NIR-II fluorescence intensity plotted as a function of absorbance at 500 nm for dye solutions. The data was fitted into a linear function. $Slope_{RHB}=3.228\times10^{6}$，$Slope_{FEB\text{-}2000}=3.649\times10^{6}$，$Slope_{FEB\text{-}2000\text{-}iRGD}=3.802\times10^{6}$.

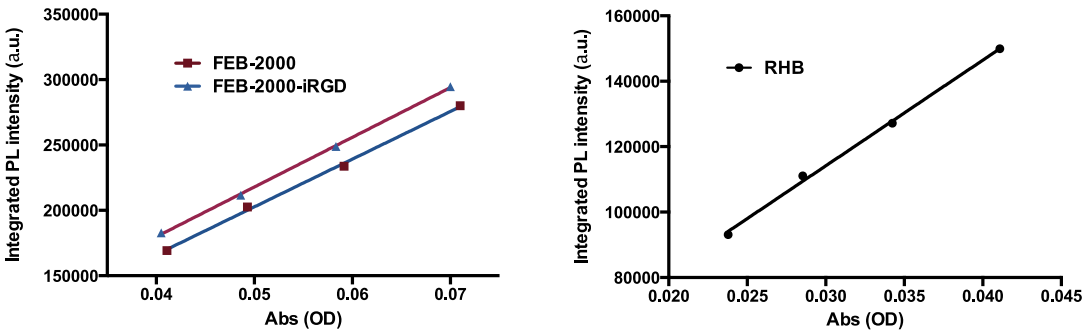


Fig. S4 Confocal laser-scanning microscopy images of MDA-MB-231 cells (a) and HEK 293T cells (b) incubated with PBS, FEB-2000, FEB-2000-iRGD and IR-783 at 310 K for 6 h. Scale bar = 5 μm.

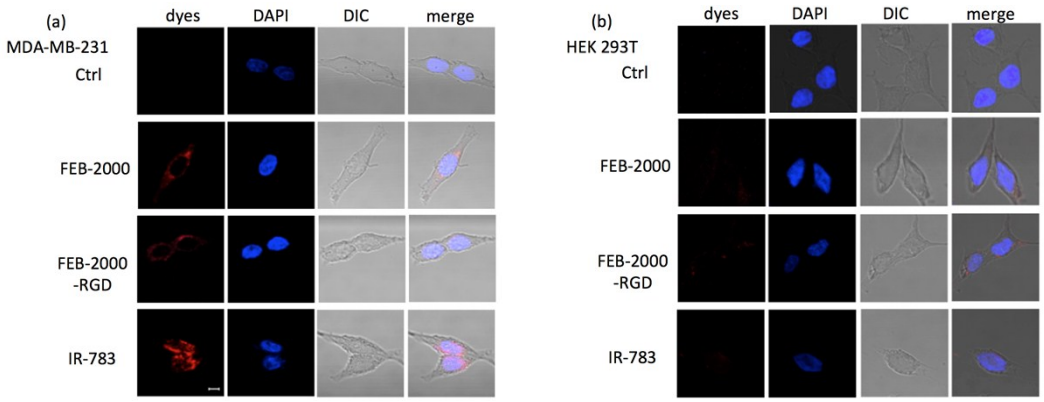


Fig. S5 a. Confocal laser-scanning microscopy images of MDA-MB-231 cells incubated with PBS, FEB-2000-iRGD at 310 K for 6 h. Scale bar = 20 μm; b. Cytotoxicity of FEB-2000-iRGD on MDA-MB-231 cell line and HeLa cell line.

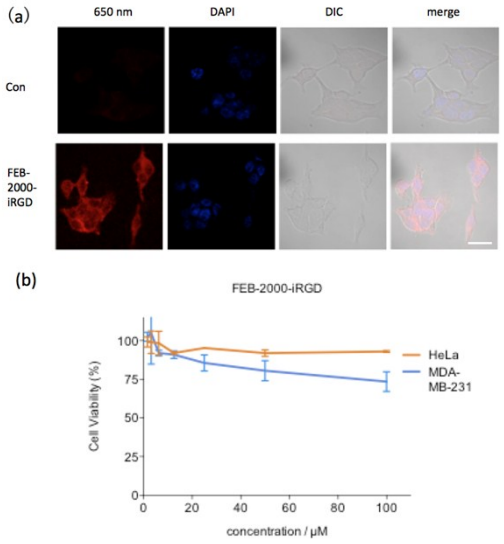
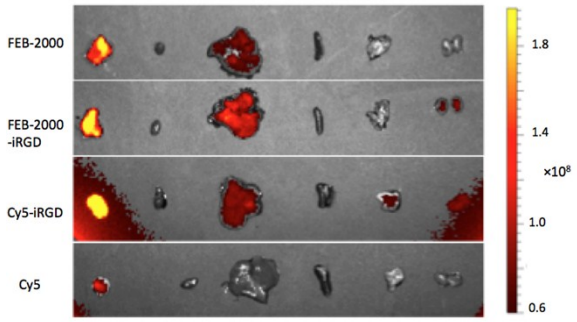
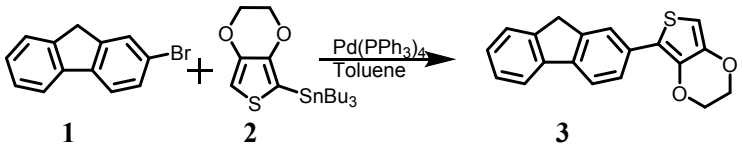


Fig. S6 Fluorescence intensity of organ and tumour samples 48 h post-injection.



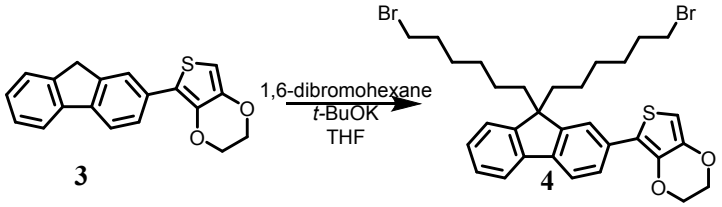
Scheme S1: Synthesis of **FEB.**

(1) Synthesis of 5-(9H-fluoren-2-yl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (3).



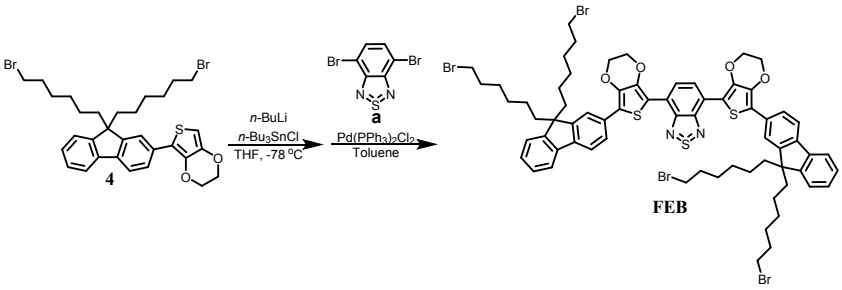
2-bromo-9*H*-fluorene **1** 5.0 g (20.4 mmol) and tributyl(2,3-dihydrothieno[3,4-*b*][1,4] dioxin-5-yl)stannane **2** 9.2 g (21.4 mmol) were dissolved in 40 mL toluene under protective gas atmosphere then $Pd(PPh_3)_4$ 200 mg was added. After reflux 6 h, the crude product was subjected to column chromatography on silica gel to afford **3** as a light yellow solid (5.8 g, 94 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.94 (dd, *J* = 3.8, 1.7 Hz, 1H), 7.82 – 7.74 (m, 3H), 7.56 (dd, *J* = 7.3, 2.0 Hz, 1H), 7.40 (td, *J* = 7.5, 2.7 Hz, 1H), 7.36 – 7.29 (m, 1H), 6.41 – 6.26 (m, 1H), 4.37 – 4.31 (m, 2H), 4.29 – 4.23 (m, 2H), 3.95 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 37.60, 65.10, 65.41, 98.00, 118.68, 120.45, 120.62, 123.15, 125.43, 125.64, 127.22, 127.41, 132.37, 138.60, 140.84, 142.11, 142.91, 144.05, 144.30. HRMS(ESI) calcd for $C_{19}H_{15}O_2S^+$, ([M+H$^+$]) 307.0793, Found 307.0787.

(2) Synthesis of 5-(9,9-bis(6-bromohexyl)-9H-fluoren-2-yl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (4).



5-(9H-fluoren-2-yl)-2,3-dihydrothieno[3,4-b][1,4]dioxine **3** 3.0 g (9.8 mmol) and 1,6-dibromohexa -ne 9.7 g (40 mmol) were dissolved in 50 mL THF at 0 ^{o}C. Then potassium tert-butanolate 2.5 g (2.1 mmol) which dissolved in THF was added dropwise. After 6 h at rt, the crude product was subjected to column chromatography on silica gel to afford **4** as a light yellow oil (4.9 g, 81 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.77 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.71 (ddd, *J* = 7.9, 4.6, 0.8 Hz, 2H), 7.69 – 7.64 (m, 1H), 7.41 – 7.29 (m, 3H), 6.35 (s, 1H), 4.42 – 4.34 (m, 2H), 4.34 – 4.26 (m, 2H), 3.30 (t, *J* = 6.8 Hz, 4H), 2.02 (dt, *J* = 11.1, 5.7 Hz, 4H), 1.74 – 1.59 (m, 4H), 1.29 – 1.17 (m, 4H), 1.16 – 1.04 (m, 4H), 0.78 – 0.56 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 23.54, 27.76, 29.06, 32.65, 34.07, 40.20, 55.01, 64.52, 64.88, 77.29, 97.35, 118.20, 119.70, 119.88, 120.23, 122.77, 124.98, 126.92, 127.02, 132.09, 138.06, 139.69, 140.86, 142.38, 150.55, 150.77. HRMS(ESI) calcd for $C_{31}H_{37}Br_2O_2S^+$, ([M+H$^+$]) 630.0803, Found 631.0861.

(3) Synthesis of FEB.

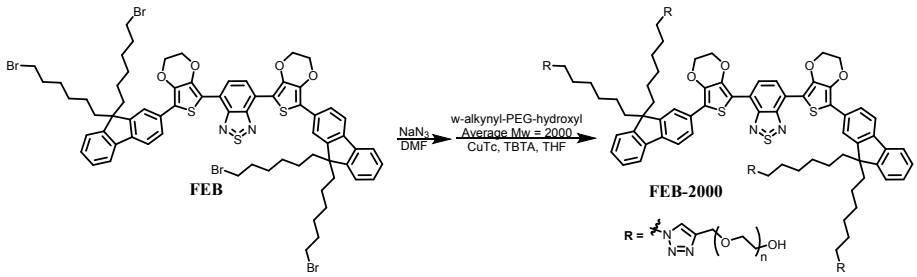


To a solution of Compound **4** 2.0 g(3.18 mmol) in 25 mL THF at -78 °C under protection gas atmosphere, *n*-BuLi(1.6 M in Hexane, 2.4 mL, 3.8 mmol)was added dropwise. After the mixturewas stirred at this temperature for another 1.5 h, trinbutyltinchloride (1.3 g, 3.8 mmol) was added to the solution. Then slowed warmed to room temperature and stirred for 8 h. After that the mixture was poured into water and extracted twice with ethyl acetate, the combined organic phase was dried with $MgSO_4$ and evaporated in vacuo without further purification. To a solution of compounds of **a** 195 mg(0.5 mmol), the crude product 1.4 g(1.5 mmol) in 15 mL toluene under protection gas atmosphere then $Pd(PPh_3)_2Cl_2$ 100 mg was added. The mixture was stirred at 110 °C for 12 h. After cooling to room temperature, the mixture was poured in to water and extracted twice with ethyl acetate, dried with $MgSO_4$ and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel and afford **FEB** as a crimson solid (435 mg, 60 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.51 (s, 2H), 7.95 (d, *J* = 8.0 Hz, 2H), 7.80 (s, 2H), 7.74 (dd, *J* = 7.7, 5.0 Hz, 4H), 7.35 (dd, *J* = 12.7, 5.9 Hz, 6H), 4.51 (d, *J* = 4.3 Hz, 8H), 3.31 (t, *J* = 6.8 Hz, 8H), 2.14 – 1.97 (m, 8H), 1.69 (dt, *J* = 14.1, 6.9 Hz, 11H), 1.39 (dd, *J* = 14.7, 7.5 Hz, 5H), 1.32 – 1.19 (m, 13H), 1.13 (dd, *J* = 15.5, 7.3 Hz, 9H), 0.77 – 0.56 (m, 8H).

^{13}C NMR (126 MHz, $CDCl_3$) δ152.44, 150.81, 150.61, 141.08, 140.88, 140.03, 138.13, 131.93, 127.10, 126.94, 126.74, 125.49, 123.38, 122.77, 120.43, 120.34, 119.89, 119.74, 111.78, 77.31, 77.06, 76.80, 64.90, 64.57, 55.12, 40.23, 34.11, 32.65, 29.73, 29.05, 28.30, 27.78, 26.81, 23.55, 17.32, 13.65.

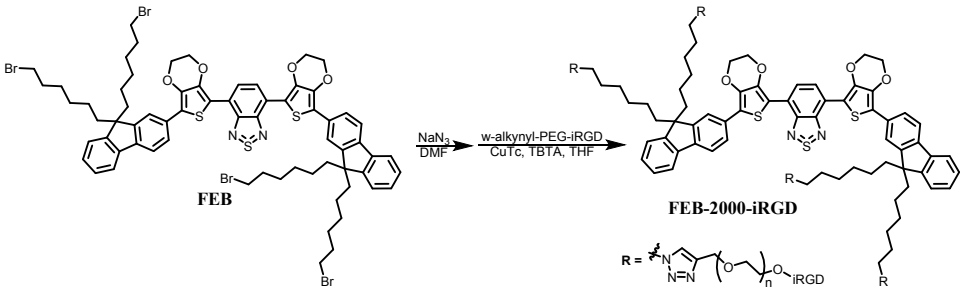
HRMS(ESI) calcd for $C_{68}H_{73}Br_4N_2O_4S_3^+$, ([M+H$^+$]) 1393.1466, Found 1393.1461.

(4)Synthesis of FEB-2000.



Compound **FEB** 100 mg (0.069 mmol) was dissolved in 10 mL DMF and sodium azide 47 mg (0.72 mmol) and heated for 3 h at 70 °C, then added just as much water as to dissolve all solids. Then it was extracted twice with ethyl acetate, the combined organic phase was dried with $MgSO_4$ and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel to afford dark crimson solid 95 mg (quant). The dark crimson solid was dissolved in 5 mL THF and CuTc10 mg, w-alkynyl-PEG-hydroxyl (average molecular weight was 2000) 560 mg, and TBTA (10 mg) was added. The system was stirred at rt for 0.5 h. Then filtered with diatomite, and the solution was evaporated in vacuo. When all the organic solvent was removed, the solid was dissolved in 15 mL water. Then transfer the water solution to the dialyzer (MWCO = 100KD). Then freeze out the water solution, **FEB-2000** (250 mg) was afforded as a crimson solid. GPC results proved the four azide groups were all substituted.

(5)Synthesis of FEB-2000-iRGD

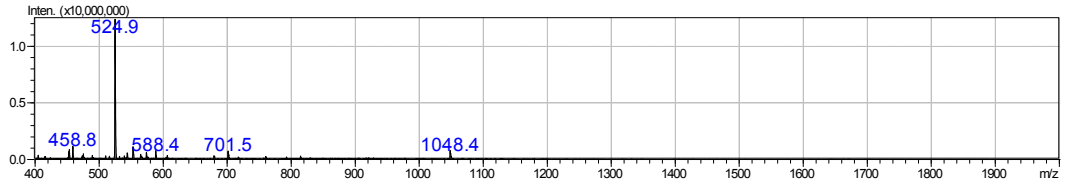


Compound **FEB** 100 mg (0.069 mmol) was dissolved in 10 mL DMF and sodium azide 47 mg (0.72 mmol) and heated for 3 h at 70 °C, then added just as much water as to dissolve all solids. Then it was extracted twice with ethyl acetate, the combined organic phase was dried with $MgSO_4$ and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel to afford dark crimson solid 95 mg (quant). The dark crimson solid was dissolved in 5 mL THF and CuTc10 mg, w-alkynyl-PEG-iRGD (average molecular weight was 3000) 840 mg, and TBTA (10 mg) was added. The system was stirred at rt for 0.5 h. Then filtered with diatomite, and the solution was evaporated in vacuo. When all the organic solvent was removed, the solid was dissolved in 15 mL water. Then transfer the water solution to the dialyzer (MWCO = 100KD). Then freeze out the water solution, **FEB-2000-iRGD** was afforded as a crimson solid.

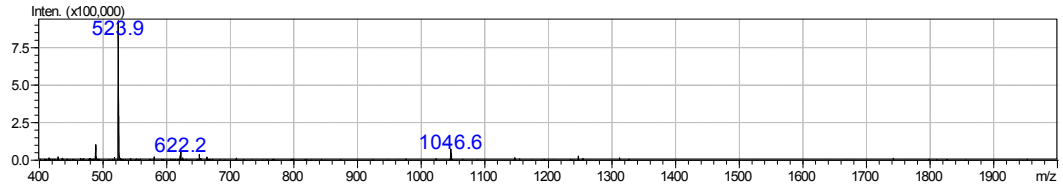
Appendix

Mass spectra data for peptides.

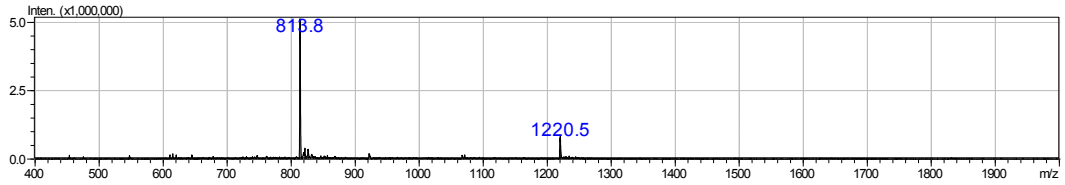
RGD: H-βAla-CRGDRGPDC-NH_2



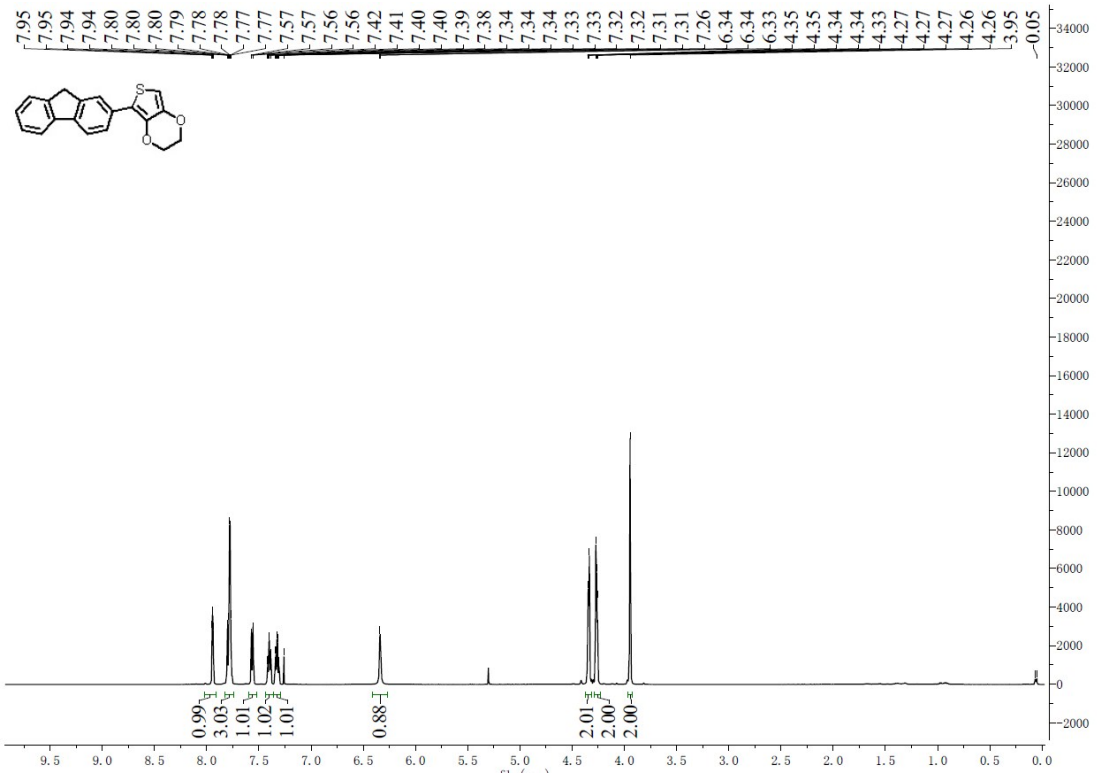
iRGD: H-βAla-c(CRGDRGPDC)-NH_2

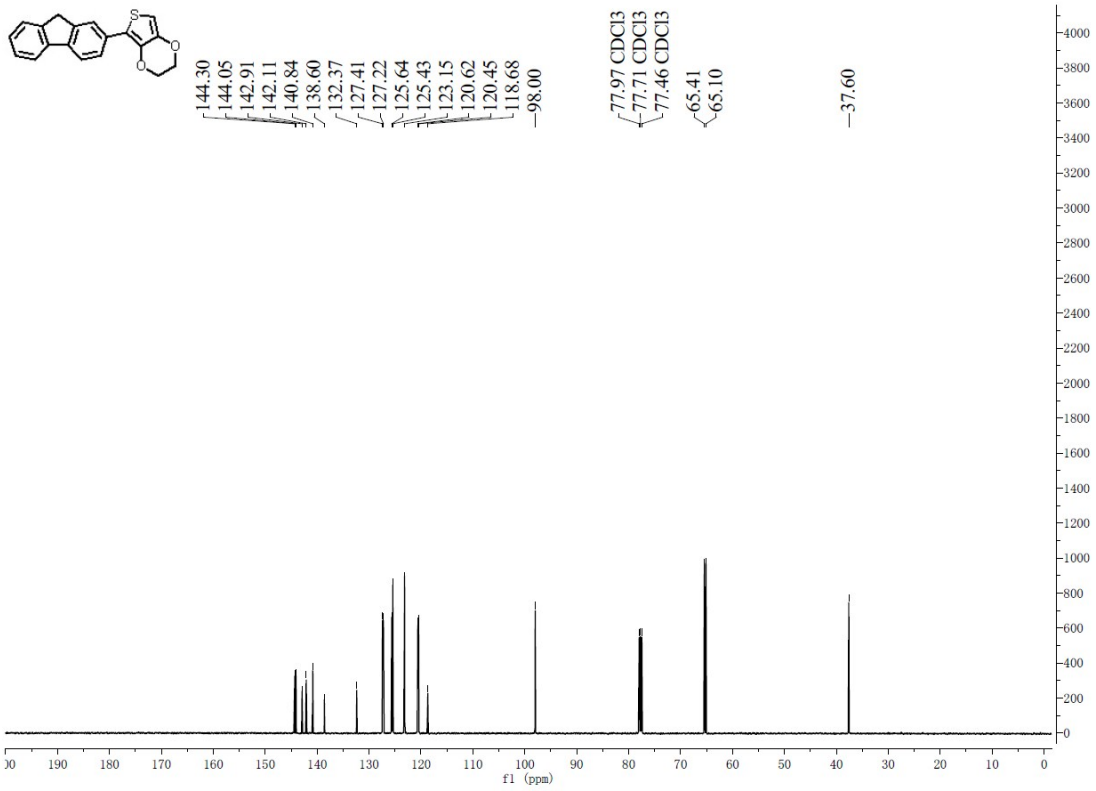


Cy5-iRGD: Cy5-βAla-c(CRGDRGPDC)-NH_2



NMR Spectrum

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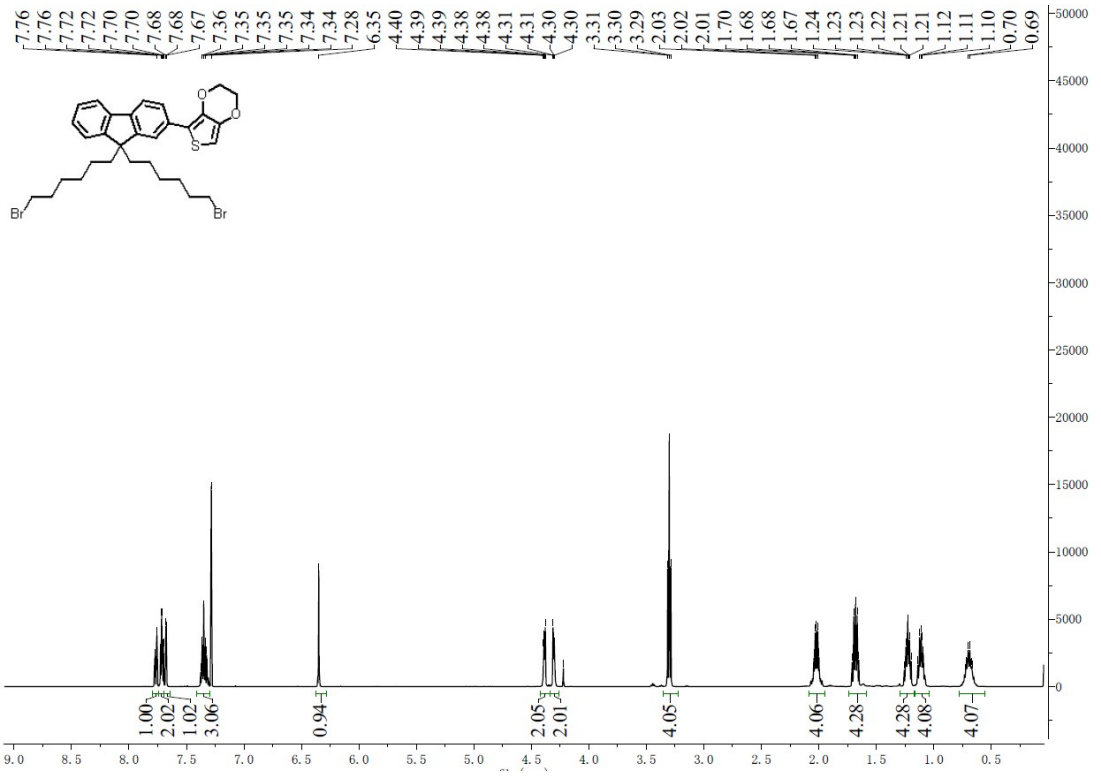
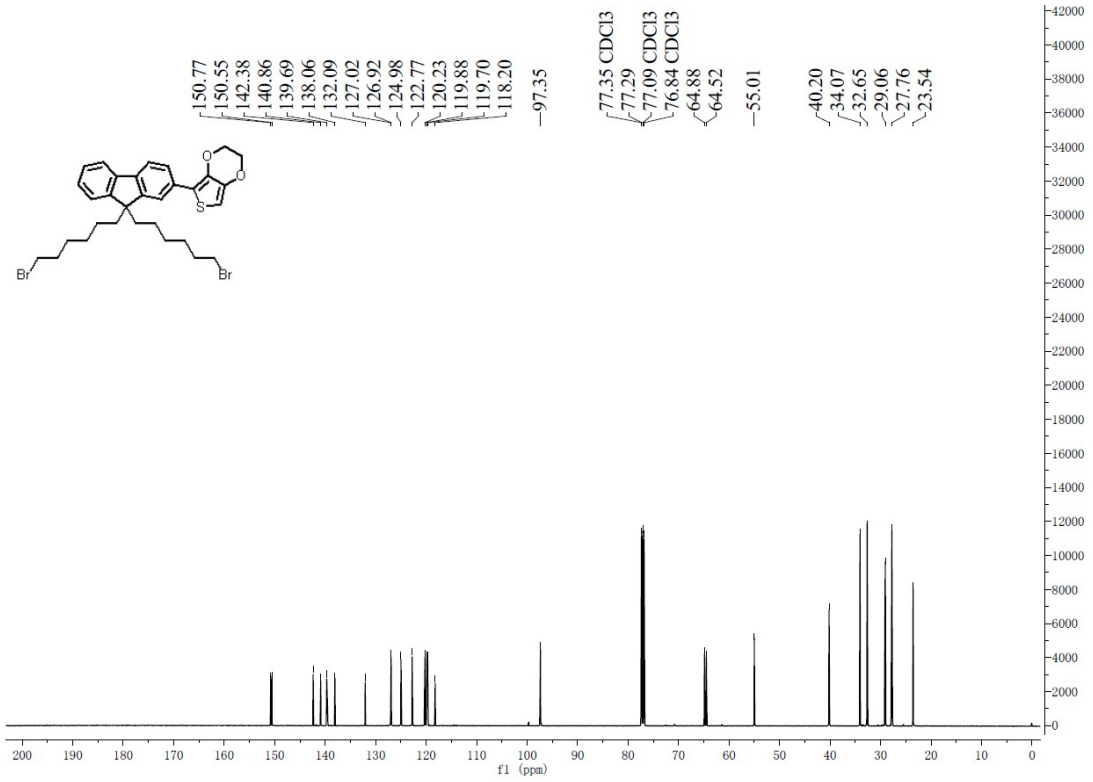
144.05
142.91
142.11
140.84
138.60
132.37
127.41
127.22
125.64
125.43
123.15
120.62
120.45
118.68
98.00
77.97 CDCl3
77.71 CDCl3
77.46 CDCl3
65.41
65.10
37.60
f1 (ppm)


Br
Br
1.00
2.02
1.02
3.06
0.94
2.05
2.01
4.05
4.06
4.28
4.28
4.08
4.07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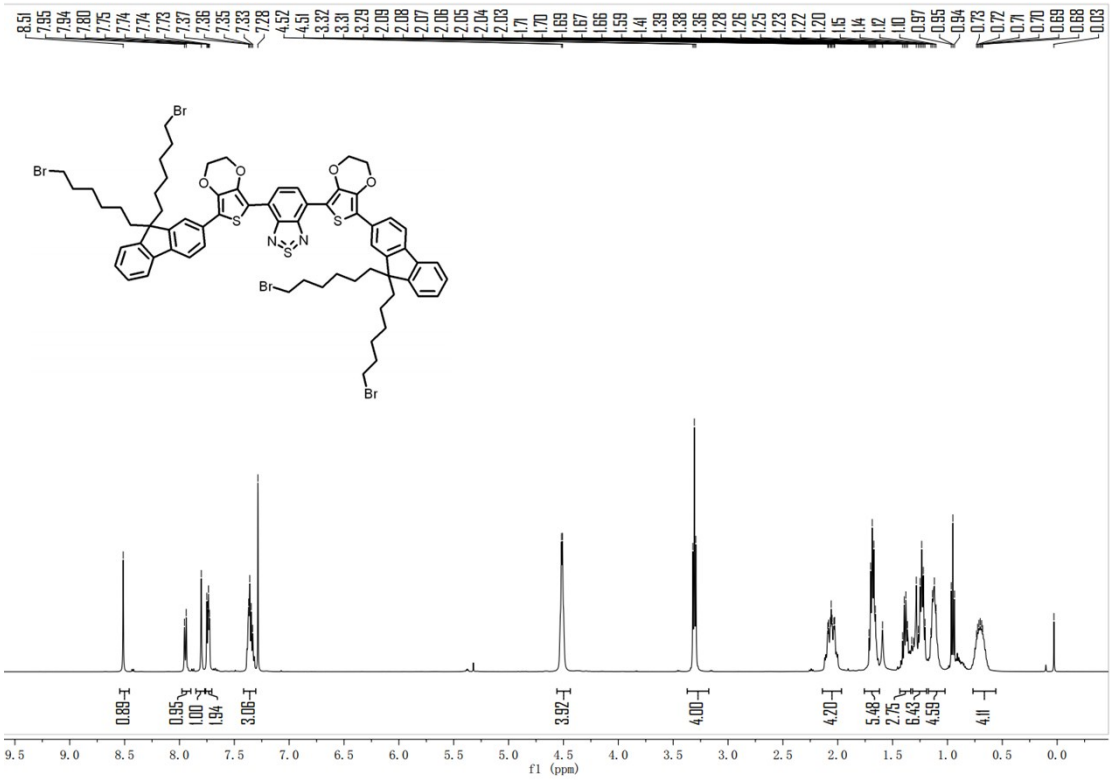
150.55
142.38
140.86
139.69
138.06
132.09
127.02
126.92
124.98
122.77
120.23
119.88
119.70
118.20
97.35
77.35 CDCl3
77.29
77.09 CDCl3
76.84 CDCl3
64.88
64.52
55.01
40.20
34.07
32.65
29.06
27.76
23.54
Br
Br
f1 (ppm)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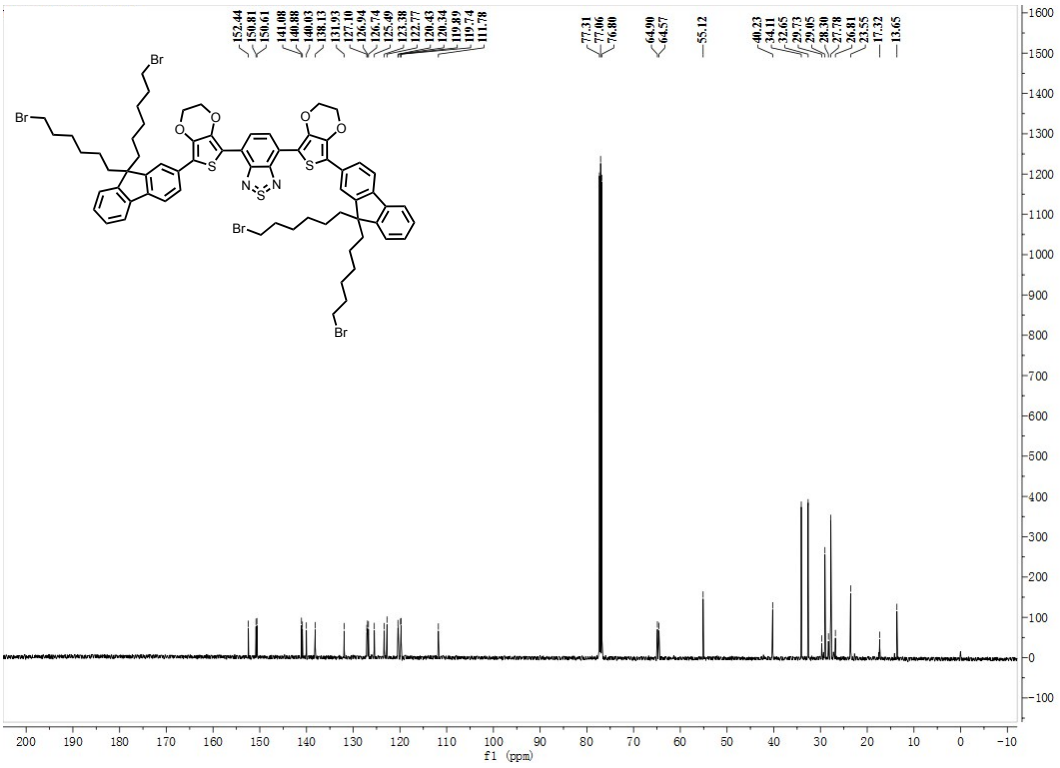
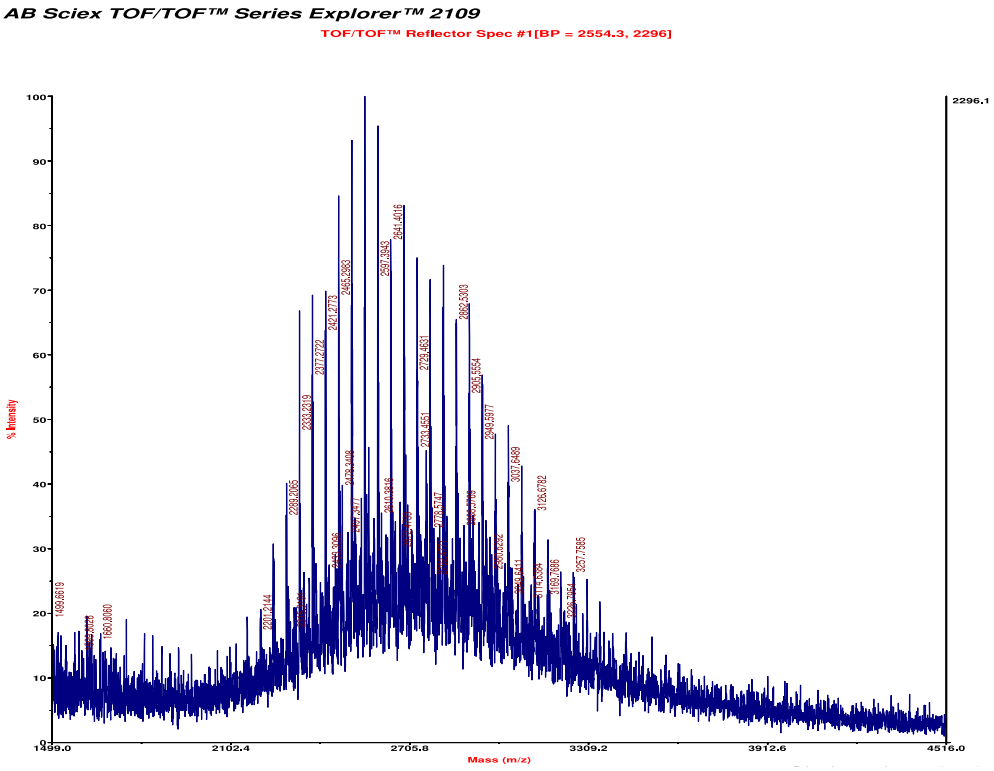
7.80
7.75
7.74
7.74
7.73
7.37
7.36
7.35
7.33
7.28
4.52
4.51
3.32
3.31
3.29
2.09
2.08
2.07
2.06
2.05
2.04
2.03
1.71
1.70
1.69
1.67
1.66
1.59
1.41
1.39
1.38
1.36
1.28
1.26
1.25
1.23
1.22
1.20
1.15
1.14
1.12
1.10
0.97
0.95
0.94
0.73
0.72
0.71
0.70
0.69
0.68
0.03
Br
Br
Br
Br
0.89
0.95
1.00
1.94
3.06
3.92
4.00
4.20
5.48
2.75
6.43
4.59
4.11
f1 (ppm)

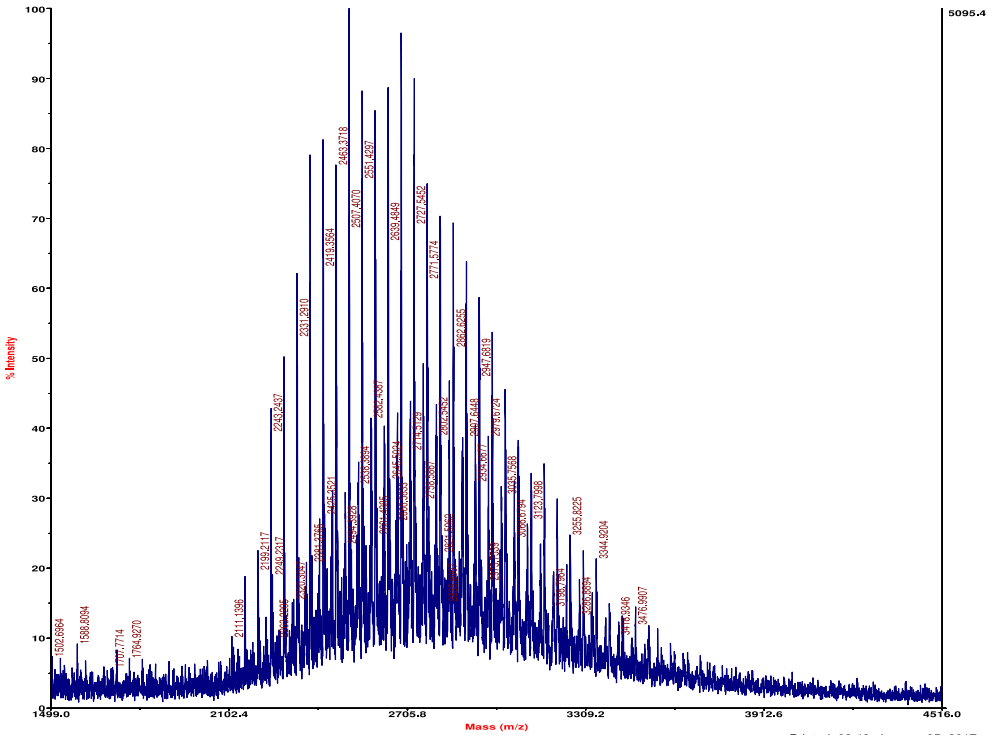


MALDI-TOF-MS data

PEG2000-RGD:

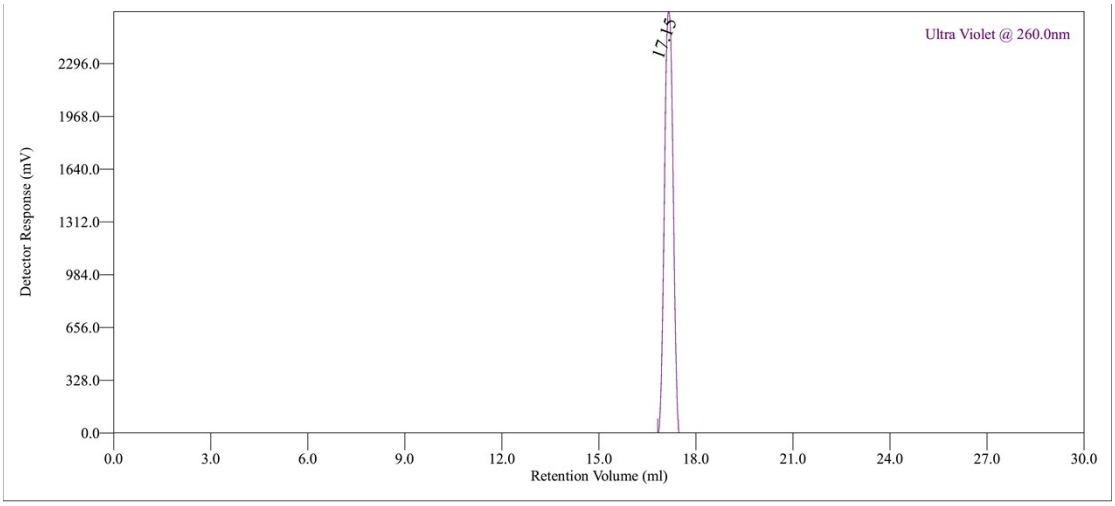


PEG2000-iRGD:



GPC data

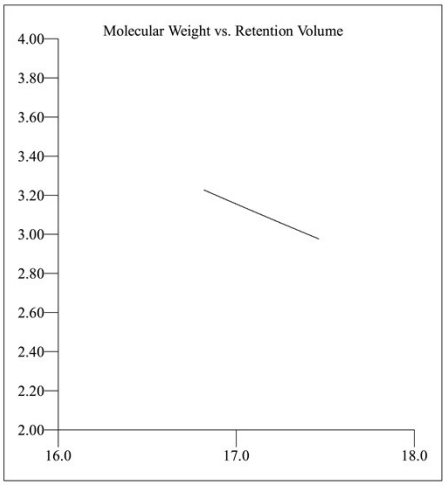
FEB-N_3:

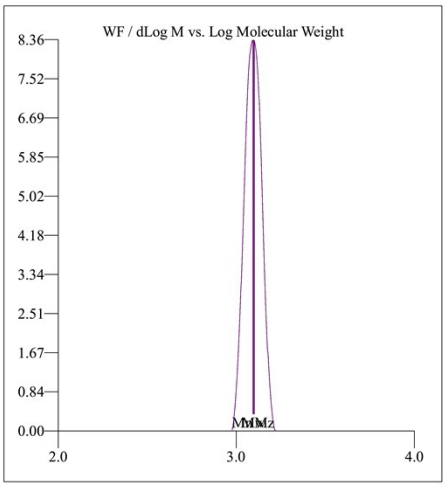


Conventional Calibration - Homopolymers : Results

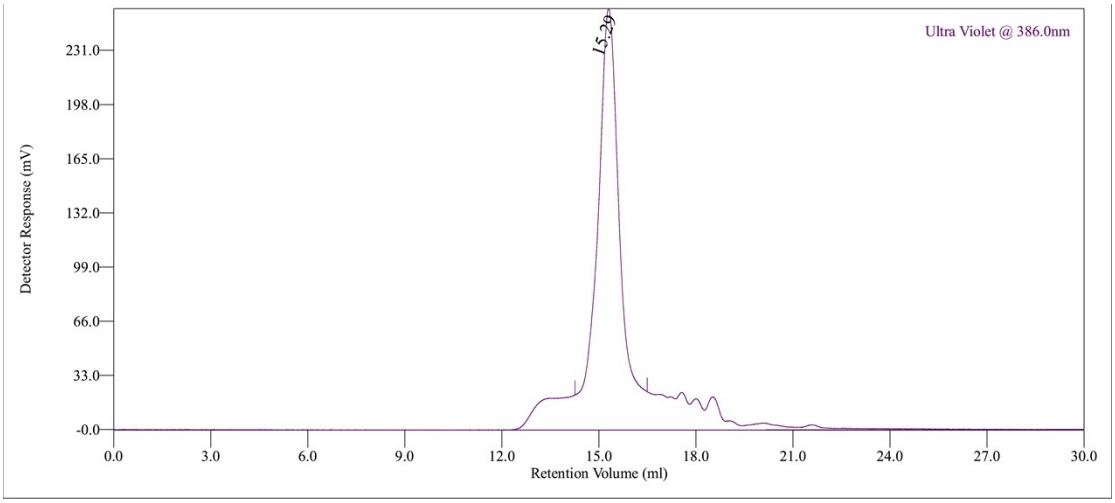
Peak RV - (ml)	17.147
Mn - (Daltons)	1,240
Mw - (Daltons)	1,252
Mz - (Daltons)	1,264
Mp - (Daltons)	1,249
Mw / Mn	1.010
Percent Above Mw: 0	100.000
Percent Below Mw: 0	0.000
Mw 10.0% Low	1,050
Mw 10.0% High	1,478
Wt Fr (Peak)	1.000
RI Area - (mvml)	0.00
UV@260nm Area - (mvml)	810.08

Annotation	
Method File	1226-0004.vcm
Limits File	
Date Acquired	Mar 01, 2017 - 14:16:01
Solvent	THF
Acquisition Operator	admin : Administrator
Calculation Operator	admin : Administrator
Column Set	GMHxl
System	System 1
Flow Rate - (ml/min)	1.000
Inj Volume - (ul)	100.0
Volume Increment - (ml)	0.00333
Detector Temp. - (deg C)	40.0
Column Temp. - (deg C)	40.0
OmniSEC Build Number	438





FEB-2000:



Conventional Calibration - Homopolymers : Results

Peak RV - (ml)	15.290
Mn - (Daltons)	6,780
Mw - (Daltons)	8,132
Mz - (Daltons)	9,710
Mp - (Daltons)	7,572
Mw / Mn	1.199
Percent Above Mw: 0	100.000
Percent Below Mw: 0	0.000
Mw 10.0% Low	3,336
Mw 10.0% High	16,203
Wt Fr (Peak)	1.000
RI Area - (mvml)	0.00
UV@386nm Area - (mvml)	208.89

Annotation	
Method File	Unsaved Method (1226-0004.vcm)
Limits File	
Date Acquired	Feb 24, 2017 - 16:54:32
Solvent	THF
Acquisition Operator	admin : Administrator
Calculation Operator	admin : Administrator
Column Set	GMHxl
System	System 1
Flow Rate - (ml/min)	1.000
Inj Volume - (ul)	100.0
Volume Increment - (ml)	0.00333
Detector Temp. - (deg C)	40.0
Column Temp. - (deg C)	40.0
OmniSEC Build Number	438