Real time monitoring of peptide delivery in vitro using high payload pH responsive nanogels

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SUPPORTING INFORMATION

Polymer Synthesis and Characterisation

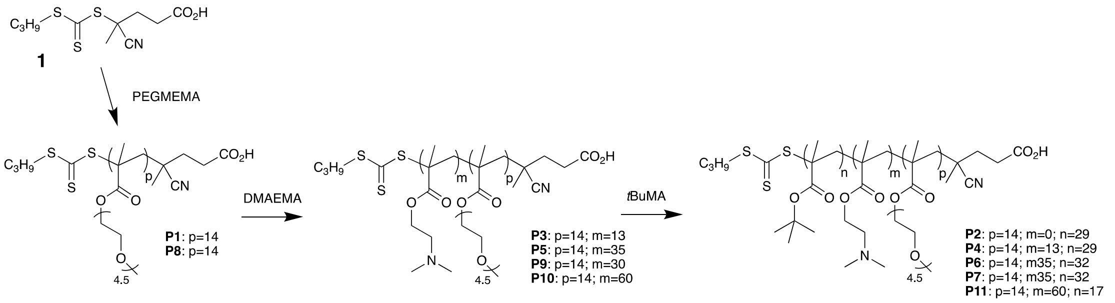


Figure S1. Scheme for the synthesis of terblock copolymers

Table S1. Terblock polymerisation conditions and characterisation data

#	Polymer	CTA	Monomer	[M] (mol.L^{-1})	[M] / [CTA]	X (%)	DP$_{theo}$	$M_{n, theo}$ (Da)	$M_{n, GPC}$ (Da)	Đ	HLB
P1	P_{14}	RAFT	PEGMEMA	1	15	92%	13.6	4359	7400	1.08	-
P2	P_{14}-B_{29}	P1	*t*BuMA	0.5	29	100%	29	8480	8250	1.07	10.6
P3	P_{14}-D_{13}	P1	DMAEMA	1	25	53%	13.1	6418	6970	1.08	-
P4	P_{14}-D_{13}-B_{29}	P3	*t*BuMA	0.5	29	100%	29	10539	8230	1.06	16.9
P5	P_{14}-D_{35}	P1	DMAEMA	1	50	53%	34.5	9783	9500	1.06	-
P6	P_{14}-D_{35}-B_{18}	P5	*t*BuMA	0.5	29	75%	18	12341	11300	1.06	15.0
P7	P_{14}-D_{35}-B_{32}	P5	*t*BuMA	0.5	48	85%	32	14330	11900	1.09	13.7
P8	P_{14}	RAFT	PEGMEMA	1	15	93%	13.9	4449	7590	1.07	-
P9	P_{14}-D_{30}	P8	DMAEMA	1	60	50%	30	9165	10550	1.1	-
P10	P_{14}-D_{60}	P8	DMAEMA	1	120	50%	60	13882	11850	1.06	-
P11	P_{14}-D_{60}-B_{17}	P10	*t*BuMA	0.5	27	62%	16.7	16255	13550	1.09	16.8

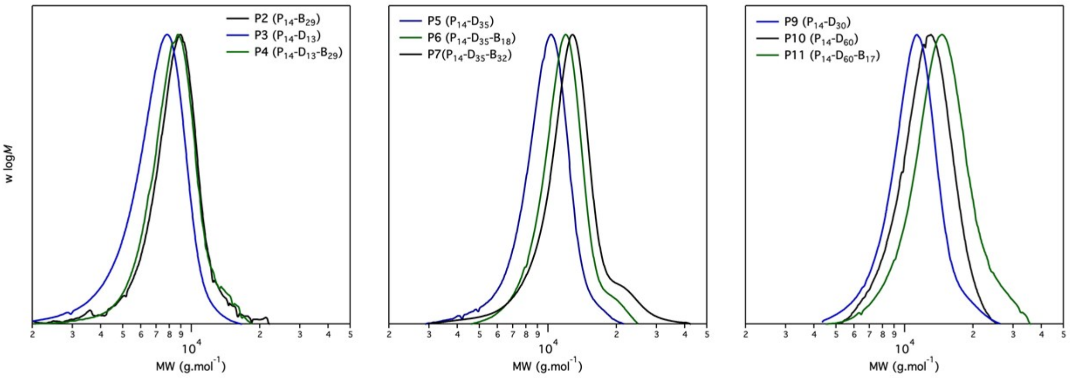


Figure S2. GPC traces of terblock copolymers. Eluent: DMF + 0.01% wt LiBr; MW reported against pMMA narrow molecular weight standards without correction

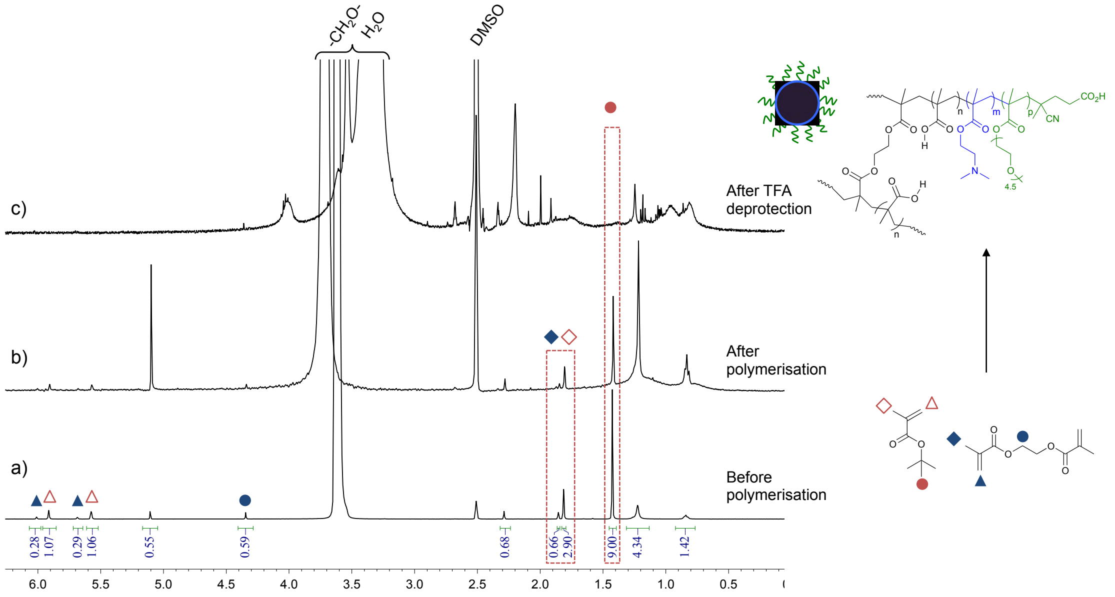
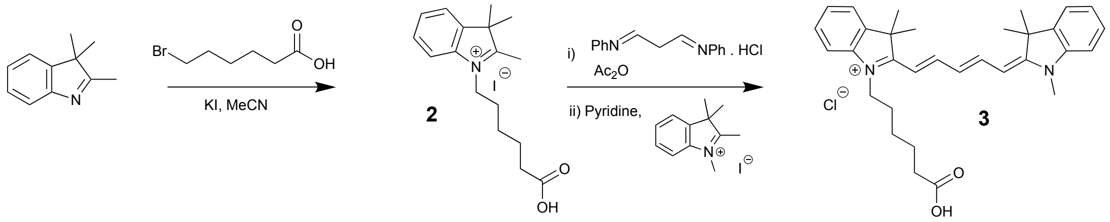


Figure S3. ^{1}H-NMR (*d6*-DMSO) of the NG3 mixture a) before miniemulsion polymerisation, b) after polymerisation and formation of the nanogel, and c) after deprotection of the tert-butyl groups with TFA, showing complete removal of the protecting group. Nanogel NMRs are taken with a long delay time (D1) of 20 s.

Synthesis of Cyanine dyes

The synthesis of the cyanine dyes was adapted from Simmons *et al.*[1] and Kyach et al.[2]



Cyanine5-CO$_2$H (3): 2,3,3-Trimethylindolenine (2.5 g, 15.5 mmol), 6-bromohexanoic acid (3.9 g, 20.2 mmol) and potassium iodide (3.4 g, 20.2 mmol) were heated to 85 ˚C in acetonitrile (30 mL) for 30 hours. After cooling to room temperature, the mixture was filtered and concentrated *in vacuo*. The residue was diluted with diethyl ether (300 mL) and left to precipitate at 4 ˚C overnight. The resultant solid was collected by filtration, washed with diethyl ether (2 x 50 mL) and dried *in vacuo*. A yield of 4.8 g, 12.1 mmol (61 %) was obtained as a red solid. Spectroscopic data were consistent with those previously reported.[1] ^{1}H NMR (400 MHz, DMSO): δ = 7.96-8.00 (1H, m, $\underline{H}$8), 7.85 (1H, dd, J = 5.9, 2.9 Hz, $\underline{H}$5), 7.61-7.67 (2H, m, $\underline{H}$6 and $\underline{H}$7), 4.46 (2H, t, J = 7.7 Hz, -C$\underline{H}_2$Ar), 2.84 (3H, s, -C$\underline{H}_3$), 2.24 (2H, t, J = 7.2 Hz, -C$\underline{H}_2$CO$_2$H), 1.84 (2H, tt, J_1 = J_2 = 7.8 Hz, -C$\underline{H}_2$CH$_2$Ar), 1.49-1.63 (8H, m, -(C$\underline{H}_3$)$_2$ and –C$\underline{H}_2$CH$_2$CO$_2$H), 1.35-1.49 (2H, m, -C$\underline{H}_2$CH$_2$CH$_2$Ar) ppm.

The indole (**2**) (0.63 g, 1.57 mmol) and malonaldehyde bis(phenylimine) monohydrochloride (0.48 g, 1.84 mmol) were dissolved in acetic anhydride (10 mL) and heated to 120 ˚C for 30 minutes. After cooling to room temperature 1,2,3,3-tetramethyl-3H-indolium iodide (0.657 g, 2.18 mmol) and pyridine (10 mL) were added and the reaction stirred for 18 hours during which time it turned dark blue. The mixture was precipitated by addition of hexane (100 mL), dissolved in chloroform (100 mL), washed with H$_2$O (2 x 100 mL) and brine (100 mL), dried with MgSO$_4$, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography with CHCl$_3$: acetone : methanol (7:2:1 v/v). The product concentrated *in vacuo* to yield a dark blue solid (0.229 g, 28%). ^{1}H NMR (400 MHz, DMSO): δ = 12.03 (1H, s, -COO$\underline{H}$), 8.29-8.40 (2H, m, b-$\underline{H}$), 7.52-7.67 (2H, m, $\underline{H}5_{a/b}$), 7.36-7.53 (4H, m, $\underline{H}7_{a/b}$ and $\underline{H}8_{a/b}$), 7.20-7.34 (2H, m, $\underline{H}6_{a/b}$), 6.51-6.63 (1H, m, c-$\underline{H}$), 6.22-6.36 (2H, m, a-$\underline{H}$), 4.09 (2H, t, J = 8.3 Hz, -C$\underline{H}_2$Ar), 3.61 (3H, s, Ar$\underline{Me}$), 2.29 (2H, m, -C$\underline{H}_2$CO$_2$H), 1.64-1.74 (14 H, m, -C$\underline{H}_2$CH$_2$Ar and Ar(C$\underline{H}_3$)$_2$), 1.50-1.60 (2H, m, -C$\underline{H}_2$CH$_2$CO$_2$H), 1.39 (2H, dt, J = 15.3, 6.4 Hz, -C$\underline{H}_2$CH$_2$CH$_2$Ar) ppm. ESI-MS (m/z): Found 483.58; calc. [M]$^+$ 483.30.

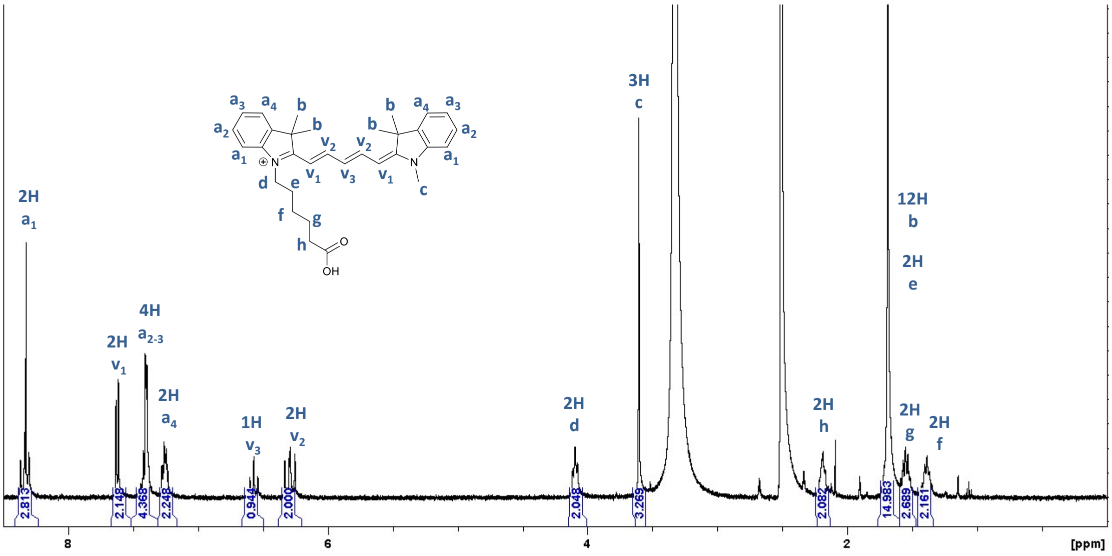
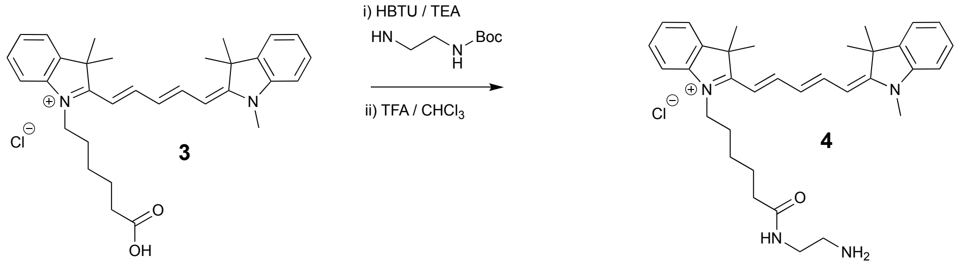
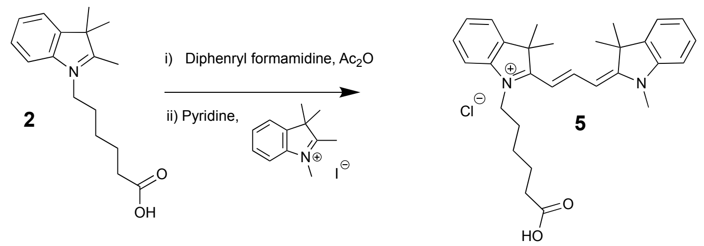


Figure S4. ^{1}H-NMR spectrum of Cy5-CO$_2$H in *d6*-DMSO (**3**)



Cyanine5-NH$_2$ (4): Ethylene diamine (6 g, 100 mmol) was mono-boc protected by dropwise addition of boc anhydride (2.18 g, 10 mmol) in dichloromethane (40 mL). After 3 h the mixture was concentrated, dissolved in saturated NaHCO$_3$ (100 mL), and extracted with dichloromethane (2 x 50 mL). The organic layer was washed with brine, dried with MgSO4 and concentrated in vaccuo to yield the product as a clear liquid (2.01 g, 12%).

The mono-boc protected diethyl amine (0.036 g, 0.22 mmol) was then mixed with Cy5-CO$_2$H (**3**, 76 mg, 0.15 mmol) in dichloromethane (3 mL), and TEA (30 mg, 0.30 mmol) and HBTU (0.084 g, 0.22 mmol) was added. The reaction was stirred overnight at RT, concentrated, and purified by flash column chromatography with CHCl$_3$: acetone : methanol (85:15:5 v/v). The boc protected product was isolated, and the structure confirmed by ESI-MS (m/z): found 621.42; calc. [M]$^+$ 621.41. The boc-protected Cy5-NH$_2$ was then dissolved in TFA / CHCl$_3$ (1:1 v/v) and diluted with CHCl$_3$ after 30 min. The TFA was removed by washing with NaHCO$_3$ (aq x 2), water (x2) and brine (x1). The product was dried with MgSO$_4$, and concentrated to a solid (47 mg, 57%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (t, *J* = 13.1 Hz, 2H), 8.05 (t, *J* = 5.6 Hz, 1H), 7.62 (d, *J* = 7.4 Hz, 2H), 7.44 – 7.32 (m, 3H), 7.25 (d, *J* = 6.7 Hz, 1H), 6.57 (t, *J* = 12.3 Hz, 1H), 6.38 – 6.18 (m, 2H), 4.09 (t, *J* = 7.5 Hz, 2H), 3.60 (s, 3H), 3.24 (q, *J* = 6.2 Hz, 1H), 2.79 (t, *J* = 6.4 Hz, 2H), 2.16 – 2.03 (m, 2H), 1.68 (s, 13H), 1.55 (q, *J* = 7.5 Hz, 2H), 1.43 – 1.27 (m, 2H).



Cyanine3-CO$_2$H (5): The indole (**2**) (0.681 g, 1.70 mmol) and *N,N*-diphenylformamidine (0.405 g, 2.06 mmol) were dissolved in acetic anhydride (10 mL) and heated to 120 ˚C for 30 minutes. After cooling to room temperature 1,2,3,3-tetramethyl-3H-indolium iodide (0.621 g, 2.06 mmol) and pyridine (11 mL) were added and the reaction stirred for 2 h at RT. The mixture was precipitated by addition of ethanol / hexane (100 mL, 1:1 v/v), and purified by flash column chromatography with CHCl$_3$: acetone : methanol (7:2:1 v/v). The product concentrated *in vacuo* to yield a dark pink solid (0.222 g, 27%). ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 8.35 (s, 1H), 7.64 (dd, *J* = 7.3, 1.2 Hz, 2H), 7.45 (d, *J* = 1.0 Hz, 4H), 7.31 (ddd, *J* = 7.4, 5.2, 3.1 Hz, 2H), 6.47 (d, *J* = 13.5 Hz, 2H), 4.11 (t, *J* = 7.5 Hz, 2H), 3.66 (s, 3H), 2.22 (s, 2H), 1.69 (s, 14H), 1.57 (s, 2H), 1.42 (s, 2H). ESI-MS (m/z): Found 457.42; calc. [M]$^+$ 457.28.

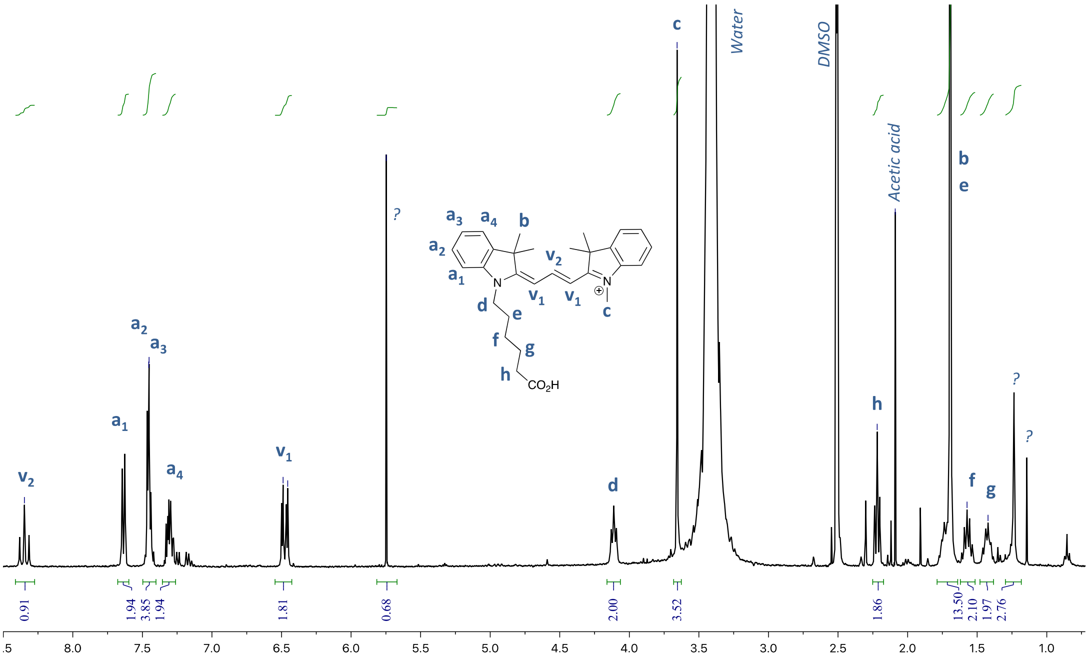


Figure S5. ^{1}H-NMR spectrum of Cy3-CO$_2$H in *d6*-DMSO

Peptide characterisation figures

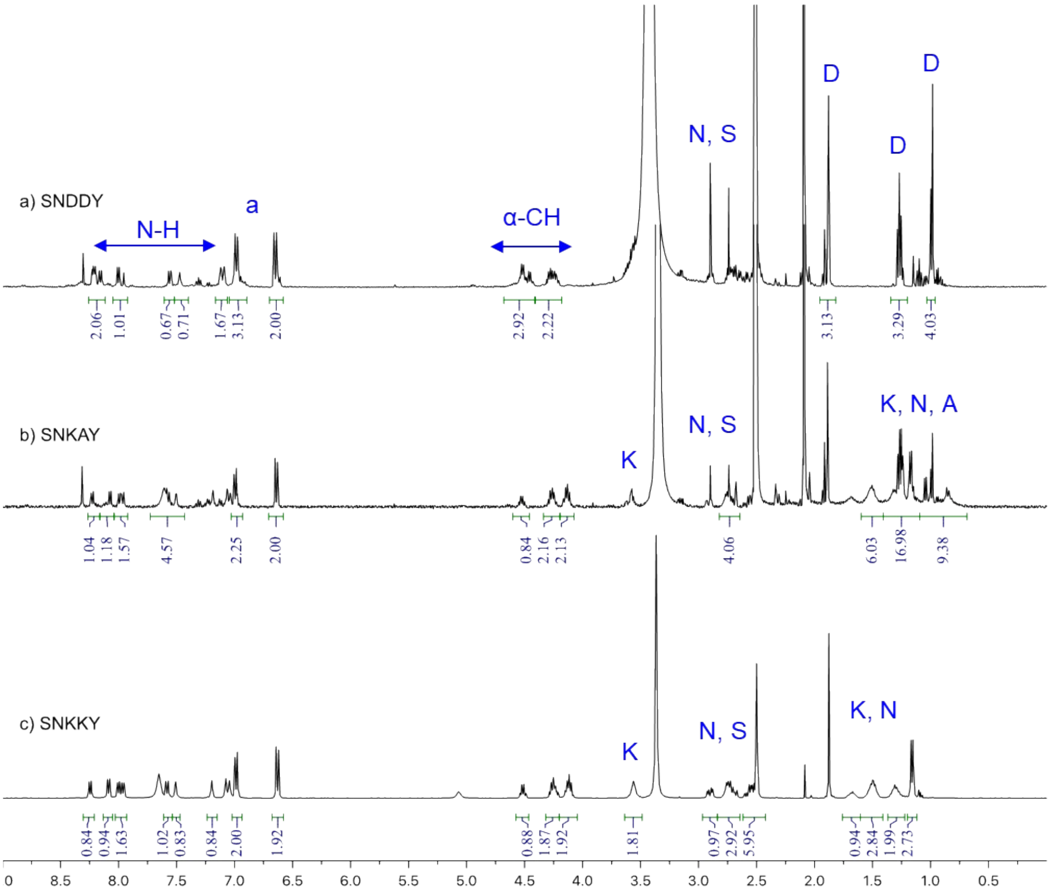


Figure S6. ^{1}HNMR of peptides synthesis in $CDCl_3$ (a) SNDDY-Ac (b) SNKAY-Ac (c) SNKKY-Ac.

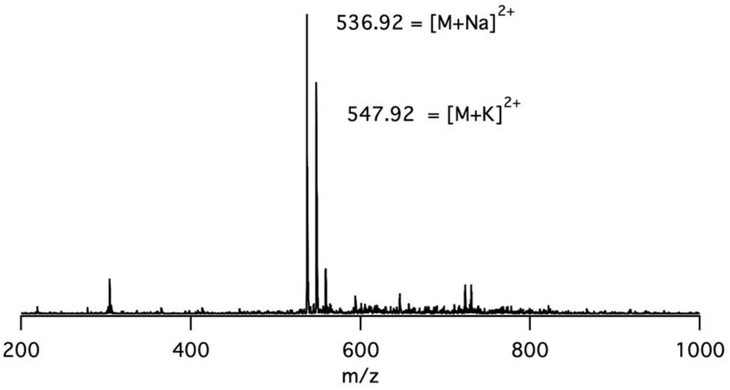


Figure S7. ESI-MS data taken in methanol in positive ion mode for the crude SNDDY-Cy3 peptide after trituration from diethyl ether.

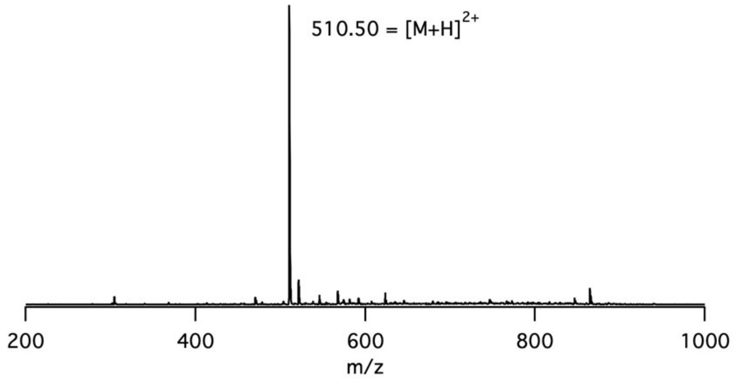


Figure S8. ESI-MS data taken in methanol in positive ion mode for the crude SNKAY-Cy3 peptide after trituration from diethyl ether.

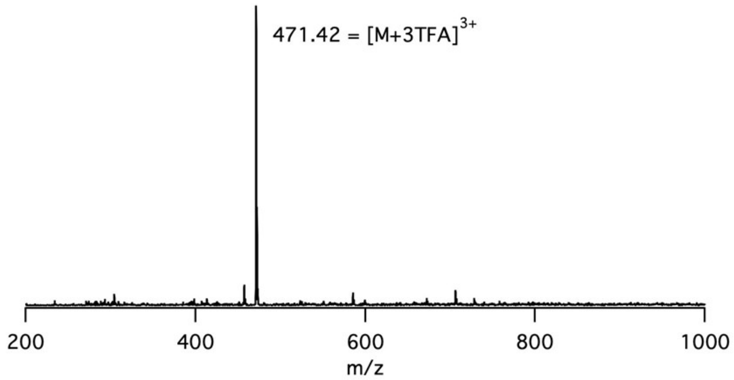


Figure S9. ESI-MS data taken in methanol in positive ion mode for the crude SNKKY-Cy3 peptide after trituration from diethyl ether.

Loading and release

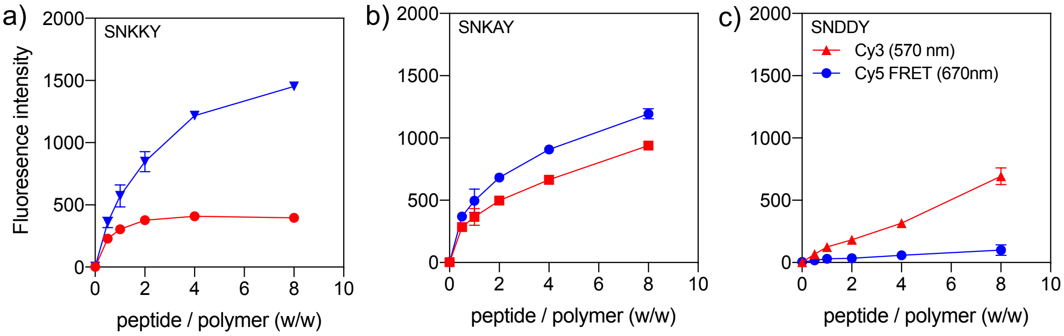


Figure S10. Raw FRET data showing Cy3 (570nm) and Cy5 (670nm) emission intensities for loaded nanogels upon excitation at 485 nm. Results are shown for loading of NG3 (0.1 mg/ml, PBS) with a) SNKKY, b) SNKAY and c) SNDDY. In all cases the ratio of labelled to unlabelled peptide is 1:9 (w/w), and the experiment was performed in 30 µL in a 384 well plate.

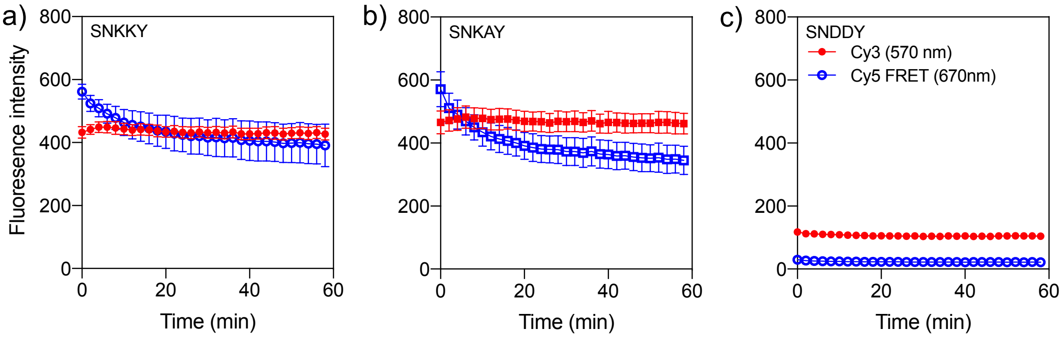


Figure S11. Raw FRET data showing Cy3 (570nm) and Cy5 (670nm) emission intensities for pre-loaded NG3 (30 µL, PBS) after switching the pH to 5.0 by addition of 10 µL of 200 mM citrate buffer (pH 4.85). All excitation is at 485 nm. Results are shown for NG3 loaded with 2 eq (w/w) of a) SNKKY, b) SNKAY and c) SNDDY. In all cases the ratio of labelled to unlabelled peptide is 1:9 (w/w), and the experiment was performed in a 384 well plate.

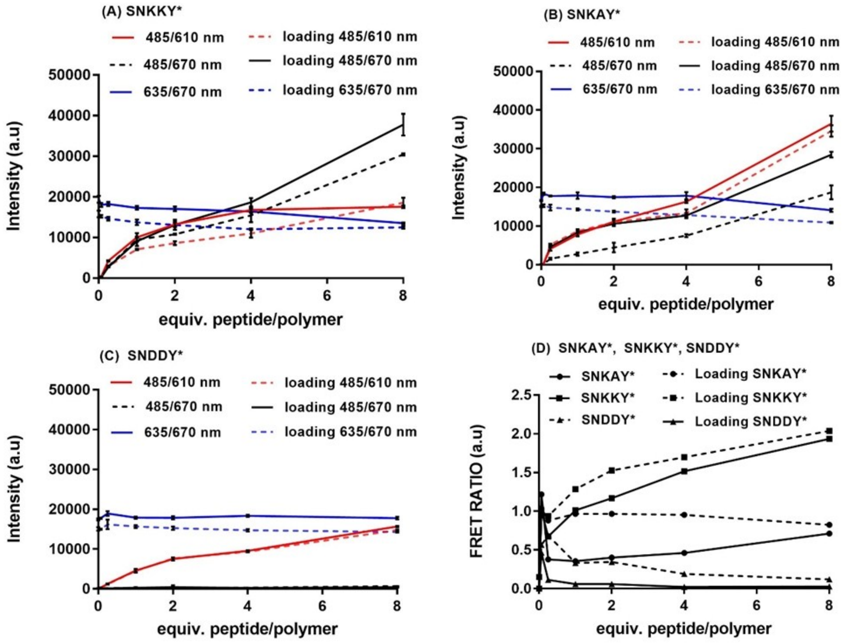


Figure S12. Release FRET results of increasing equivalents of peptide to NG3. (A) Release FRET of SNKKY* (1 : 9) in comparison with its loading FRET by the polymer (0.1 mg/mL). (B) Release FRET of SNKAY* (1 : 9) in comparison with its loading FRET by the polymer (0.1 mg/mL). (C) Release FRET of SNDDY* (1 : 9) in comparison with its loading FRET by the polymer (0.1 mg/mL). (D) FRET ratios of the peptides during loading versus release.

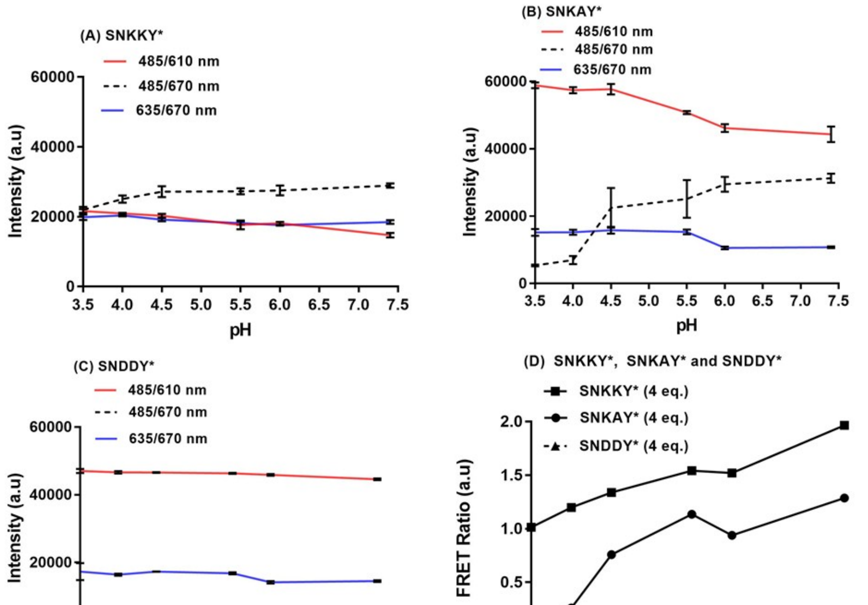


Figure S13. FRET release results of peptides by preloaded NG3 in different pH buffers (A) FRET release results of SNKKY*/SNKKY (1 : 9) (400 wt%) by polymer (0.1 mg/mL) after pH is gradually decreased with citrate buffers (200 mM). (B)) FRET release results of SNKAY*/SNKAY (1 : 9) (400 wt%) by polymer (0.1 mg/mL) after pH is gradually decreased with citrate buffers (200 mM).) (C) FRET release results of SNDDY*/SNDDY (1 : 9) (400 wt%) by polymer (0.1 mg/mL) after pH is gradually decreased with citrate buffers (200 mM). (D) Release FRET ratios of peptides by polymer in different pH buffers.

Fluorescence Lifetime Imaging Microscopy (FLIM)

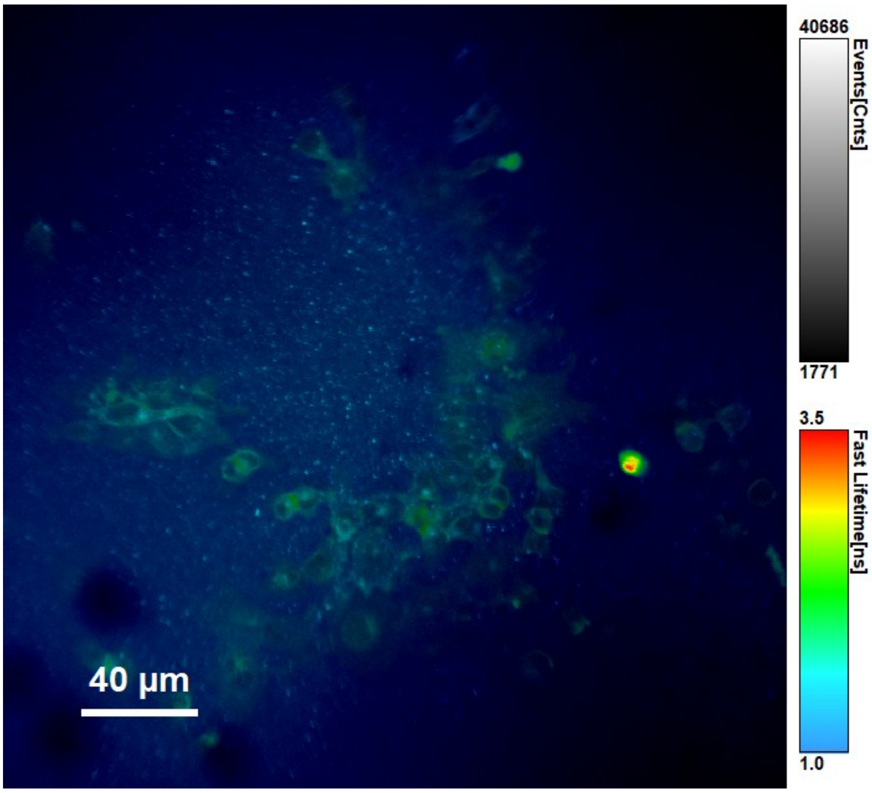


Figure S14. FLIM image after 10 min of incubation of FRET tagged NG3/SNKAY (1:2 wt) with MCF-7 cells.

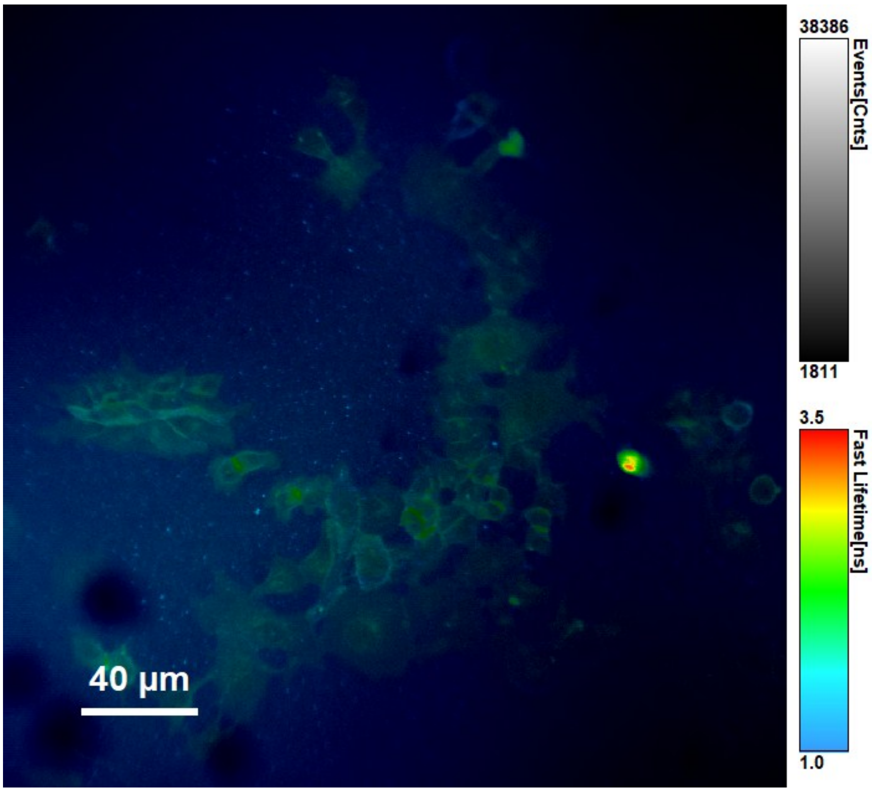


Figure S15. FLIM image after 60 min of incubation of FRET tagged NG3/SNKAY (1:2 wt) with MCF-7 cells.

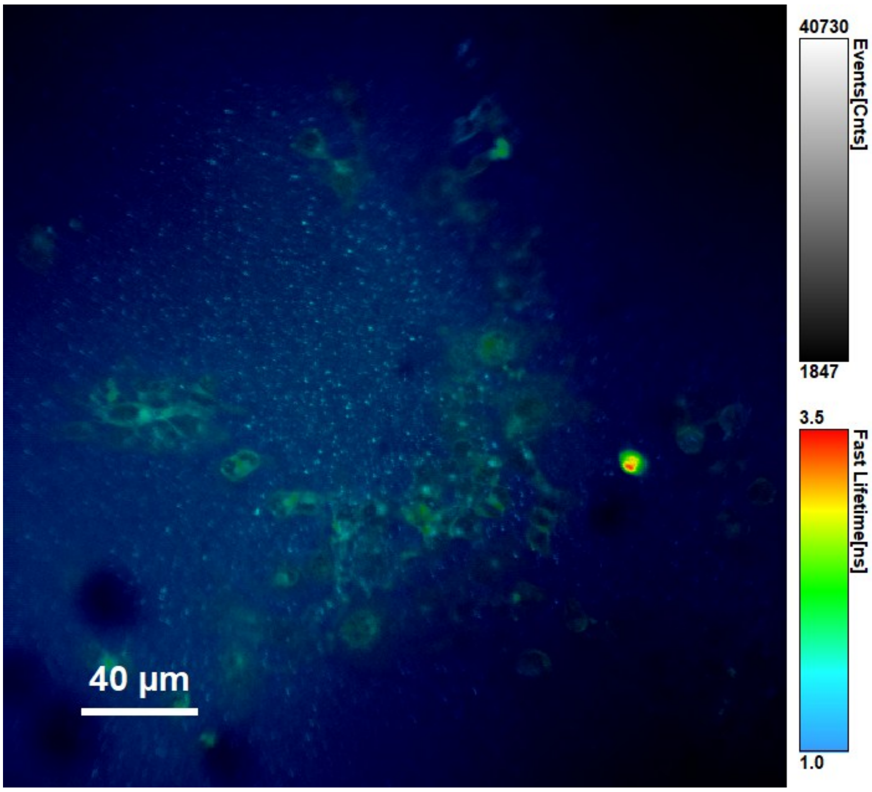


Figure S16. FLIM image after 90 min of incubation of FRET tagged NG3/SNKAY (1:2 wt) with MCF-7 cells.

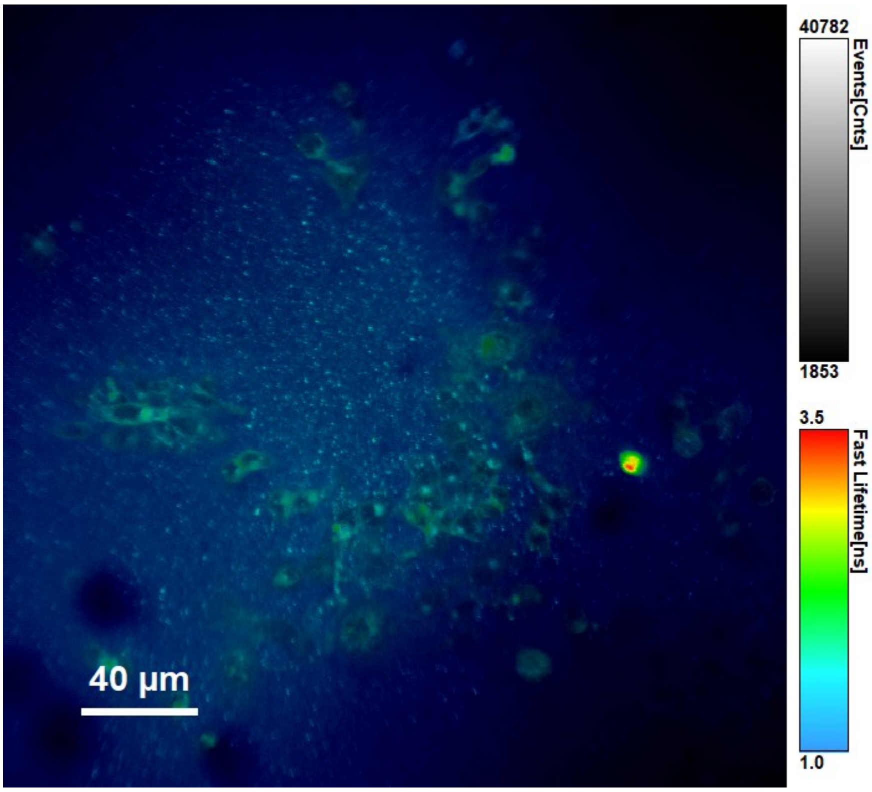


Figure S17. FLIM image after 120 min of incubation of FRET tagged NG3/SNKAY (1:2 wt) with MCF-7 cells.

References:

[1] R. L. Simmons, R. T. Yu, A. G. Myers, *J. Am. Chem. Soc.,* **2011**, *133*, 15870.

[2] M. V. Kvach, A. V. Ustinov, I. A. Stepanova, A. D. Malakhov, M. V. Skorobogatyi, V. V. Shmanai, V. A. Korshun, *Eur. J. Org. Chem.,* **2008**, 2107.