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Moving into the red – A near infra-red optical probe for analysis of human neutrophil elastase in activated neutrophils and Neutrophil Extracellular Traps

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1. Supplementary Figures

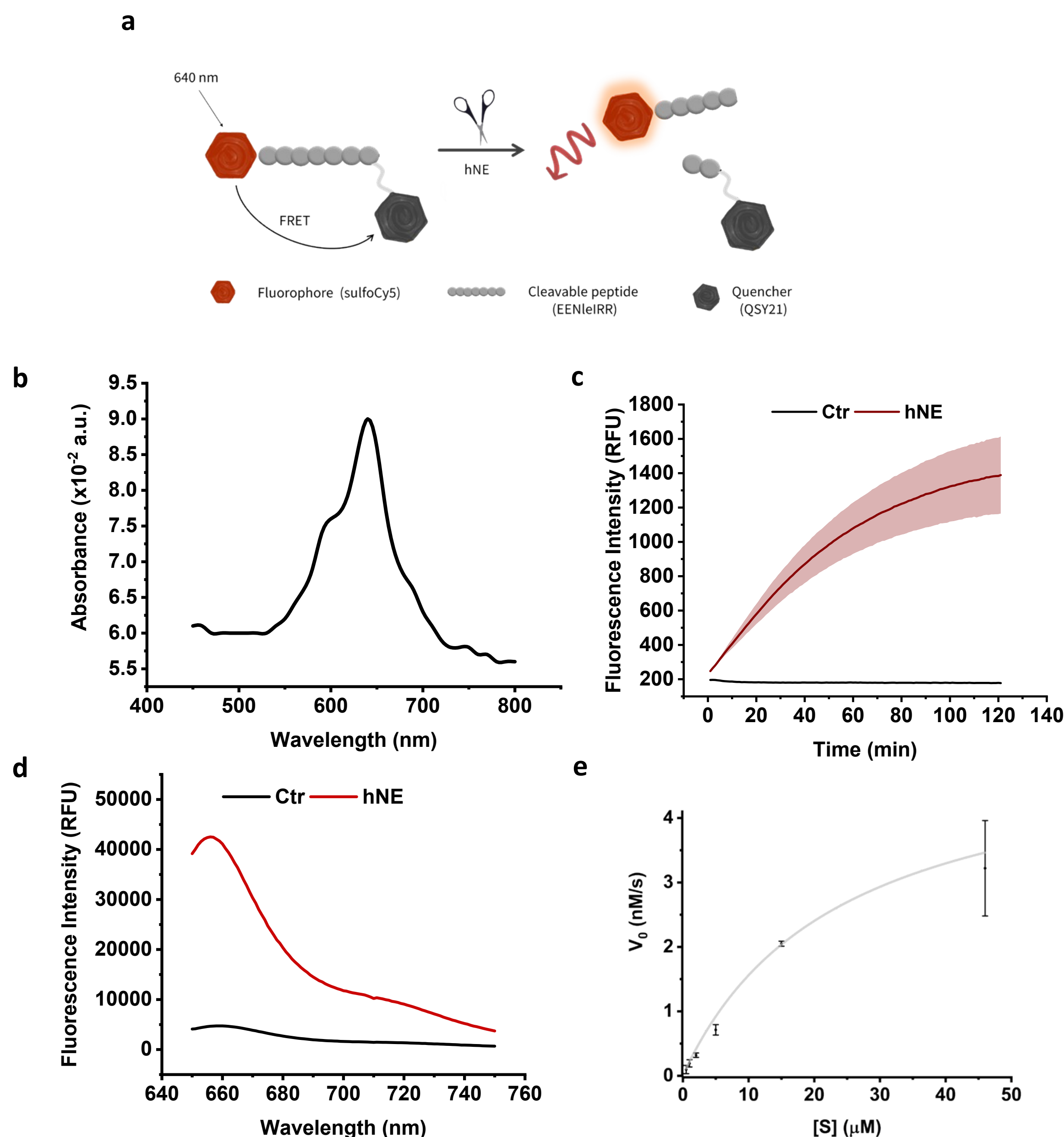


Figure S1. Probe **1 (HNE-1F1Q)** characterisation**. a)** Schematic representation of probe **1. b)** Absorption spectrum of **1** (38 μM); **c)** Time-dependent activation of **1** (38 μM) by hNE (100 nM), n = 3; **d)** Emission spectrum of the probe **1** (38 μM) in the presence and absence of hNE (100 nM), n = 3; **e)** Michaelis–Menten plot was generated from calculated initial velocities over the first 15 minutes of reaction at increasing concentrations of **1** in the presence of a constant concentration of hNE (100 nM). The kinetic values for Km displayed in **d** were calculated by analysing the Michaelis-Menten saturation curve and Eadie-Hofstee plots. Ctr: Control, no hNE, n = 3. Full structure of compound in main manuscript figure 1 or supponting information page 18.

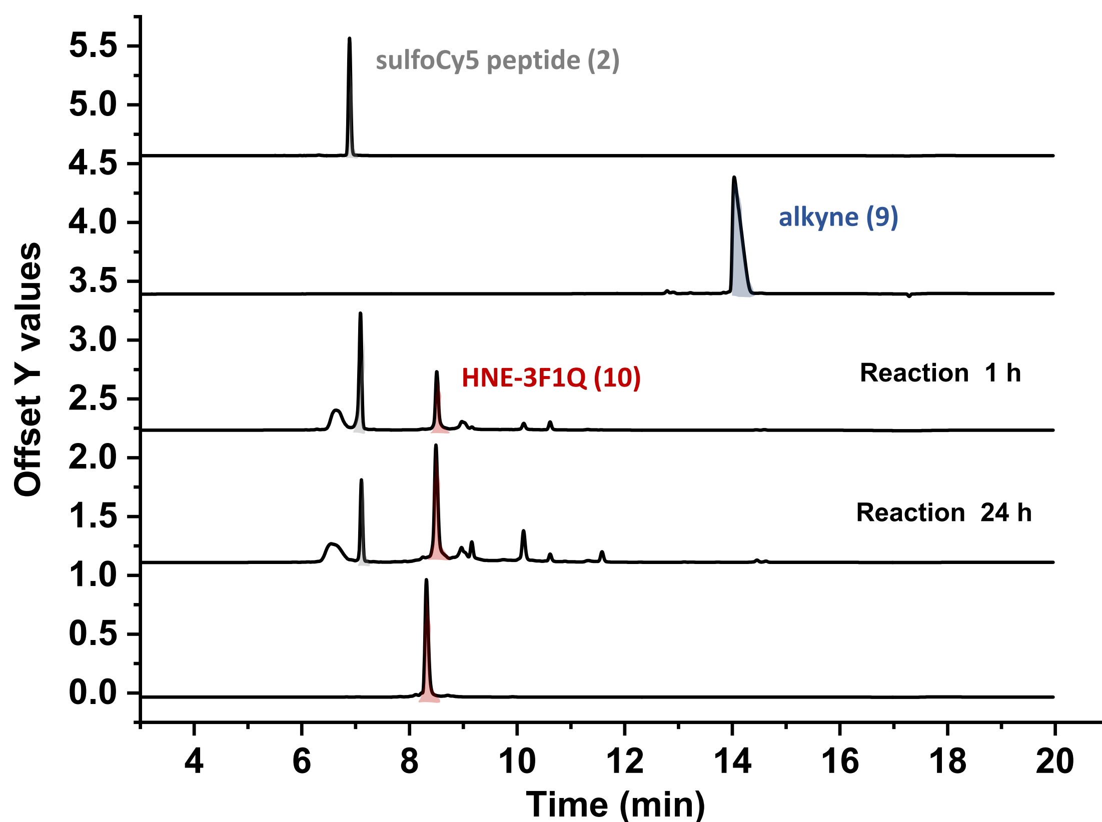


Figure S2. Reaction monitoring of the Copper-catalysed click reaction for the synthesis of **10** (**HNE-3F1Q**, red peak). Monitored by RP HPLC (650 nm). Four equivalents of sulfoCy5 labelled peptide (**2**, grey peak) were reacted with 1 equivalent of the alkyne **9** (blue peak) in the presence of CuI/THPTA. Progress was monitored by changes in the peaks (**2** and **9**), which are consumed over time (Note: 1 equivalent of peptide **2**, will remain after 100% conversion of the alkyne). As the alkyne/peptide are consumed, the appearance of a new peak corresponding to the product **10** (red peak) is observed.

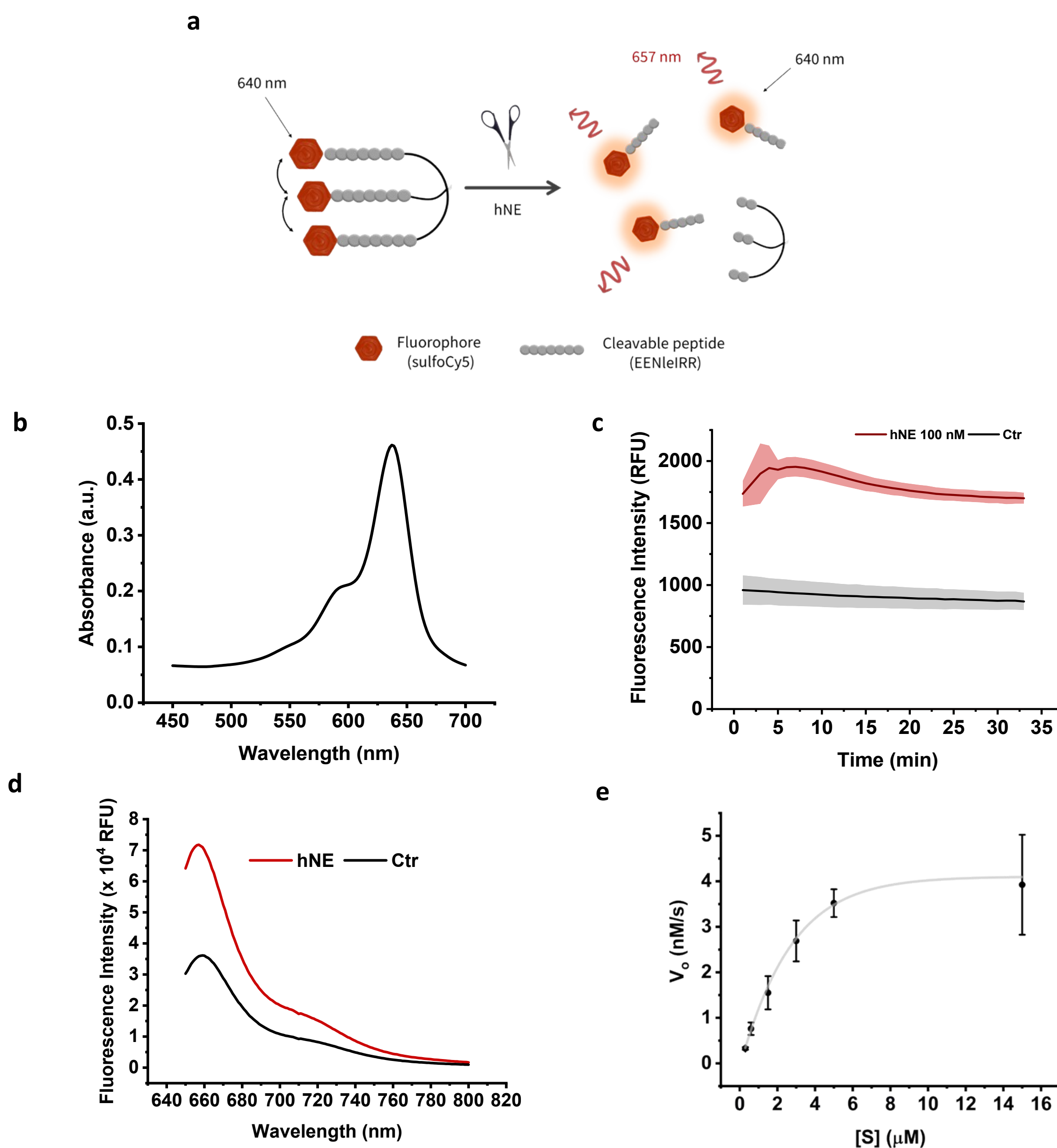


Figure S3. Characterisation of the self-quenching probe **11** (**HNE-3F0Q**). **a)** Schematic representation of probe **11**. **b)** Absorption spectrum of **11** (12 µM) shows a maximum absorption at 640 nm**; c)** Activation of **11** (12 µM) happens within seconds in the presence of hNE (100 nM) but the background fluorescence of the uncleaved probe is high, only giving an approximately 2-fold increase in fluorescence; **d)** Fluorescence emission of **11** in the presence and absence of hNE (100 nM) (maximum emission at 657 nm); **e)** Michaelis–Menten saturation curve for activation of increasing concentrations of **11** in the presence of hNE (10 nM). Kinetic values were calculated by analysing the Michaelis-Menten saturation curve and Eadie-Hofstee plots. Ctr = no hNE. Full structure of compound in page 22.

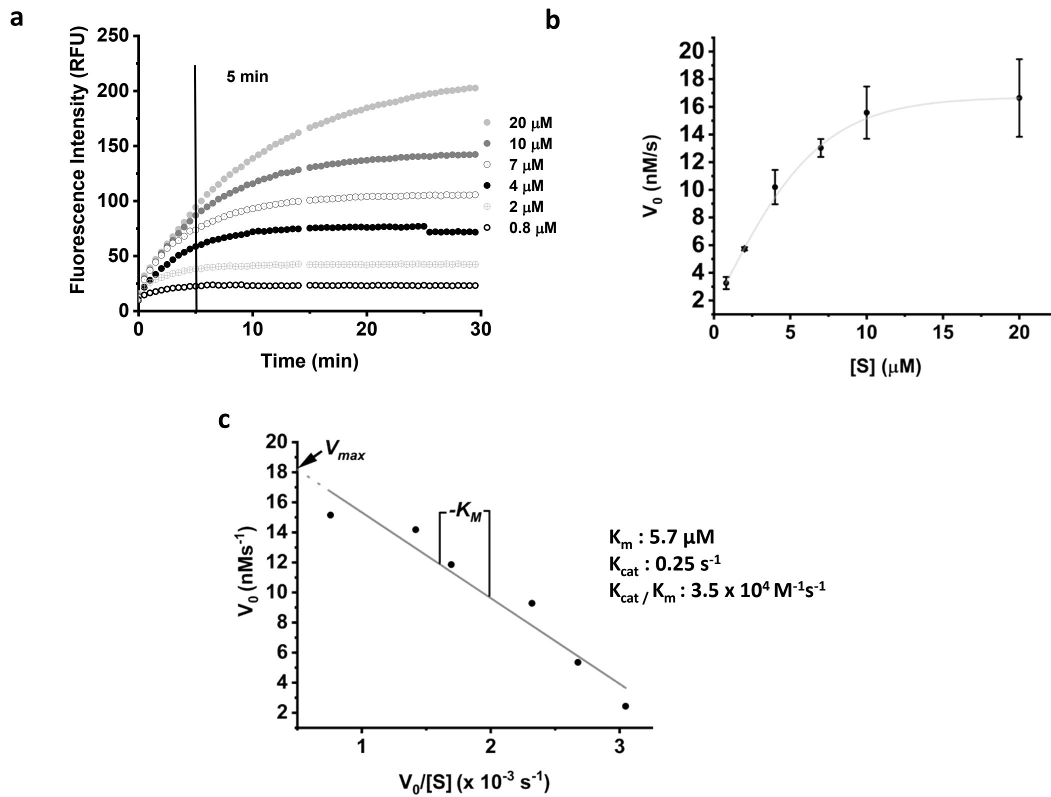


Figure S4 Kinetic parameters were obtained for **10 (HNE-3F1Q**), with the initial velocities plotted using Michaelis Menten, Lineweaver Burk or Eadie Hofstee graphs to obtain the corresponding kinetic parameters K_m, k_{cat} and turnover number (k_{cat}/K_m). **a)** Activation at increasing concentrations of probe **10** (0.8 µM to 20 µM) in presence of a constant concentration of protease hNE (100 nM) and calculation of initial velocities over the first 5 minutes of incubation. **b)** Michaelis Menten curves generated from the initial velocities calculated in the range of 0.8 to 20 µM allows: **c)** Eadie Hofstee plot (V vs V/[S]). All datapoints are n=3

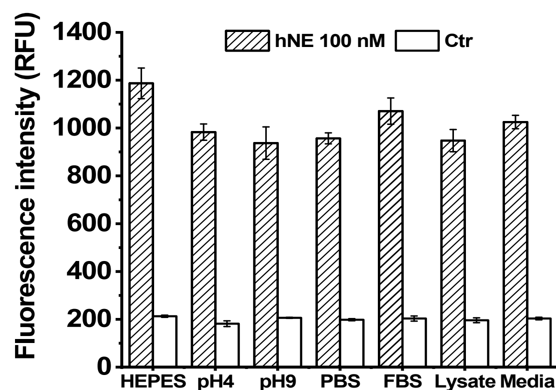


Figure S5 Stability of **10** (HNE-3F1Q) signal (10 µM) in different environments. The fluorescence of the cleaved and uncleaved probe was monitored in these different conditions for 1.5 h. No activation of the probe or changes in fluorescence was detected in the absence of the protease in the different environments. Ctr: no hNE.

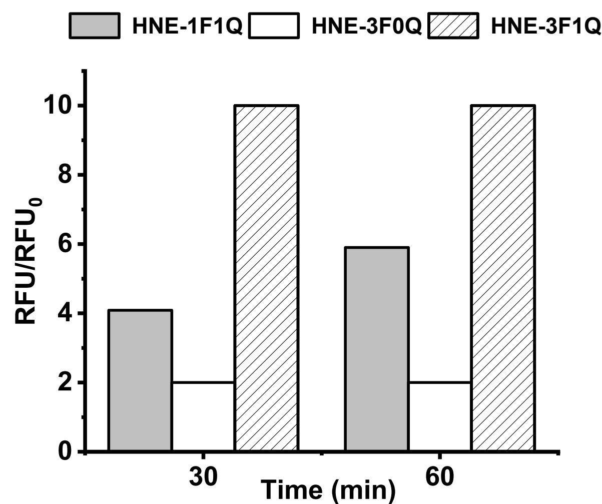


Figure S6 Comparison of activation profiles of the linear FRET-peptide probe **11** (HNE-1F1Q, 35 µM), the self-quenching probe **11** (HNE-3F0Q, 12 µM) and the super silent probe **10** (HNE-3F1Q, 12 µM) in the presence of hNE (100 nM). Three equivalents of the linear probe **1** were used referent to probes **10** and **11** (35 µM and 12 µM, respectively) to make the sulfonated cyanine 5 concentration equivalent. The graph shows the fold increases in fluorescence following 30 or 60 min incubation with hNE.

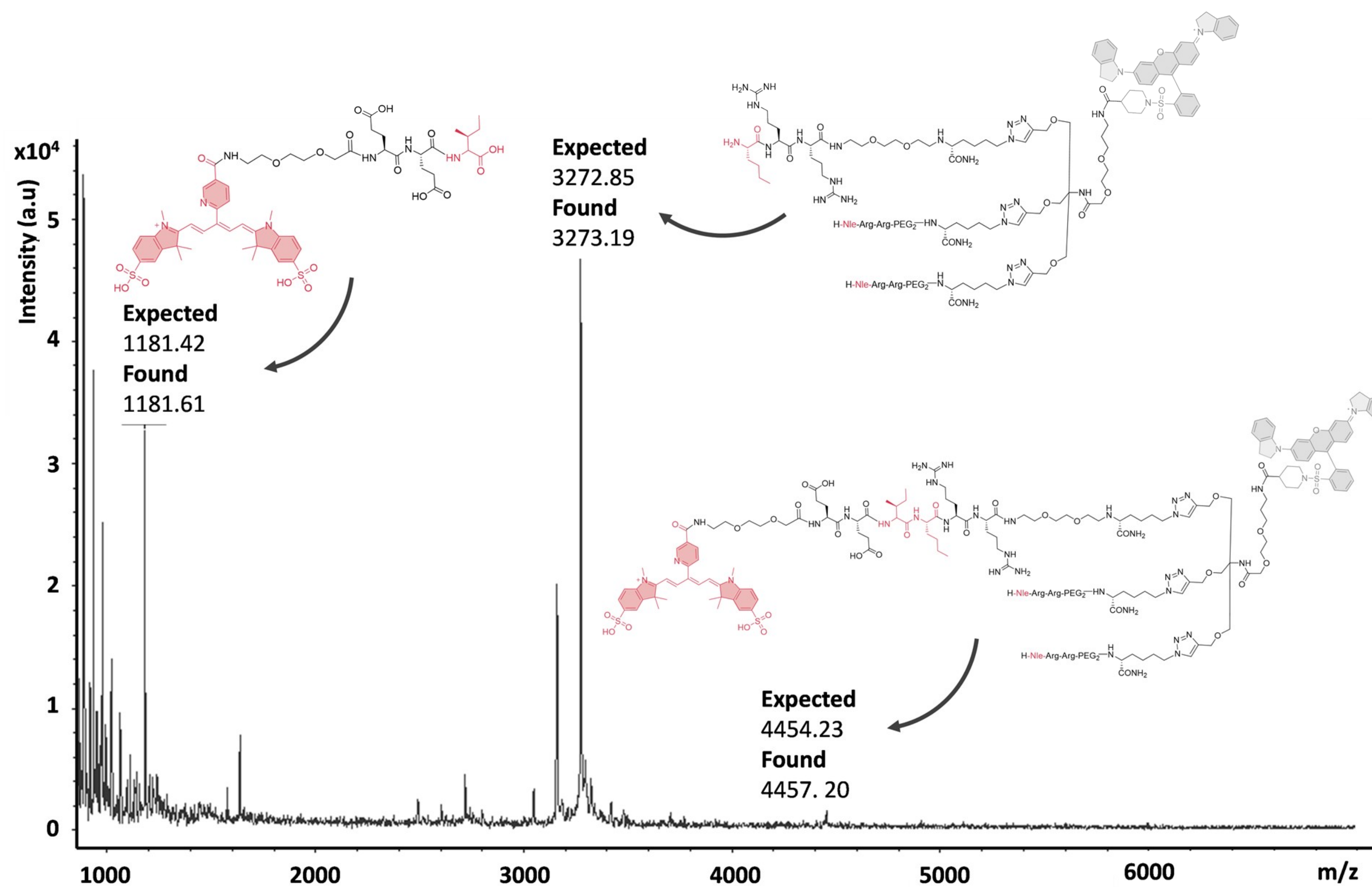


Figure S7. Cleavage site determination following cleavage of probe **10** (HNE-3F1Q). MALDI-TOF MS analysis of the activated probe **10.** Fully cleaved C-terminal fragment of HNE-3F1Q (m/z 3273), N-terminal fragment (m/z 1181) as well as the probe with one branch intact (m/z 4457) were detected in the crude.

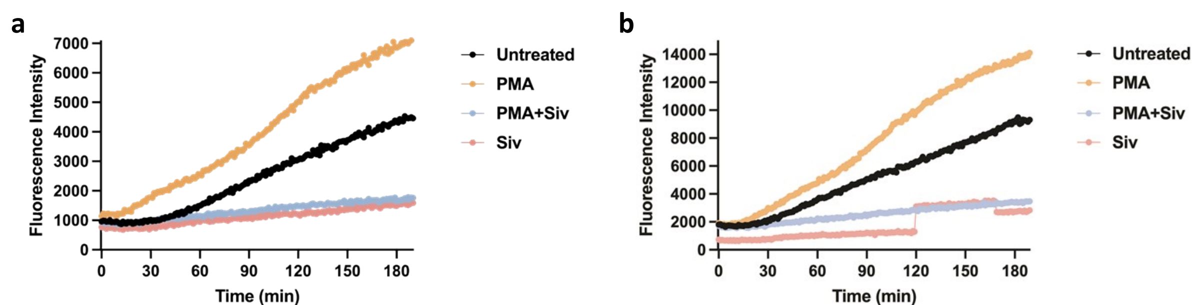


Figure S8. Neutrophils (10 x 10^5/well) were plated in a 96 well round bottom plate and pre-treated with or without Sivelestat (100 µM) for 30 minutes and with or without PMA (10 nM). A time dependent increase in fluorescence signal was observed over 3 hours (λ_{ex} 640/10, λ_{em} 680/20). **a)** 3 µM **b)** 5 µM of probe **10** (HNE-3F1Q).

2. Experimental Procedures. Chemistry

General

All Fmoc-amino acids, DIC, Oxyma and aminomethyl polysterene resin (0.75 mmol/g) and Fmoc-Rink Amide Linker were purchased from GL Biochem, Sigma, Fluorochem or Apollo Scientific and used without further purification. The sulfonated cyanine 5 and QSY21-NHS ester was prepared according to a previously reported procedure.[1,2] Electrospray ionization mass spectrometry (ESI-MS) analyses were carried out on an Agilent Technologies LC/MSD quadrupole 1100 series mass spectrometer (QMS) in an ESI mode. High-resolution mass spectra (HR-MS) were recorded on a Bruker SolariX Fourier transform ion cyclotron resonance mass spectrometer (FT-MS). MALDI-TOF spectra were acquired on a Bruker Ultraflextreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in H_2O/CH_3CN/TFA (69.9/30/0.1) or α-cyano-4-hydroxycinnamic acid (10 mg/mL) in H_2O/CH_3CN/TFA (49.9/50/0.1). NMR spectra were recorded using Bruker AC spectrometers operating at 500 MHz for ^1H and ^{13}C NMR. Chemical shifts are reported on the δ scale in ppm and are referenced to residual non-deuterated solvent resonances (proton) or deuterated solvent (carbon). Compounds **5** and **6** were prepared according to a previously reported procedure.[4]

Analytical reverse-phase high-performance liquid chromatography (RP HPLC) was performed on an HP1100 system equipped with a Kinetex 5 µm XB-C18 reverse-phase column (5 cm ×

4.6 mm, 5 µm) with a flow rate of 1 mL/min and eluting with $H_2O/CH_3CN/HCOOH$ (95/5/0.1) to $H_2O/CH_3CN/HCOOH$ (5/95/0.1). Method A was used as default unless otherwise stated.

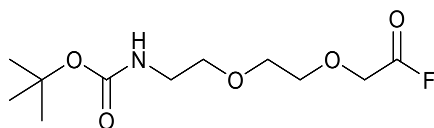
- **Method A:** 10 minutes. With a gradient from 5 % to 95% CH_3CN over the first 6 min then holding at 95% for 3 min, with detection at 495 and/or 650 nm and by evaporative light scattering.
- **Method B:** 20 minutes. With a gradient from 5 % to 95% CH_3CN over the first 15 min then holding at 95% for 4 min, with detection at 495 and/or 650 nm and by evaporative light scattering.

The preparative system was equipped with a Kinetex 5 µm XB-C18 100 Å, reverse-phase column (150 × 21.2 mm, AXIA packed). The separation was achieved with a method with a flow rate of 10 mL/min and eluting with $H_2O/CH_3CN/HCOOH$ (95/5/0.1) to $H_2O/CH_3CN/HCOOH$ (5/95/0.1) over a gradient of 21 minutes.

The semipreparative system was equipped with an Aeris 5 µm XB-C18 100 Å, reverse-phase column (250 × 10 mm). Separation was achieved with a flow rate of 10 mL/min and eluting with $H_2O/CH_3CN/HCOOH$ (95/5/0.1) to $H_2O/CH_3CN/HCOOH$ (5/95/0.1) with a gradient over 30 or 35 minutes.

2.1 Alkyne building block synthesis

***N*-Boc-3-[2-(2-aminoethoxy)ethoxy]ethanoic acid fluoride (3)**

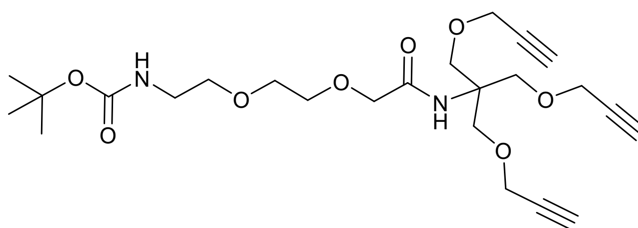


8-*Tert*-butyloxycarbonylamino-3,6-dioxaoctanoic acid (264 mg, 1.0 mmol, 1 eq) was dissolved in anhydrous DCM (5 mL) and cyanuric fluoride (172 µL, 2 eq, 2.0 mmol) was added followed by pyridine (81 µL, 2 eq) and the reaction stirred for 2 h. Conversion was monitored by TLC (MeOH:DCM 1:9) and LC-MS by adding a small amount of the reaction mixture into anhydrous

MeOH to form the corresponding methyl ester for analysis). Upon completion, ice water (100 mL) was added to the reaction mixture and the organic layer was dried with $MgSO_4$ and evaporated *in vacuo*. The product was used immediately without further purification (223 mg, 80%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.1 (s, 1H) 4.34 (d, *J* = 3.5 Hz, 2H), 4.16 (s, 2H), 3.79 – 3.73 (m, 2H), 3.68 – 3.63 (m, 2H), 3.53 (t, J = 5.2 Hz, 2H), 1.44 (s, 9H). **^{13}C NMR** (126 MHz, Chloroform-*d*) δ 162.2, 159.3, 156.2, 79.5, 71.5, 71.4, 70.5, 66.8, 66.3, 40.5, 28.4. **LC-MS (ESI)**: calculated for $C_{12}H_{23}NO_6$ (methyl ester) $[M-F+OMe+Na]^+$ 300.1, found 300.1 **HPLC-ELSD (Method A)** ($MeOH/H_2O$, 15 min): 6.9 min, purity 85 %.

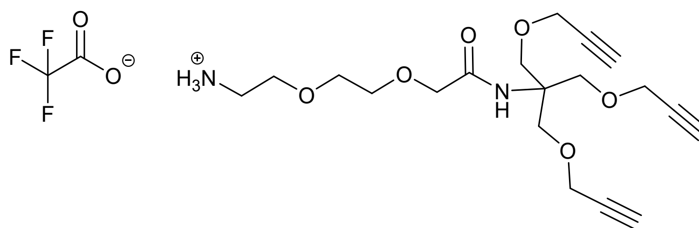
***N*-Boc-2-[2-(2-aminoethoxy)ethoxy]ethanamide, *N*-[2-(2-propyn-1-yloxy)-1,1-bis[(2-propyn-1-yloxy)methyl]ethyl] (7)**



Compound **3** (70 mg, 1 eq, 0.3 mmol) was dissolved in anhydrous DMF (5 mL) and DIPEA (0.5 mL, 3 eq. 3.0 mmol) was added followed by *tris*(hydroxymethyl)propargyl **6**[3] (80 mg, 1 eq, 0.3 mmol) and the reaction stirred overnight. Conversion was monitored by TLC and RP-HPLC. The solvent was evaporated *in vacuo* and the product was purified by column chromatography (eluted with DCM:MeOH 9:1) (110 mg, 75 %).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.84 (br s, 1H), 5.03 (br s, 1H), 4.17 (d, *J* = 2.4 Hz, 6H), 3.92 (s, 2H), 3.88 (s, 6H), 3.69 – 3.59 (m, 4H), 3.56 (t, *J* = 5.2 Hz, 2H), 3.34 (quartet, *J* = 5.4 Hz, 2H), 2.45 (t, *J* = 2.4 Hz, 3H), 1.46 (s, 9H). **^{13}C NMR** (126 MHz, Chloroform-*d*) δ 169.6, 156.0, 79.6, 79.3, 74.7, 71.0, 70.9, 70.5, 70.1, 68.5, 59.1, 58.7, 40.4, 28.4. **LC-MS (ESI)** $[M + Na]^+$ m/z 503.1; **FT-HRMS :** calculated for $C_{24}H_{37}N_2O_8$ $[M+Na]^+$ m/z 503.2380 Found: $[M+Na]^+$ 503.2364**; HPLC-ELSD (Method A): t_R** 5.08 min, purity 86 %.

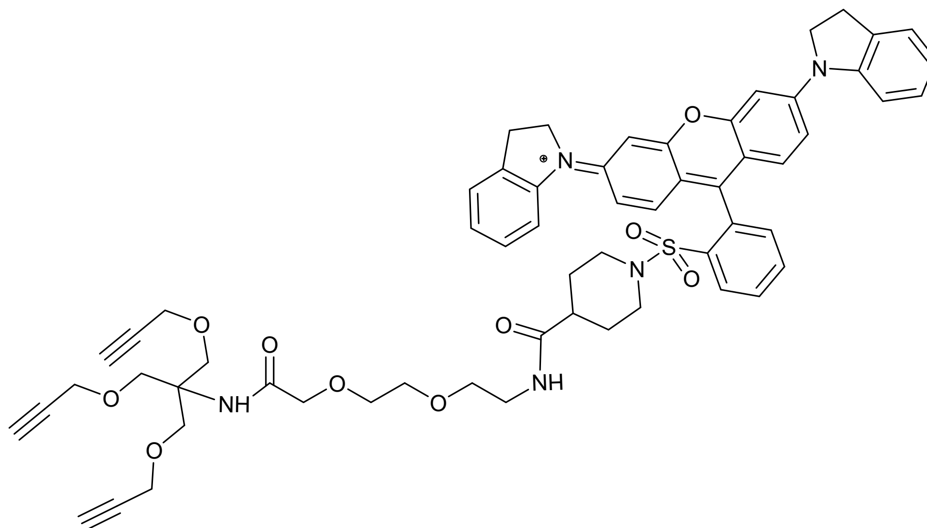
2-[2-(2-aminoethoxy)ethoxy]-ethanamide, *N*-[2-(2-propyn-1-yloxy)-1,1-bis[(2-propyn-1-yloxy)methyl]ethyl] 2,2,2-trifluoroacetate (8)



Compound **7**[3] (110 mg, 0.3 mmol) was dissolved in 20% TFA in DCM (5 mL) and stirred for 2 h. The solvent was evaporated under vacuum and the product used without further purification (quantitative).

^{1}H NMR (500 MHz, MeOD-*d4*) δ 4.19 (d, *J* = 2.4 Hz, 6H), 3.98 (s, 2H), 3.85 (s, 6H*)* 3.79-3.74 (m, 6H), 3.19 – 3.13 (m, 2H), 2.89 (t, *J* = 2.4 Hz, 3H), 1.31 (br s, 3H).**^{13}C NMR** (126 MHz, MeOD-*d4*) δ 170.7, 79.1, 74.8, 70.5, 70.0, 69.9, 67.9, 66.6, 59.3, 58.1, 39.3. **LC-MS (ESI):** $[M + Na]^+$ 403.1; **FT-HRMS:** calculated for $C_{19}H_{29}N_2O_6$ $[M+H]^+$ m/z 381.2020 Found: $[M+H]^+$ 381.2022; **HPLC-ELSD (Method A):** t_R 3.29 min, purity 93%.

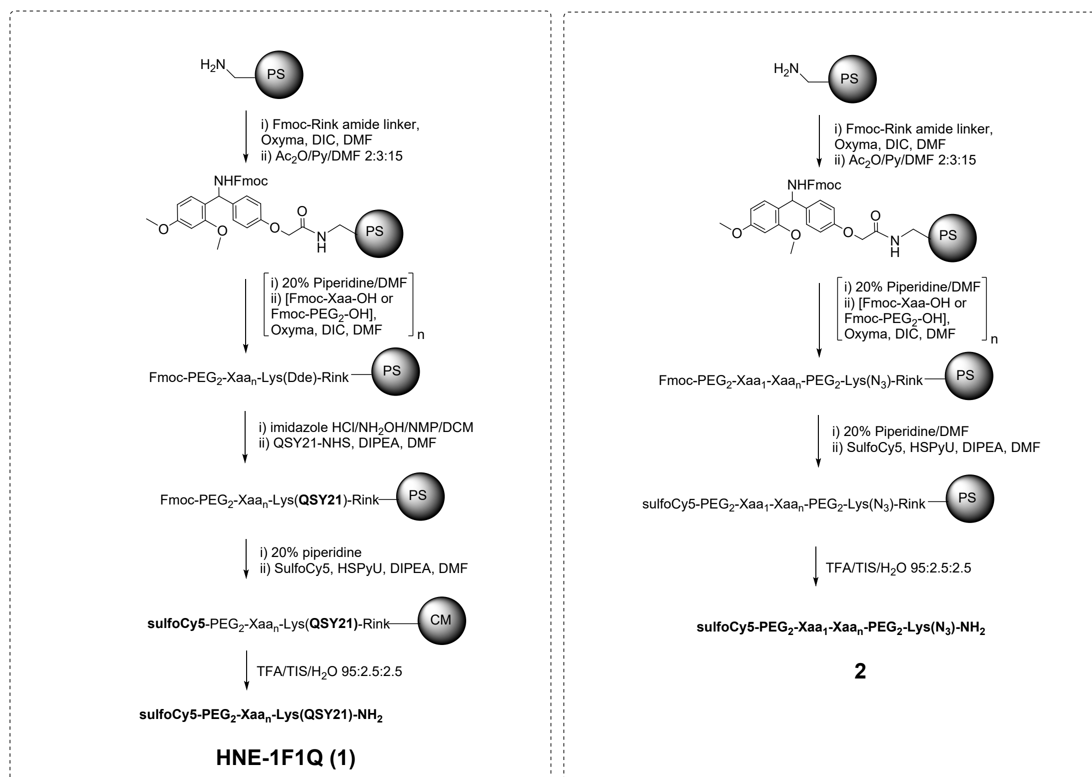
***N*-QSY21-2-[2-(2-ethoxiamide)ethoxy]-ethanamide, *N*-[2-(2-propyn-1-yloxy)-1,1-bis[(2-propyn-1-yloxy)methyl]ethyl] (9)**



QSY21-NHS[2] (130 mg, 1 eq, 0.17 mmol) was dissolved in anhydrous DMF and **8** (90 mg, 1.5 eq, 0.23 mmol) was added followed by DIPEA (100 µL, 2.5 eq, 0.42 mmol), and the reaction stirred at 50 ^{o}C for 6 h. Conversion was monitored by TLC and RP-HPLC. The solvent was removed *in vacuo* and the mixture was purified by column chromatography (eluting with DCM:MeOH 9:1) to give the product **9** as a dark blue film (80 mg, 45%).

^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.65 (br s, 1H), 8.17 (d, *J* = 7.4 Hz, 1H), 7.96 – 7.84 (m, 2H), 7.61 (d, *J* = 8.1 Hz, 1H), 7.55 (d, *J* = 9.3 Hz, 1H), 7.48 – 7.41 (m, 2H), 7.38 (d, *J* = 7.4 Hz, 1H), 7.36 – 7.29 (m, 1H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.19 – 7.14 (m, 2H), 7.10 (t, *J* = 8.9 Hz, 1H), 6.97 (d, 1H), 6.88 (dd, *J* = 8.5, 2.3 Hz, 1H), 6.86 – 6.81 (m, 1H), 6.77 (t, *J* = 7.3 Hz, 1H), 6.40 – 6.33 (m, 1H), 6.25 (br s,1H), 4.40 (q, *J* = 8.2 Hz, 1H), 4.31 (q, *J* = 8.2 Hz, 1H), 4.16 (dd, *J* = 9.2, 2.3 Hz, 6H), 4.01 – 3.91 (m, 4H), 3.87 (d, *J* = 13.2 Hz, 6H), 3.72 – 3.68 (m, 1H), 3.68 – 3.60 (m, 3H), 3.58 (t, J = 5.2 Hz, 1H), 3.55 (t, J = 6.0 Hz, 1H), 3.49 (q, J = 5.3 Hz, 1H), 3.43 (d, J = 12.1 Hz, 1H), 3.38 (q, *J* = 5.6 Hz, 1H), 3.33 (t, *J* = 7.9 Hz, 2H), 3.13 (t, *J* = 8.4 Hz, 3H), 3.10 – 3.03 (m, 1H), 2.65 – 2.56 (m, 1H), 2.47 (dt, *J* = 11.5, 2.3 Hz, 3H), 2.45 – 2.38 (m, 1H), 2.05 – 2.00 (m, 1H), 1.99 – 1.92 (m, 1H), 1.82 – 1.76 (m, 1H), 1.61 (qd, J = 12.1, 4.1 Hz, 1H). **^{13}C NMR** (126 MHz, Chloroform-*d*) δ 173.9, 169.9, 157.6, 152.5, 151.3, 146.5, 143.9, 142.2, 136.6, 134.7, 133.9, 132.3, 131.3, 130.7, 128.4, 127.8, 127.1, 126.3, 125.2, 125.0, 119.1, 116.5, 113.9, 99.9, 79.6, 79.5, 74.8, 71.0, 70.9, 70.8, 70.3, 69.9, 69.8, 68.5, 59.2, 59.1, 58.7, 53.2, 52.0, 45.4, 45.0, 39.2, 38.8, 37.3, 29.7. **LC-MS (ESI):** $[M+H]^{+2}$ 522.9.; **FT-HRMS:** calculated for $C_{60}H_{62}N_5O_{10}S$ $[M]^+$ m/z 1044.4212 Found: $[M]^+$ 1044.4212; **MALDI-TOF MS:** calculated for $C_{60}H_{62}N_5O_{10}S$ $[M]^+$ m/z $[M]^+$ 1046.77; **HPLC-ELSD (Method A): t_R** 7.63 min, purity 92 %.

2.2 General solid-phase synthesis methods for Fmoc SPPS



Scheme S1. Synthesis of the linear peptides.

Rink amide linker attachment to ChemMatrix or Polystyrene resin: The Fmoc-Rink-amide linker (3 eq) was dissolved in DMF (10 mL) and Oxyma (3 eq) was added and the mixture was stirred for 10 min. DIC (3 eq) was added and the mixture was stirred for further 1 min. The solution was added to the resin (0.7 mmol/g PS or 1.0 mmol/g CM, 1 eq, pre-swollen in DCM) and shaken for 2 h. The resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The coupling reaction was monitored by a Kaiser test.

Dde deprotection[4]: To the resin (pre-swollen in DCM), 2% hydrazine in DMF was added and the reaction mixture was shaken for 2h. The resin was filtered and washed with DMF (3 × 20 mL), DCM (3 × 20 mL) and MeOH (3 × 20 mL). Dde deprotection in the Fmoc or sulfoCy5 containing peptides was achieved with a solution containing imidazole (1.35 mmol) and hydroxylamine hydrochloride (1.80 mmol) in NMP (5 mL). After complete dissolution 5 volumes of this solution were diluted with 1 volume of CH_2Cl_2 and the resin was treated with the final mixture for 3 h at room temperature. The solution was drained and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL).

Fmoc deprotection: To the resin (pre-swollen in DCM), 20% piperidine in DMF was added and the reaction mixture was shaken for 10 min. The solution was drained and the resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). This procedure was reaped twice.

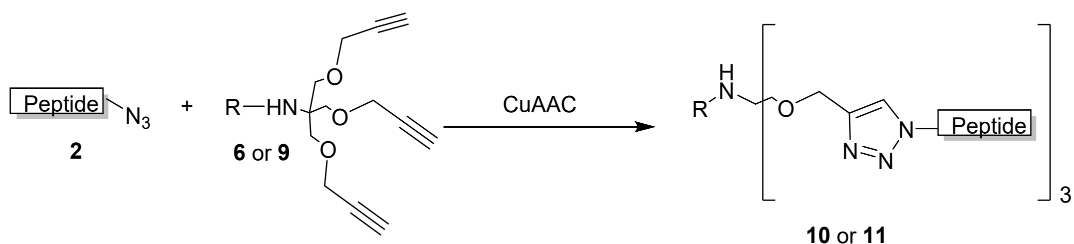
Amino acid and dye couplings: A solution of the *N*-Fmoc-protected amino acid, Fmoc-$(EG)_2$-OH (3 eq), Fmoc-Lys(Dde)-OH with Oxyma (3 eq) in DMF (0.1 M per amino) was stirred for 10 min. DIC (3 eq) was added and the solution was stirred for further 1 min. The solution was added to the resin (1 eq, pre-swollen in DCM) and the reaction mixture was shaken for 30 min at 50° C, except for Fmoc-Lys(MR)-OH that was shaken for 1 h at 50° C. The solution was drained and the resin washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The coupling reactions were monitored by a Kaiser test.

N-terminal capping with sulfo-Cy5 dye: A solution containing sulfo-Cy5[1] (1.0 eq per amine) in anhydrous DMF (10 mg/mL) was activated with *N,N,N',N'*-bis(tetramethylene)-*O*-(*N*-succinimidyl)uranium hexafluorophosphate (HSPyU) (1.0 eq) and DIPEA (3 eq) at 40^0 C for 1 h. Once the activation was complete, as confirmed by LC-MS and HPLC, the solution was added to the resin together with DIPEA (3 eq) and shaken overnight. The solution was drained and the resin washed with DMF until the wash solution was colourless, then DCM (3 × 5 mL) and MeOH (3 × 5 mL).

QSY21 coupling: QSY21-NHS ester[2] (1.0 eq per amine) was coupled in anhydrous DMF (0.1 M) containing DIPEA (3 eq) for 12 h. The solution was drained and the resin washed with DMF until the wash solution was colourless, then DCM (3 × 5 mL), MeOH (3 × 5 mL) and finally ether (3 × 5 mL).

Cleavage and deprotection: The resin (pre-swollen in DCM) was shaken 3 h in TFA/TIS/H_2O (95:2.5:2.5). The solution was collected by filtration and the resin was washed with the cleavage cocktail. The combined filtrates were added to ice-cold ether, and the precipitated solid was collected by centrifugation, and washed repeatedly with cold ether (3 × 50 mL).

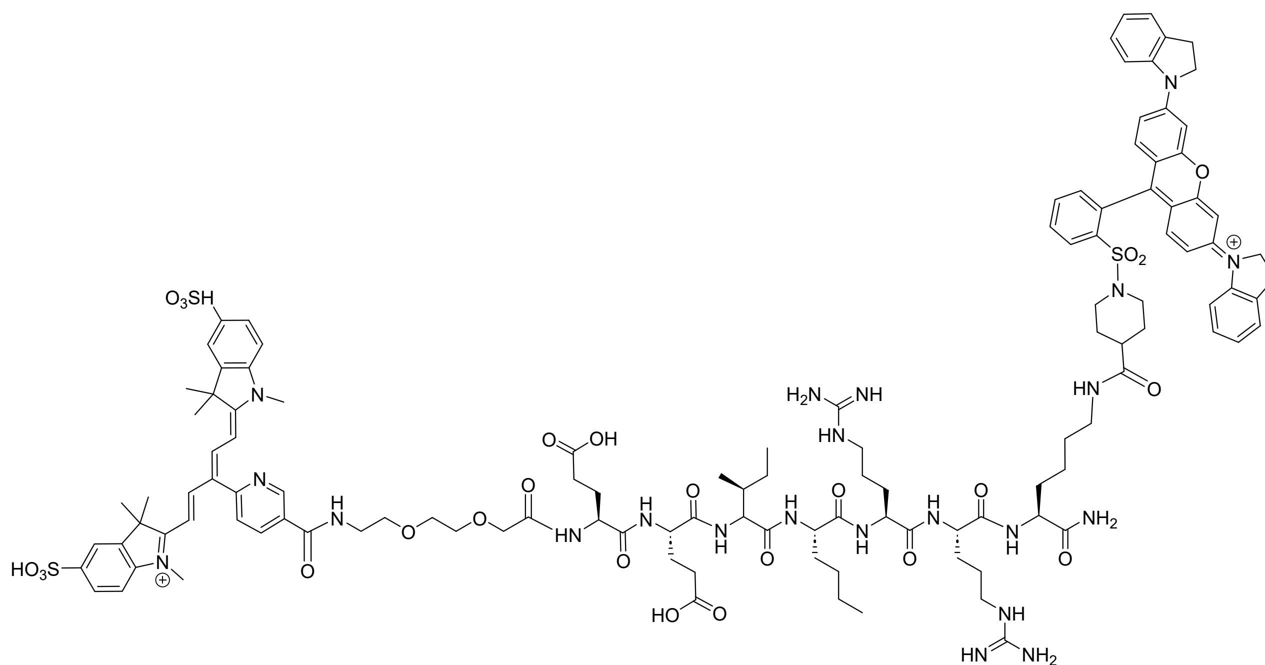
2.3 Procedure for Click Chemistry:

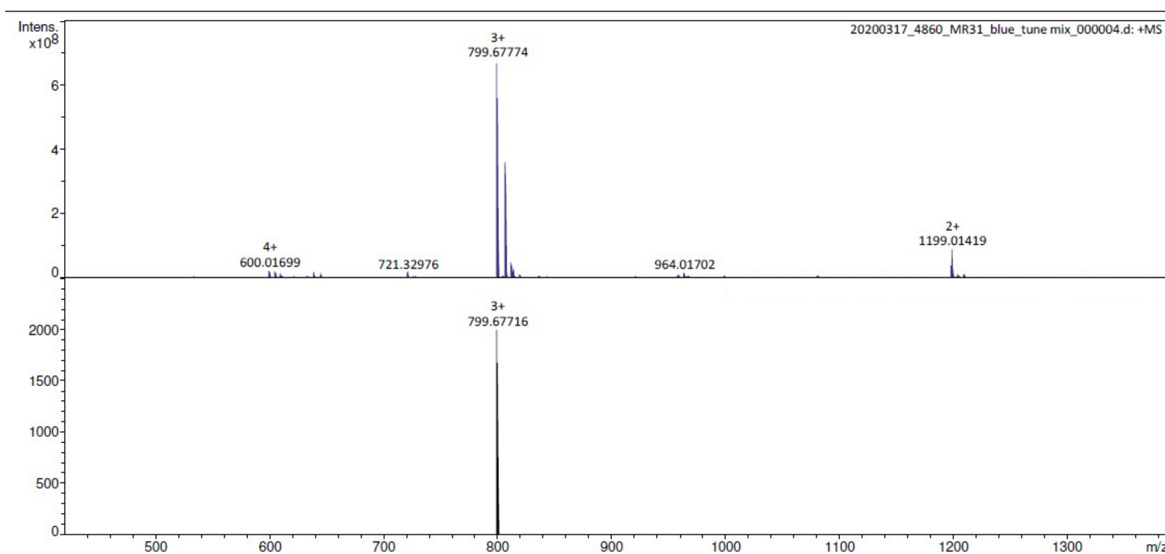


R = H or QSY21-EG$_2$, The azide-peptide **2** (4 eq, final concentration 3 mM), and alkynes **6** or **9** (1 eq, final concentration 0.7 mM) were dissolved in anhydrous DMF and a solution of premixed CuI/THPTA (0.5/2.5 eq, final concentration 1.4 mM/7.1 mM) was added. The reaction was allowed to proceed at 50 ^{0}C overnight, under N_2. Monitoring was carried out by RP-HPLC until reaction completion. The solvent was evaporated under vacuum and the resulting product purified by semipreparative RP-HPLC

3. Final compound characterisation

3.1 Probe 1 (HNE-1F1Q)

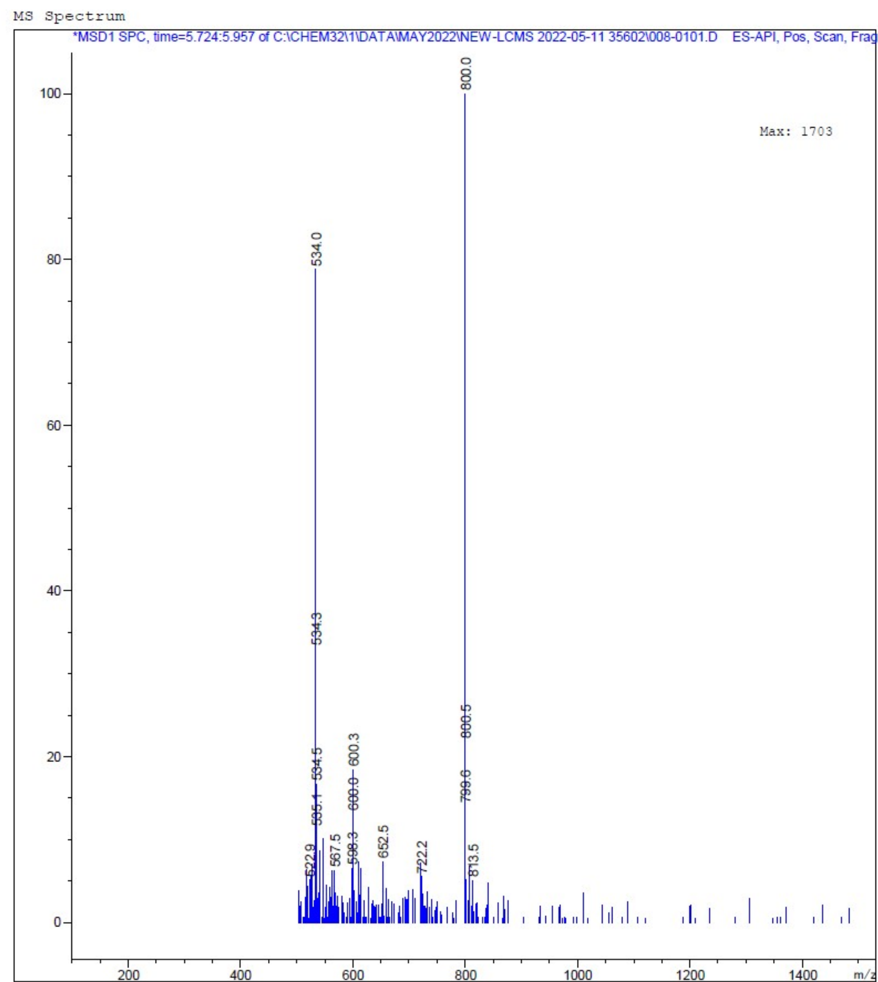


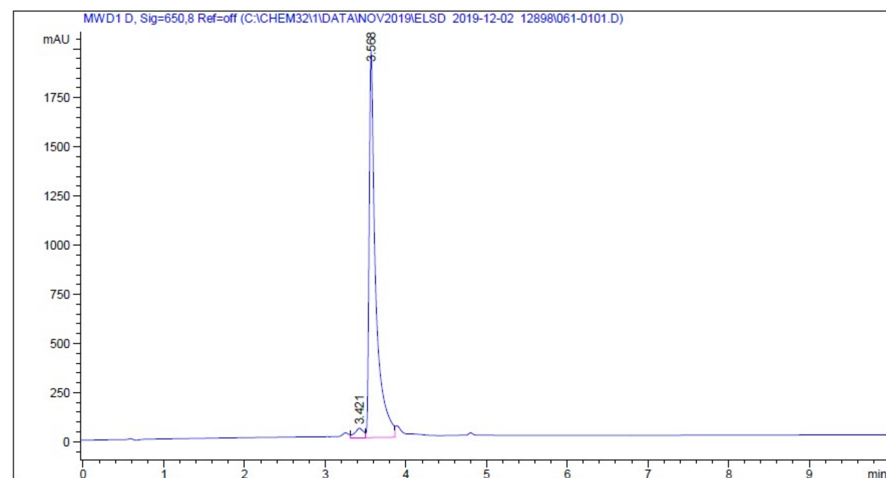


FT-HRMS: Calculated m/z for $C_{120}H_{153}N_{22}O_{26}S_3^{+3}$ $[M+H]^{+3}$ Expected: 799.67716 Found: 799.67774;

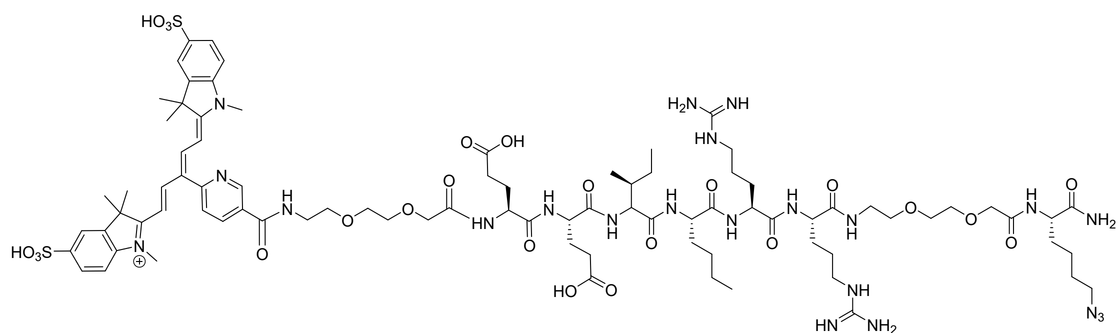
LC-MS : Found m/z $[M+H]^{3+}$ 800.0

HPLC-UV (Method A, 650 nm): r_t 3.6 m

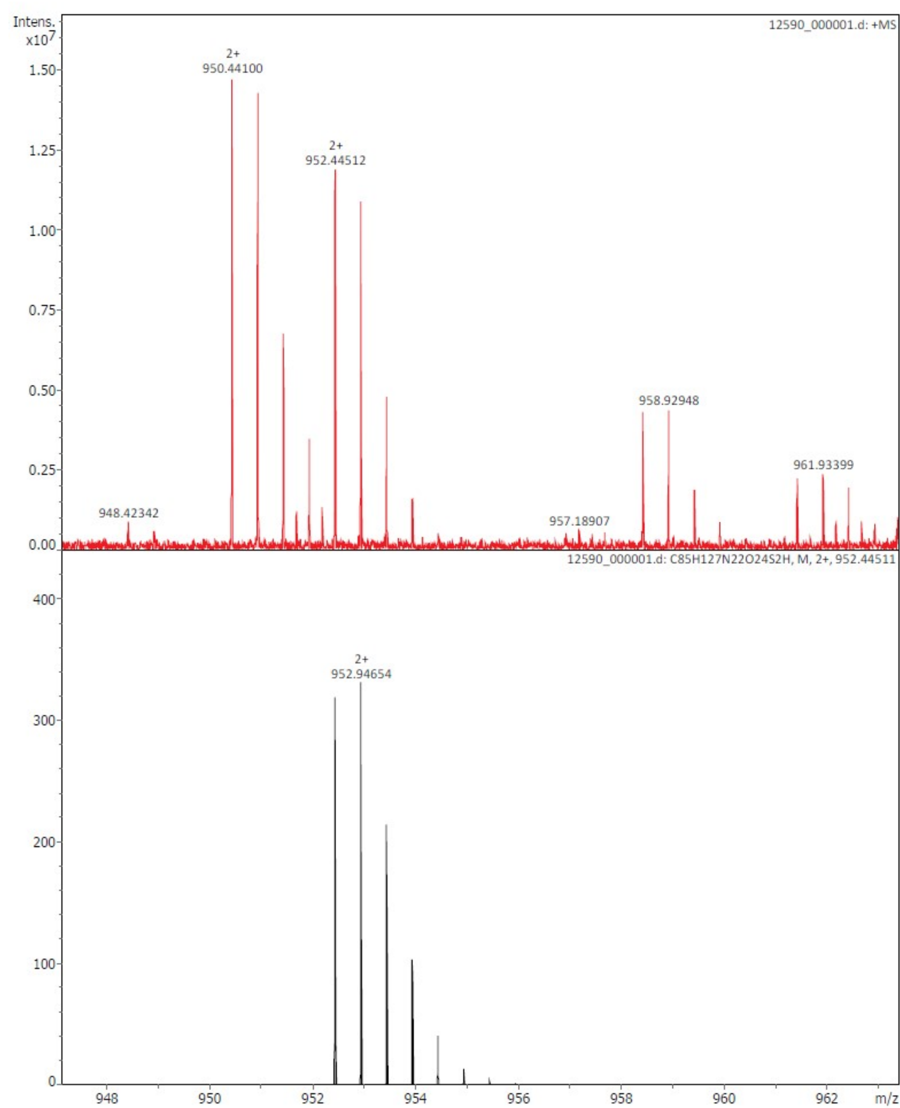




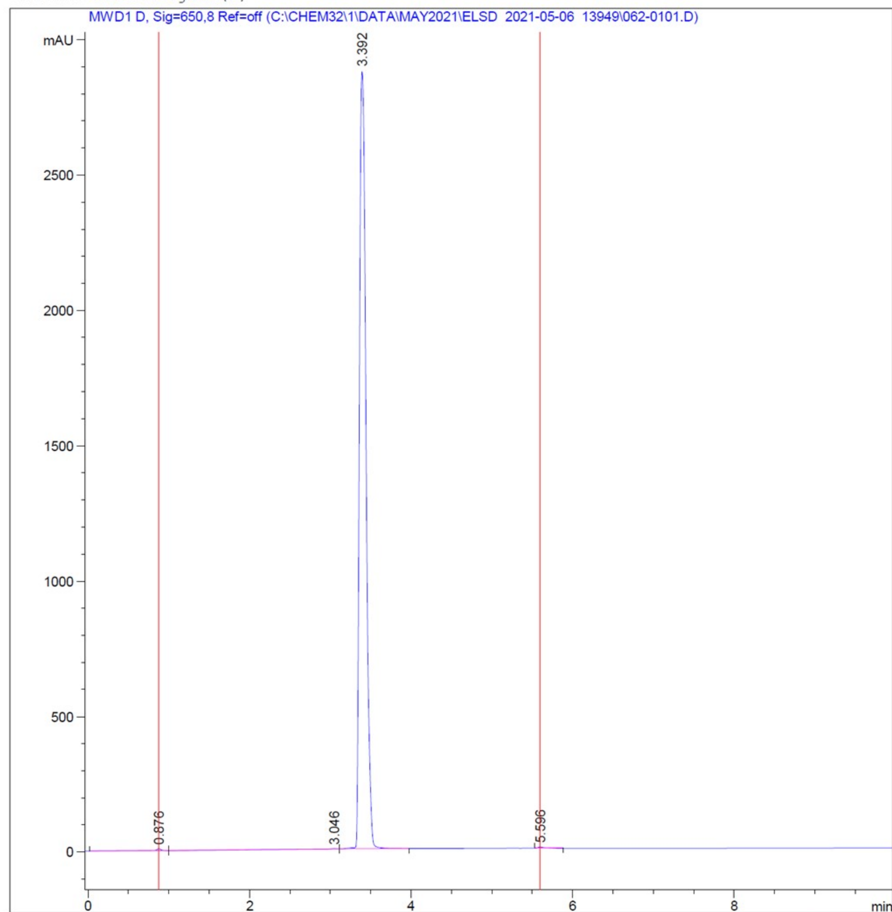
3.2 Azide peptide 2



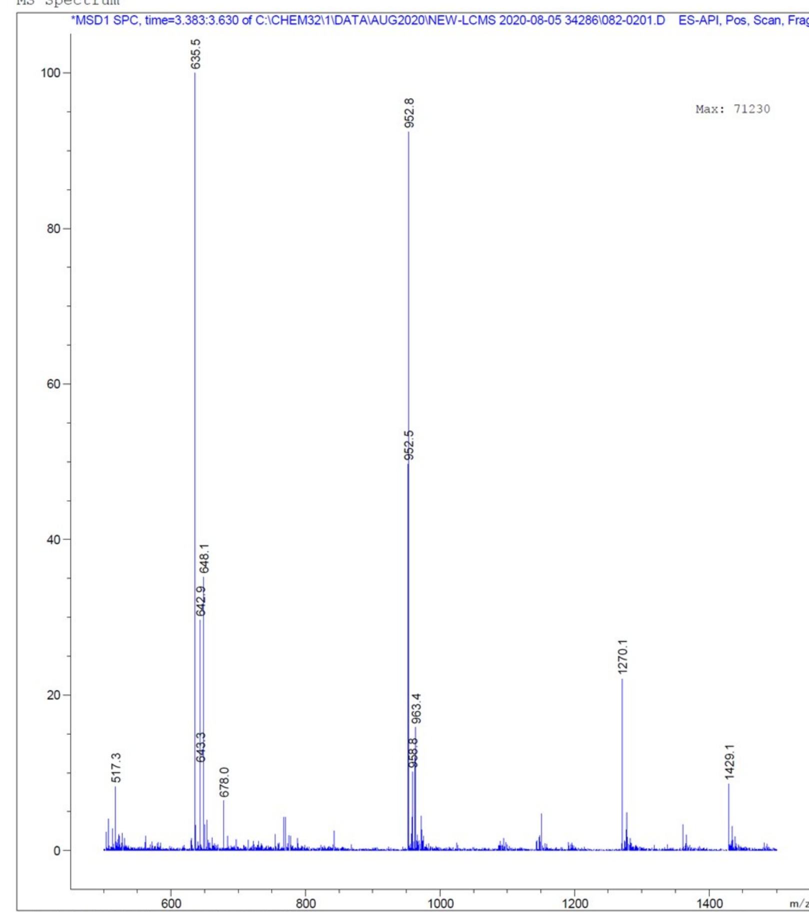
FT-HRMS: Calculated m/z for $C_{85}H_{127}N_{22}O_{24}S_2$ $[M+H]^{2+}$ Expected: 952.944511 Found: 952.44512



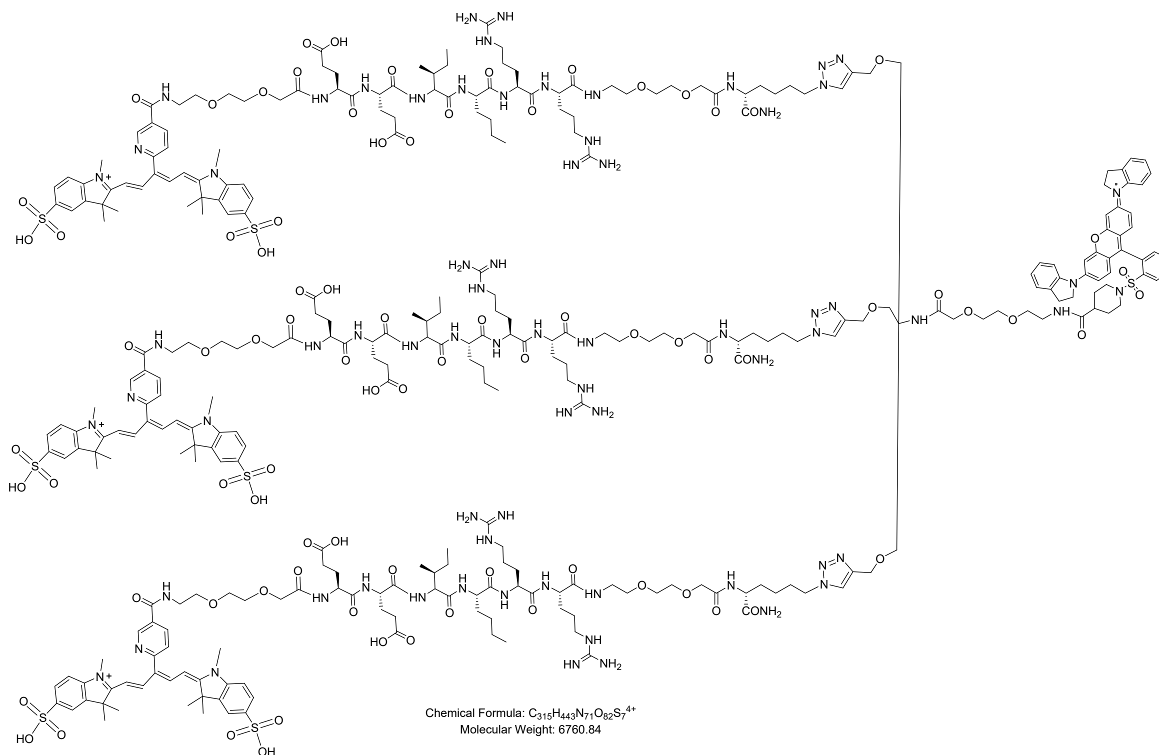
HPLC ELSD (Method A, 650 nm) : r_t 3.4 min



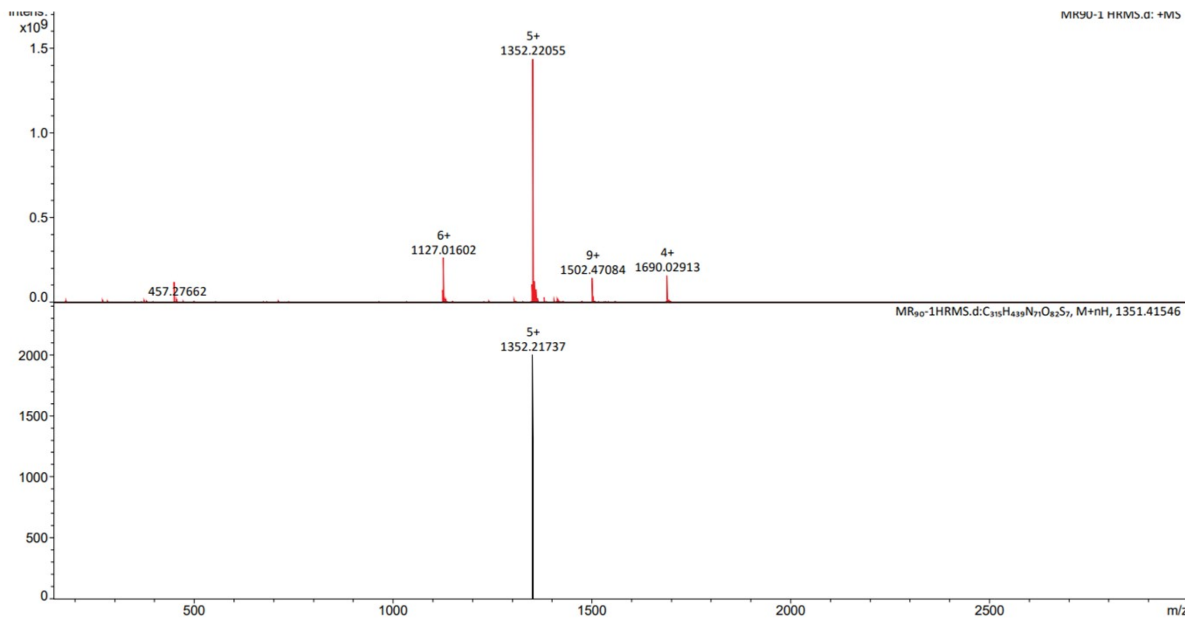
LC-MS : m/z [M+H]$^{+2}$ 952.8



3.3 Probe 10 (HNE-F31Q)

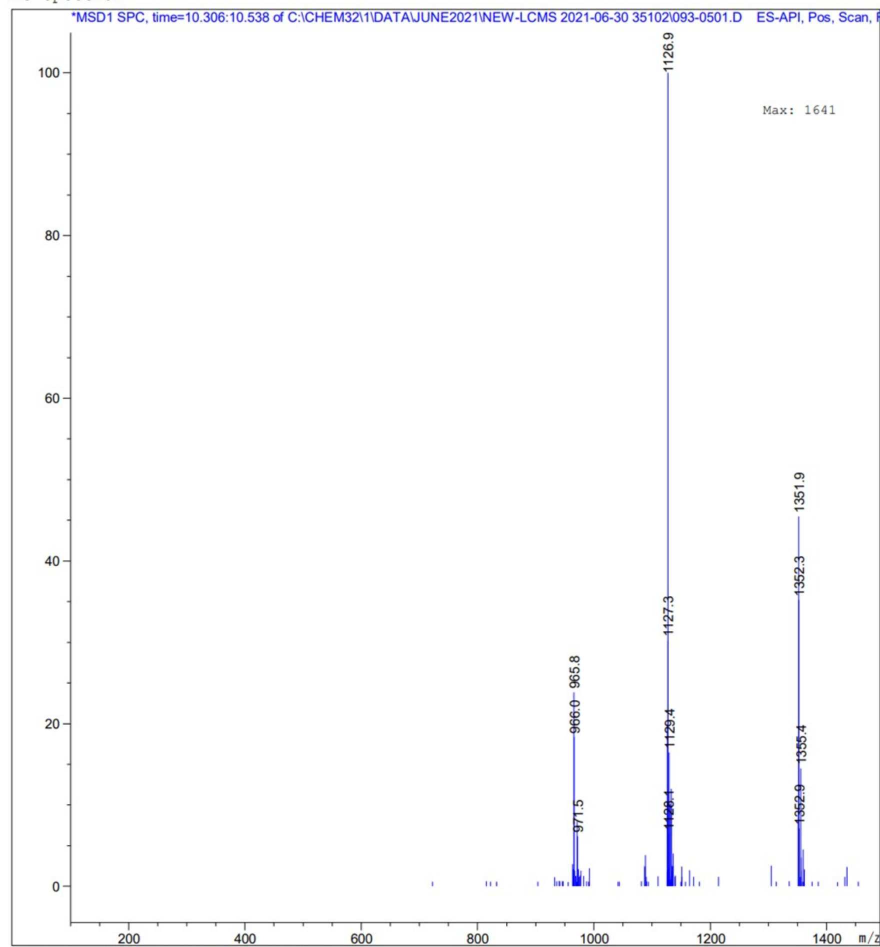


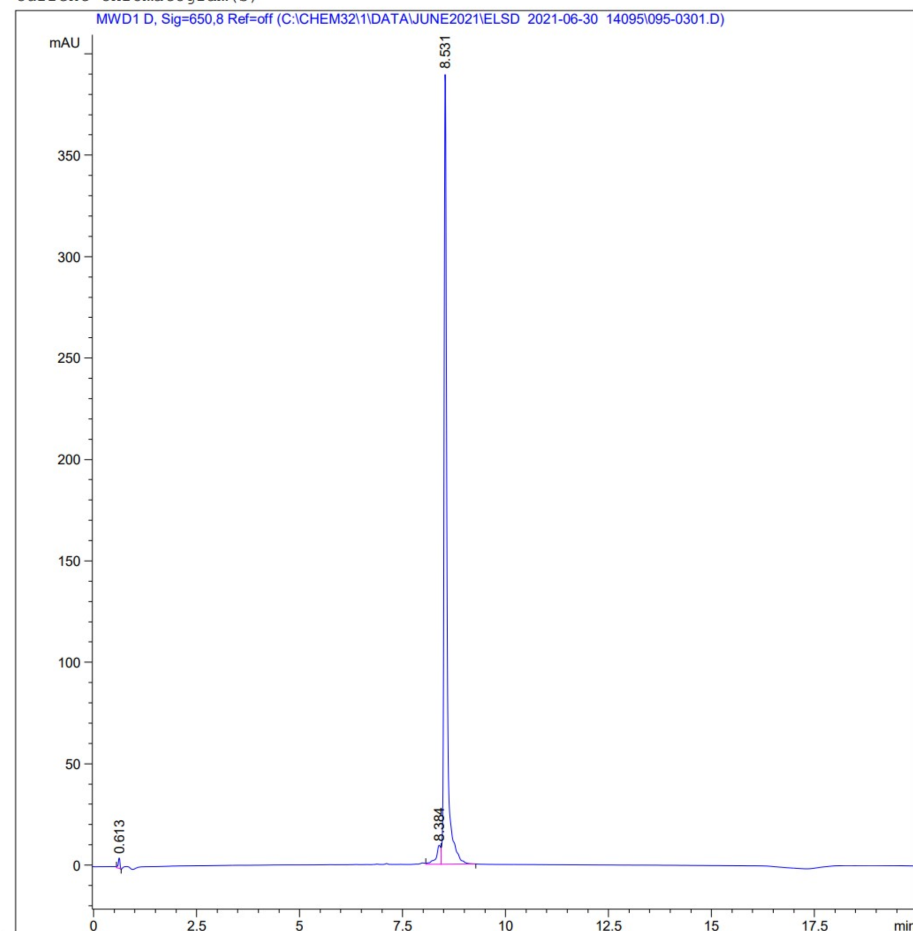
FT-HRMS: Calculated m/z for $C_{315}H_{443}N_{71}O_{82}S_7$ $[M+H]^{5+}$ Expected: 1352.21737 Found: 1352.22055.



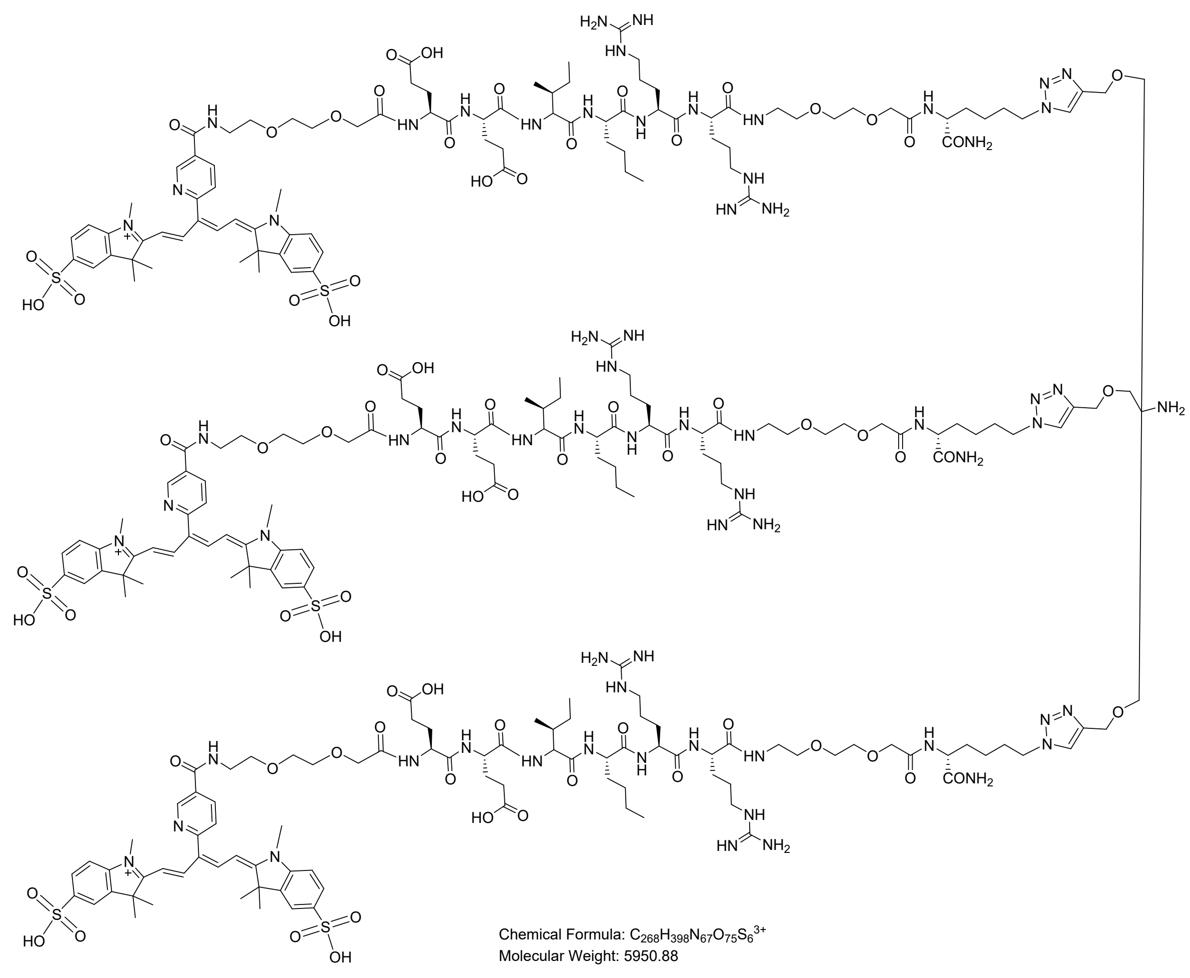
LC-MS : m/z $[M+H]^{5+}$ 1126.9

HPLC ELSD (Method B, 650 nm) : r_t 8.5 min

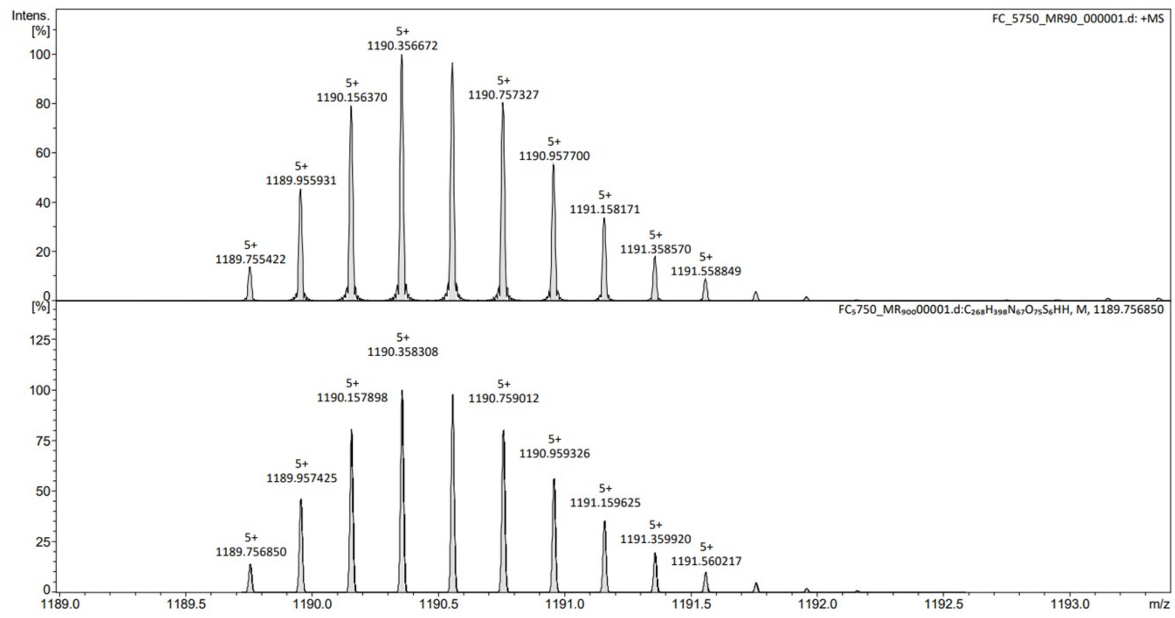




3.4 Probe 11 (HNE-3F0Q)

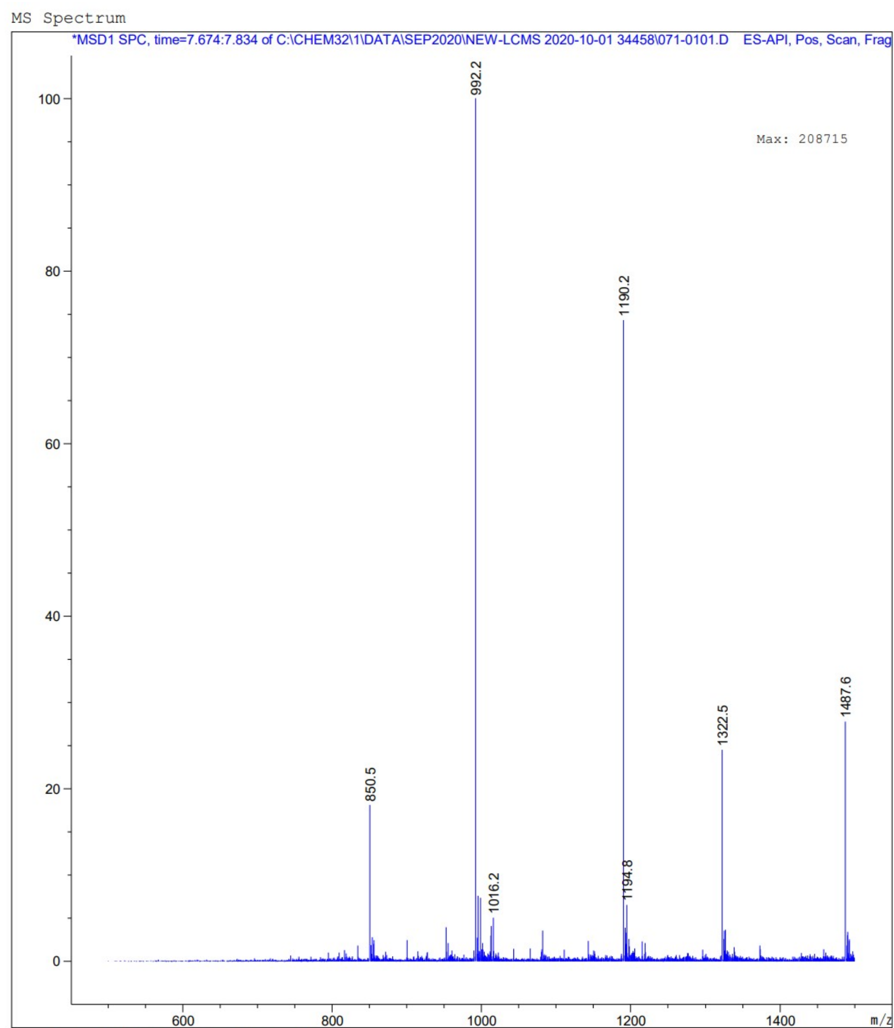


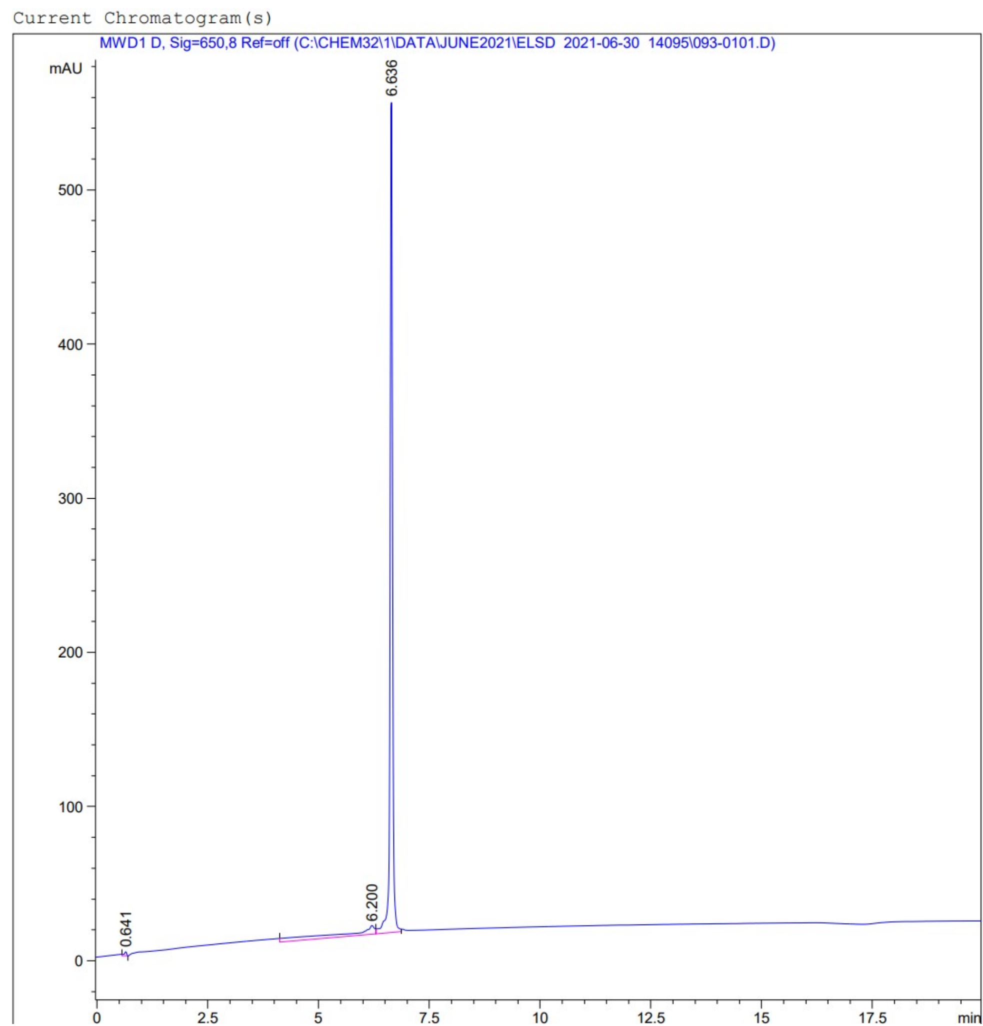
FT-HRMS: Calculated m/z for $C_{268}H_{400}N_{67}O_{75}S_{65}$ $[M+2H]^{5+}$: 1190.358308 Found: 1190.356672



LC-MS : Found m/z $[M+H]^{4+}$: 992.2

HPLC ELSD (Method B, 650 nm): r_t : 6.6 min





4. Experimental Procedures. Biology

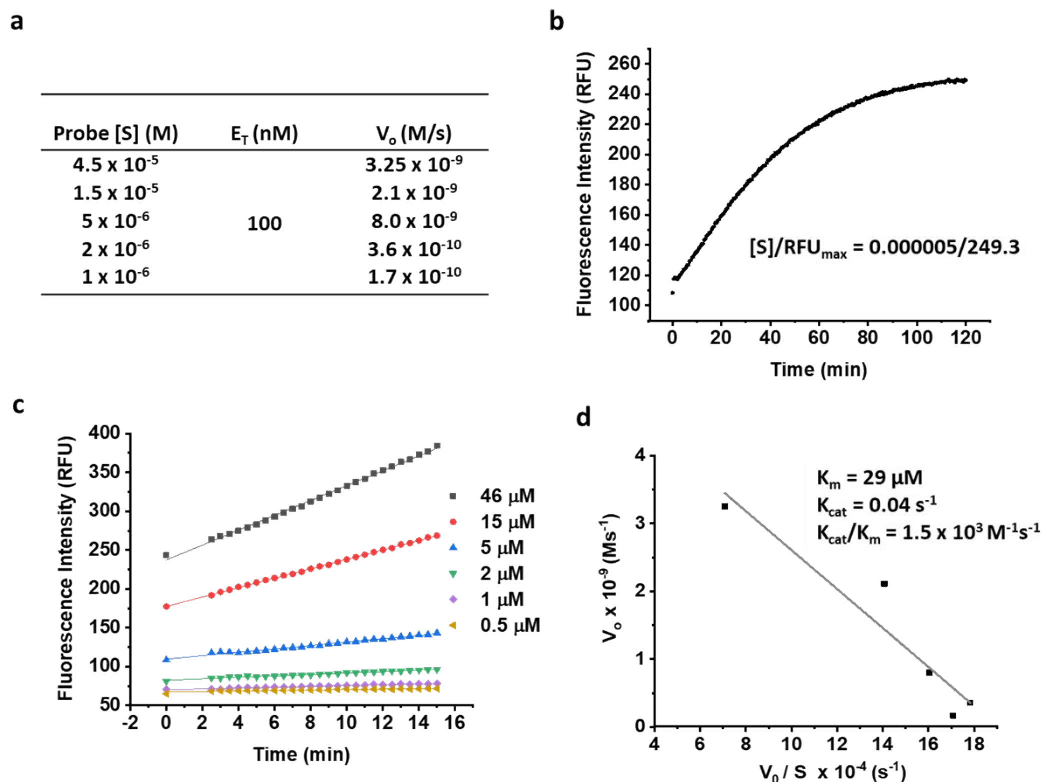
4.1 Probe activation *in vitro*

Probes (12 µM for probe **10** and **11**, 36 µM for probe **1**) were incubated with hNE (10, 50 or 100 nM), Cathepsin G (100 nM) or PR3 (100 nM) (Athens Biotechnology) in reaction buffer (50 mM HEPES, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 50 µL in a 96-well plate (Life Technologies). The time-dependent increase in fluorescence was monitored over 1 or 2 h using a fluorescence microplate reader (Biotek Synergy HT multi-mode reader) ($\lambda_{ex/em}$ 640/10, emission 680/20) at 37 °C. Buffer, enzymes and inhibitor where appropriate were incubated in the wells for 30 min at 37 °C before adding the probe. Readings were taken immediately after addition of the probe every 30 s and the plate was shaken for 10 s before the start of the readings. Data was normalised to buffer alone and the fold-change in signal (Relative Fluorescent Units) compared to enzyme-free controls was calculated. Data was plotted using Origin.

4.2 Kinetic parameters determination (K_m, k_{cat}, k_{cat}/K_m)

hNE was added to solutions of increasing concentrations of the probe (within a range of 1- 45 µM for probe **1**, 0.7-20 µM for probe **10** and 0.3-15 µM for probe **11**) in a 96-well plate (n=3) and fluorescence intensity values monitored every 30 second or 1 minute recorded on a Biotek Synergy HT multi-mode reader. Control samples (n = 3) had the same composition but no enzyme. The RFU was plotted against time (min) to obtain initial velocity values (V_o) on the first 5, 10 or 15 min of reaction. Velocity units (RFU/s) were converted to M/s by the factor $[S]/RFU_{max}$. Eadie-Hofstee and or Lineweaver Burk plots were used to generate values of K_m, V_{max} and k_{cat}/K_m.

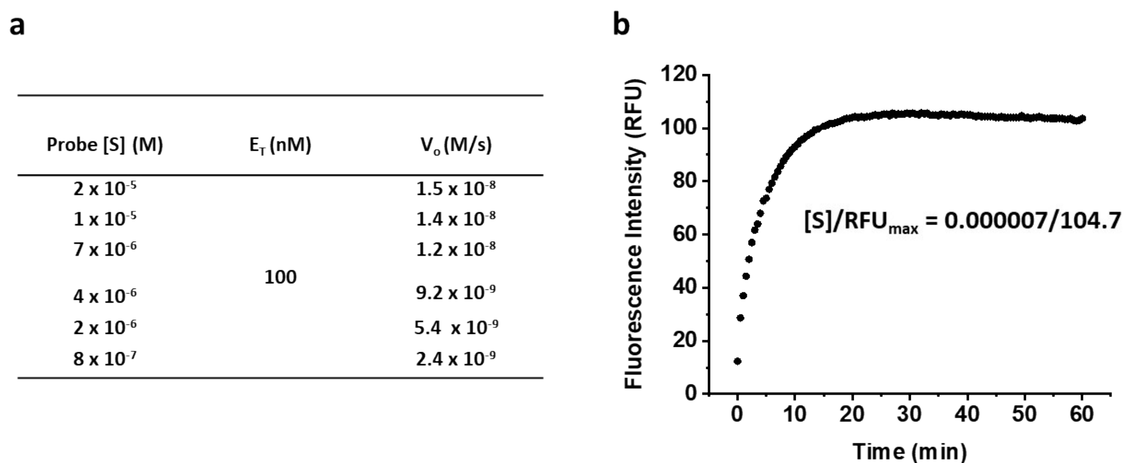
4.2.1 Probe 1 – linear substrate HNE-1F1Q



Probe [S] (M)	E_T (nM)	V_o (M/s)
4.5×10^{-5}		3.25×10^{-9}
1.5×10^{-5}		2.1×10^{-9}
5×10^{-6}	100	8.0×10^{-9}
2×10^{-6}		3.6×10^{-10}
1×10^{-6}		1.7×10^{-10}

Figure S9. Kinetic parameter determination for linear NIR FRET probe **1** (HNE-1F1Q). **a)** The initial velocities obtained at increasing concentrations of probe at a constant concentration of enzyme of 100 nM. **b)** Normalisation factor ($[S]/RFU_{max}$) was obtained from the activation profile of the probe at 5 M, where RFU_{max} was 249.3. **c)** Initial velocity plots at increasing concentrations of the **HNE-1F1Q. d)** Eadie-Hofstee plot provided a K_m value of 29 μM and a turnover number (k_{cat}) of 0.04 s^{-1}. Values of fluorescence were obtained with a Biotek Synergy HT multi-mode reader with 30 seconds intervals during 15 minutes, λ_{ex} = 640/10 , λ_{em} = 680/20 (fluorescence gain 50). See figure S1.

4.2.2 Probe 10 – Three fluorophore one quencher probe HHNE-3F1Q

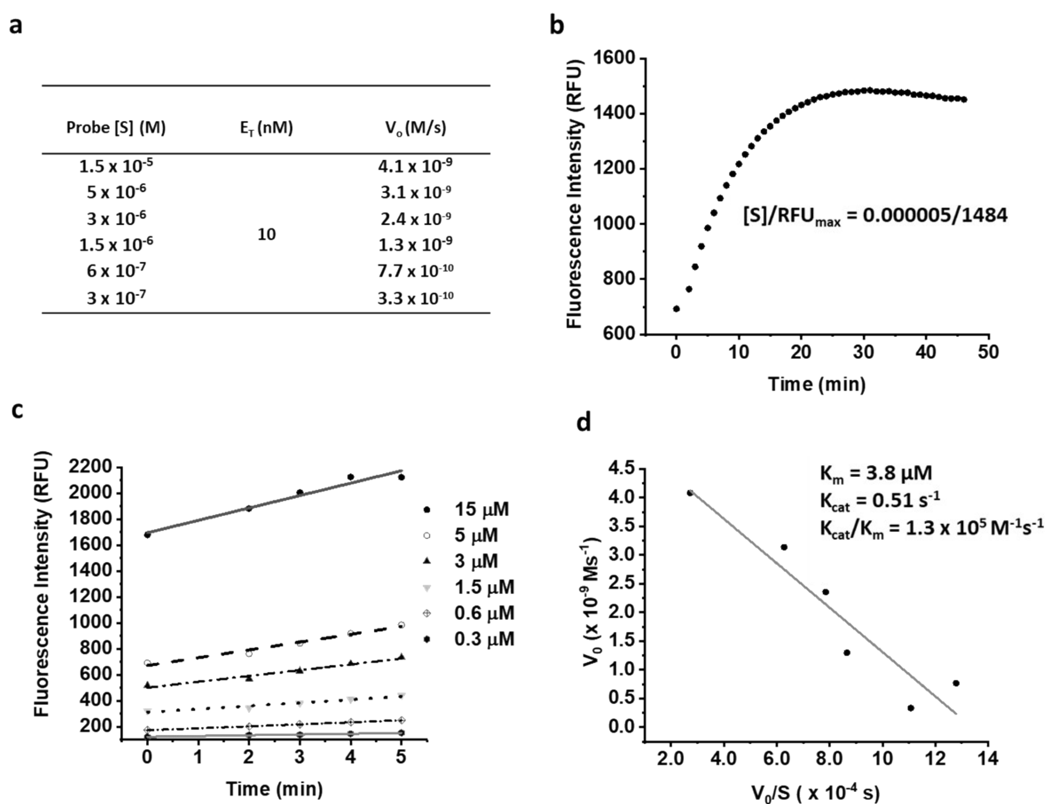


a

Probe [S] (M)	E_T (nM)	V_o (M/s)
2×10^{-5}	100	1.5×10^{-8}
1×10^{-5}		1.4×10^{-8}
7×10^{-6}		1.2×10^{-8}
4×10^{-6}		9.2×10^{-9}
2×10^{-6}		5.4×10^{-9}
8×10^{-7}		2.4×10^{-9}

Figure S10. Kinetic parameters determination for probe 10 (HNE-3F1Q). a) The initial velocities obtained at increasing concentrations of probe at a constant concentration of enzyme of 100 nM. **b)** Normalisation factor ($[S]/RFU_{max}$) was obtained from the activation profile of the probe at 5 uM, where RFU_{max} was 1484. Values of fluorescence were obtained with a Biotek Synergy HT multi-mode reader, with 1 minute intervals during 5 minutes and λ_{ex} = 640/10, λ_{em} = 680/20 (with a fluorescence gain 35). See figure S5.

4.2.3 Probe 11 - self-quenching probe HNE-3F0Q



Probe [S] (M)	E_T (nM)	V_o (M/s)
1.5×10^{-5}		4.1×10^{-9}
5×10^{-6}		3.1×10^{-9}
3×10^{-6}	10	2.4×10^{-9}
1.5×10^{-6}		1.3×10^{-9}
6×10^{-7}		7.7×10^{-10}
3×10^{-7}		3.3×10^{-10}

Figure S11. Kinetic parameter determination for NIR probe **11** (**HNE-3F0Q**). **a)** The initial velocities obtained at increasing concentrations of probe at a constant concentration of enzyme of 10 nM. **b)** Normalisation factor ([S]/RFU_{max}) was obtained from the activation profile of the probe at 5 uM, where RFU_{max} was 1484. **c)** Initial velocity plots at increasing concentrations of the HNE-3F0Q. **d)** Eadie-Hofstee plot provided a K_M value of 3.8 μM and a turnover number (k_{cat}) of 0.51 s^{-1}. Values of fluorescence were obtained with a Biotek Synergy HT multi-mode reader with 1 minute intervals during 5 minutes, λ_{ex} = 640/10, λ_{em} = 680/20 (fluorescence gain 50). See figure S3.

4.3 Confocal images of neutrophils stained with probe 10 (HNE-3F1Q)

Neutrophils were isolated from peripheral blood collect from healthy volunteers by using dextran sedimentation and Percoll gradient separation.[5] Human neutrophil experiments were carried out under ethics approval from the Lothian Research Ethics Committee (AMREC Reference number 20/HV/069 and 21-EMREC-041) and with informed consent from all the blood donors of the Centre for Inflammation Research (University of Edinburgh).

4.4 Plate reader experiments of neutrophils stained with probe 10 (HNE-3F1Q)

Neutrophils were plated in monolayer in an ibidi µ-Slide 8 wells chamber slide (5 x 10^5/well in phenol red RPMI media). Neutrophils were stained with 3 µM **HNE-3F1Q** (**10**) and treated with or without (untreated) fMLF (200 nM, 30 min, 37°C, 5% CO_2) or PMA (10 nM, 3 h, 37°C, 5% CO_2). Before imaging each well was stained with Hoechst 33342 (100 nM, 20 min, RT dark). Images were taken with a SP8 Confocal Leica microscope. Hoechst: 405 laser; HNE-F3Q1: 633 laser, 10x objective, 10 µm scale bar.

Neutrophils were isolated from peripheral blood collect from healthy volunteers by using dextran sedimentation and percoll gradient separation. Human neutrophil experiments were carried out under ethics approval from the Lothian Research Ethics Committee (AMREC Reference number 20/HV/069 and 21-EMREC-041) and with informed consent from all the blood donors of the Centre for Inflammation Research (University of Edinburgh).

After isolation, neutrophils (10 x 10^5/well in phenol red free RPMI) were plated in a 96 well round bottom plate and pre-treated with or without (untreated) Sivelestat (100 µM, 30 min, 37°C, 5% $_{CO2}$) and following stimulation with PMA (10 nM). Time and dose dependent increase of fluorescence was monitored over 3 h with a plate reader (Cytation 5) ($\lambda_{ex}/_{em}$ 640/10, emission 680/20).

4.5 Fluorometric experiments

Probes **1** (36 µM), **10 or 11** (12 µM) were incubated with or without hNE (100 nM), in reaction buffer (50 mM Hepes buffer, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume

of 150 µL for 1 hour. The solution (50 µL) was then diluted by a factor of 3 and transferred into a quartz cuvette. Emission spectrum readings were recorded in a spectrofluorometric range from 650 to 800 nm after 1 h incubation, with the excitation wavelength of 640 nm.

4.6 Absorbance spectrum of probes

Probes **1** (36 µM), **10 o**r **11** (12 µM) were incubated in reaction buffer (50 mM HEPES, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 50 µL in a 96-well plate (Life Technologies) and absorbance was measured across the spectral range 300 to 700 nm.

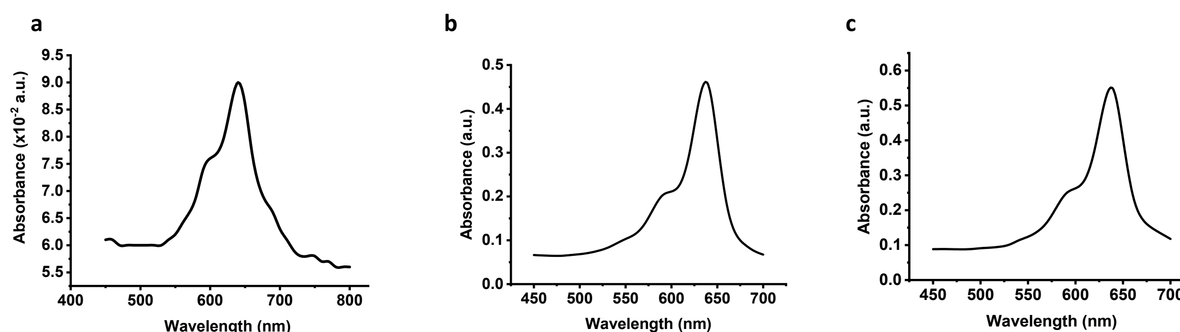


Figure S12. Absorbance spectrum of **a)** Probe **1** (HNE-1F1Q); **b)** Probe **11** (HNE-3F0Q) and **c)** Probe **10** (HNE-3F1Q).

4.7 Effect of pH, temperature and different biological environments on fluorescence signal.

The fluorescence intensity of the cleaved *vs* uncleaved probe was assessed at pH 4, 7 and 9, or in HEPES buffer (control, pH 7.4), complete cell media , 10% Fetal Bovine Serum (FBS) or in a Hela cell lysate and incubated for 2 hours at 37ºC . Stocks of cleaved and uncleaved probe were generated by incubating the probe in reaction buffer (50 mM HEPES pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v)) in a final volume of 200 µL at 10 µM , with or without hNE (Athens Biotechnology). Cleaved or uncleaved probe solutions were added to the different environments (to give final concentration on the well 5 µM in a 50 µL volume) and fluorescent intensities measured using a fluorescence microplate reader.

For the Hela cell lysate, cells were seeded and grown in a T25 flask over 48 hours. For lysis, cells were resuspended in sterile water (5 mL), approximately 1 million cells/mL, and incubated for 30 min at 37°C. The resulting lysate was centrifuged at 13000 g for 10 min and the supernatant collected and used immediately.

References

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