Supporting Information For:

Biodistribution Analysis of NIR-Labeled Nanogels using *in Vivo* FMT Imaging in Triple Negative Human Mammary Carcinoma Models

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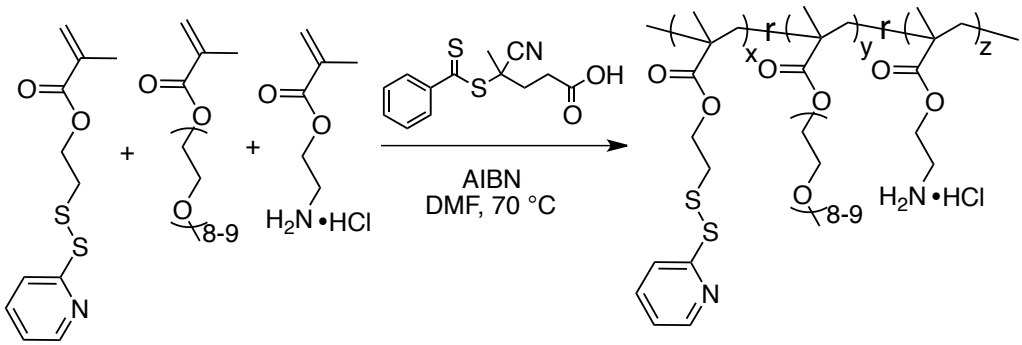
Experimental Procedures



Synthesis of random copolymer p(OEGMA-*co*-PDSMA-*co*-AEMA) P1:

To a Schlenk-flask, monomers PDSMA (1.160 g, 4.54 mmol), OEGMA (800 mg, 1.68 mmol), AEMA (23.6 mg, 0.20 mmol), chain transfer agent 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (47.2 mg, 0.17 mmol), and AIBN (5.6 mg, 0.034 mmol) were dissolved in DMF (4 mL). The mixture was degassed by performing three freeze-pump-thaw cycles and filled with argon. The reaction mixture was then sealed and transferred into a pre-heated oil bath at 70 °C and stirred for 24 h. The reaction flask was submerged in an ice bath to quench the polymerization, then dialyzed against dichloromethane in MWCO 3500 membrane for 48 hours to remove unreactive monomers. The solution was dried to yield the random copolymer **P1** as a waxy oil. GPC (THF) M_n: 6 kDa.

Đ: 1.5. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.49, 7.71, 7.15, 4.35-4.01, 3.76-3.51, 3.39, 3.05, 2.26-1.73, 1.18-0.78. The molar ratio of the three monomers in the polymer were determined by relative integrations of the aromatic protons of PDS, methoxy protons of PEG, and methylene protons of AE to give 29:68:3 (OEG:PDS:AE).

Synthesis of random copolymer p(OEGMA-*co*-PDSMA-*co*-AEMA) P2:

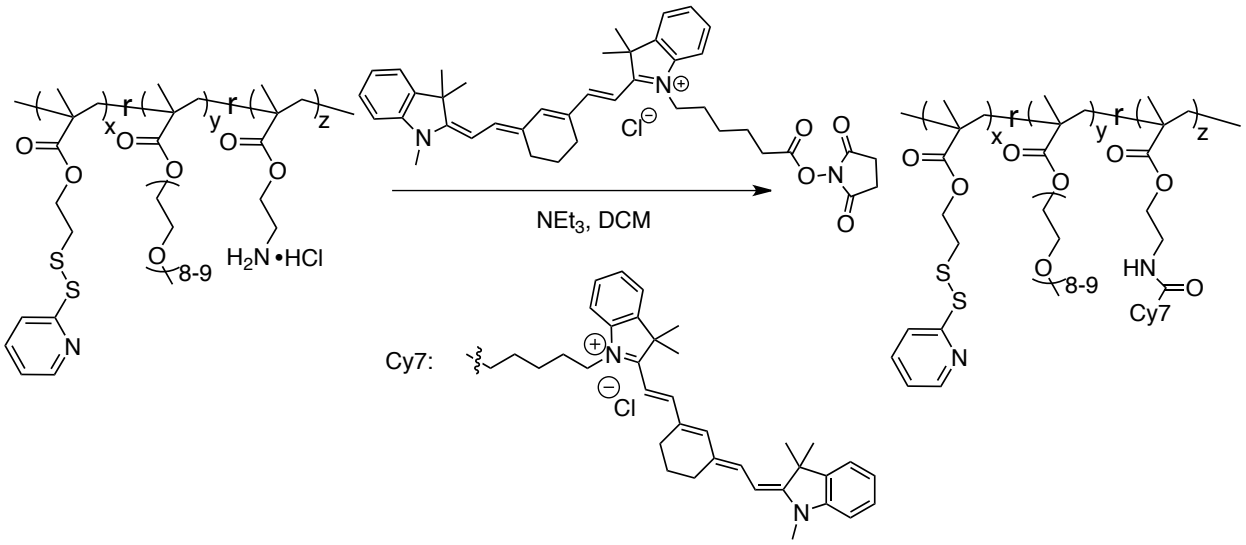
To a Schlenk-flask, monomers PDSMA (510 mg, 2 mmol), OEGMA (352 mg, 0.74 mmol), AEMA (14.7 mg, 0.089 mmol), chain transfer agent 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (10.3 mg, 0.037 mmol), and AIBN (1.2 mg, 0.0037 mmol) were dissolved in DMF (1.8 mL). The mixture was degassed by performing three freeze-pump-thaw cycles and filled with argon. The reaction mixture was then sealed and transferred into a pre-heated oil bath at 65 °C and stirred for 18 h. The reaction flask was submerged in an ice bath to quench the polymerization, then dialyzed against dichloromethane in MWCO 3500 membrane for 48 hours to remove unreactive monomers. The solution was dried to yield the random copolymer **P2** as a waxy oil. GPC (THF) M_n: 13 kDa. Đ: 1.2. ^{1}H NMR (400 MHz, Acetone-D_6) δ (ppm): 8.45, 7.66, 7.10, 4.35-3.97, 3.77-3.49, 3.34, 2.95, 2.12-1.67, 1.14-0.77. The molar ratio of the three monomers in the polymer were determined by relative integrations of the aromatic protons of PDS, methoxy protons of PEG, and methylene protons of AE to give 28:70:2 (OEG:PDS:AE).

Synthesis of random copolymer p(OEGMA-*co*-PDSMA-*co*-AEMA) P3:

To a Schlenk-flask, monomers PDSMA (402 mg, 1.57 mmol), OEGMA (269 mg, 0.57 mmol), AEMA (11.4 mg, 0.07 mmol), chain transfer agent 4-cyano-4-(phenylcarbonothioylthio)pentanoic

acid (6.9 mg, 0.025 mmol), and AIBN (0.8 mg, 0.005 mmol) were dissolved in DMF (1.2 mL). The mixture was degassed by performing three freeze-pump-thaw cycles and filled with argon. The reaction mixture was then sealed and transferred into a pre-heated oil bath at 65 °C and stirred for 18 h. The reaction flask was submerged in an ice bath to quench the polymerization, then dialyzed against dichloromethane in MWCO 3500 membrane for 48 hours to remove unreactive monomers. The solution was dried to yield the random copolymer **P3** as a waxy oil. GPC (THF) M_n: 22 kDa. Đ: 1.3. ^{1}H NMR (400 MHz, Acetone-D_6) δ (ppm): 8.42, 7.63, 7.07, 4.37-3.93, 3.78-3.46, 3.34, 2.99, 2.10-1.60, 1.13-0.74. The molar ratio of the three monomers in the polymer were determined by relative integrations of the aromatic protons of PDS, methoxy protons of PEG, and methylene protons of AE to give 27:69:4 (OEG:PDS:AE).

Synthesis of p(OEGMA-*co*-PDSMA-*co*-Cy7) P1-P3:



To round bottom flasks, the p(OEGMA-*co*-PDSMA-*co*-AEMA) polymers **P1-P3** (200 mg, 0.022 mmol AE) separately, each with Cy7 NHS ester (22.2 mg, 0.032 mmol), were dissolved in DCM (3 mL) and purged with argon. Then triethylamine (6.14 uL, 0.044 mmol) was added and the mixtures were stirred for 12 hours at ambient temperature. Mixtures were purified by dialysis

against dichloromethane in a MWCO 3500 regenerated cellulose membrane for 48 hours. The solutions were dried to yield the Cy7-labeled polymers p(OEGMA-*co*-PDSMA-*co*-Cy7) **P1-P3** as waxy oils.

Cy7 Conjugation Quantification of p(OEGMA-*co*-PDSMA-*co*-Cy7):

The presence of unreacted amine of AEMA monomer was evaluated by using the Fluorescamine reaction. Nanogels solutions (1 mg/mL, 93 nM amine/AE monomer) of precursor polymer p(OEGMA-*co*-PDSMA- *co*-AEMA), or NG-AE, and Cy7 reacted polymer p(OEGMA-*co*-PDSMA-*co*-Cy7), or NG-Cy7, were prepared in PBS buffer pH 7.4. In a 96 well (flat-bottomed) plate PBS buffer pH 7.4 (150 μL) and sample solutions NG-AE or NG-Cy7 (20 uL) were added to each well. A blank control was prepared containing PBS buffer pH 7.4 (170 μL). Then, fluorescamine solution in DMSO (12 uL, 465 nM) was added to each well and the fluorescence was obtained using a Molecular Devices Spectramax M5 plate reader (excitation: 390 nm; emission 465 nm). Average fluorescence values for NG-AE and NG-Cy7 were obtained from replicate readings (n=3) and normalized. Fluorescence of NG-Cy7 compared to NG-AE suggests 12.8% amines on NG-Cy7 were unreacted, a negligible 0.04% remaining of total monomer (**Figure S1**).

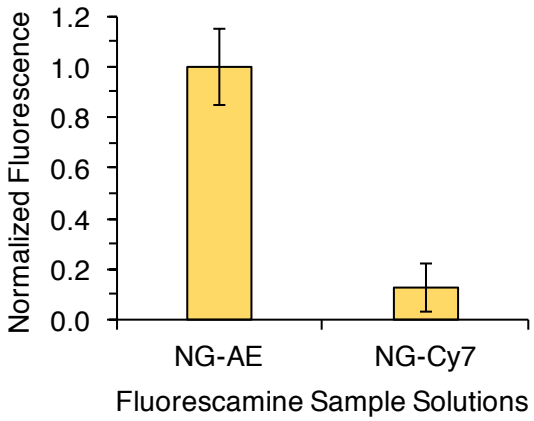


Figure S1. Fluorescamine normalized fluorescence of free amine reaction of nanogel NG-AE from precursor polymer p(OEGMA-*co*-PDSMA- *co*-AEMA) and NG-Cy7 of polymer p(OEGMA-*co*-PDSMA-*co*-Cy7), or NG-Cy7.

Nanogel Crosslinking PEG Post-Modification:

The crosslinking density was determined using the previously reported procedure (*J. Am. Chem. Soc*., **2012**, *134*, 6964-6967) by calculating the amount of 2-pyridinethione byproduct using its molar extinction coefficient (8.08 x 10^3 M^{-1} cm^{-1} at 343 nm) (*Bioconjugate Chem*. **2006**, *17*, 1376-1384). UV-vis absorption measurements were performed on 1000-fold dilutions of crosslinking reaction solutions. Crosslinking percentage was calculating by assumption that cleavage of two PDS units would produce two 2-pyridinethione byproduct and one disulfide bond. The functionalization of the nanogels with poly(ethylene glycol) methyl ether thiol (average M_n 1000, 2000, and 5000) was also quantified by the further formation of byproduct 2-pyridinethione from post functionalization with thiol moieties as previously reported (*Biomacromolecules*, **2015**, *16 (10)*, 3161–3171). Typically, PEG(1000)-SH, PEG(2000)-SH, or PEG(5000)-SH were reacted with crosslinked nanogel solution at PDS molar equivalencies or 1, 1.2, and 2, respectively, and stirred for 24 hours. For example, PEG(1000)-SH (27.5 mg, 0.0275 mmol) was dissolved in a minimum volume of water (170 μL) then added to the crosslinked nanogel solution (11.55 mg, 0.0275 mmol) and stirred for 24 hours. 2-Pyridinethione concentration calculations using its molar

extinction coefficient was supported by comparison with addition of excess DTT, to obtain 100% pyrdinethione generation, which was also used to calculate conjugation efficiencies (**Table S1**).

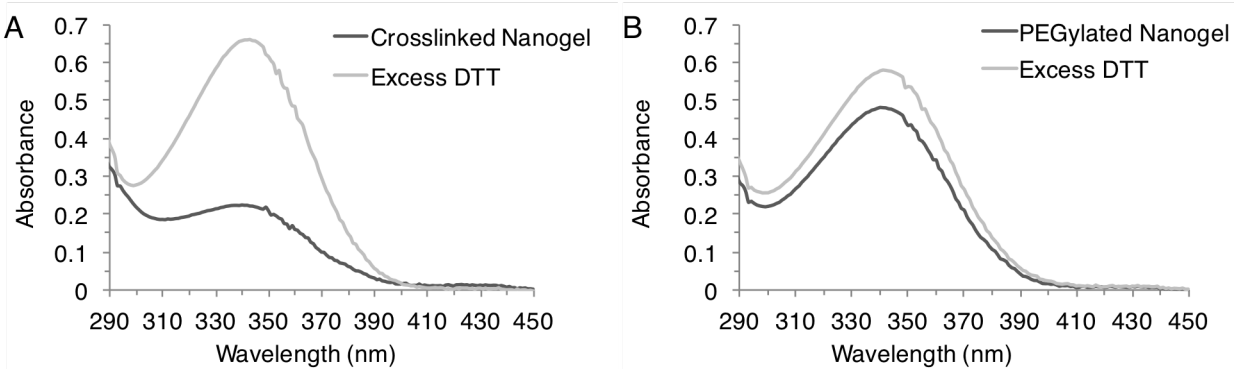


Figure S2. UV-vis absorption Spectra of 2-pyridinethione byproduct at 343 nm (A) crosslinking reaction with DTT and (B) PEG conjugation with poly(ethylene glycol) methyl ether thiol M_n 2000, each with comparison to 100% 2-pyridinethione from reaction with excess DTT.

Table S1. Nanogels' formulation, mPEG-thiol conjugation lengths, reaction efficiencies, and conjugation extents.

Name	Polymer	Formulation Conditions	Crosslink (PDS%)	Crosslink (mole%)	PEG (M_n)	PEG rxn Efficiency (PDS%)	PEG (mole%)
28 nm	P2 (13 K)	5 mg/mL, 25 ºC	60	42	1000	97	27
50 nm	P1 (6 K)	10 mg/mL, 15 mM Na_2SO_4, 25 ºC	40	28	1000	95	40
80 nm	P2 (13 K)	5 mg/mL, 1 mM Na_2CO_3 50 ºC	20	14	1000	80	45
135 nm	P2 (13 K)	5 mg/mL, 2.5 mM Na_2CO_3, 50 ºC	20	14	1000	80	45
36 nm PEG-1K	P1 (6 K)	10 mg/mL, 1.5 mM Na_2SO_4, 25 ºC	23	16	1000	94	51
56 nm PEG-2K	P1 (6 K)	10 mg/mL, 1.5 mM Na_2SO_4, 25 ºC	23	16	2000	91	49
58 nm PEG-5K	P1 (6 K)	10 mg/mL, 1.5 mM Na_2SO_4, 25 ºC	23	16	5000	68	37
78 nm PEG-1K	P1 (6 K)	5 mg/ml, 1 mM Na_2CO_3, 50 ºC	20	14	1000	94	53
78 nm PEG-2K	P1 (6 K)	5 mg/ml, 1 mM Na_2CO_3, 50 ºC	20	14	2000	91	51
79 nm PEG-5K	P1 (6 K)	5 mg/ml, 1 mM Na_2CO_3, 50 ºC	20	14	5000	72	40
49 nm 29% PEG	P2 (13 K)	10 mg/mL, 40 ºC	30	21	2000	63	29
44 nm 24% PEG	P2 (13 K)	10 mg/mL, 40 ºC	37	26	2000	48	24
42 nm 18% PEG	P2 (13 K)	10 mg/mL, 40 ºC	51	36	2000	54	18
34 nm 6% PEG	P2 (13 K)	10 mg/mL, 40 ºC	83	58	2000	43	6

31 nm 0% PEG	P2 (13 K)	10 mg/mL, 40 °C	98	68	2000	N/A	0
36 nm 46% PEG	P3 (22 K)	10 mg/mL, 2 mM Na_2CO_3, 25 °C	20	14	2000	82	46
35 nm 43% PEG	P3 (22 K)	10 mg/mL, 2 mM Na_2CO_3, 25 °C	30	21	2000	88	43

Final Nanogel Cy7 Probe Concentration:

The Cy7 probe concentration in final nanogel solutions were obtained by UV-vis absorption measurements using its molar extinction coefficient (199000 M^{-1} cm^{-1} at 759 nm) at Near-IR probe was quantified using a NanoDrop 2000C spectrophotometer. Concentrations were calculated assuming a path length of 1 mm.

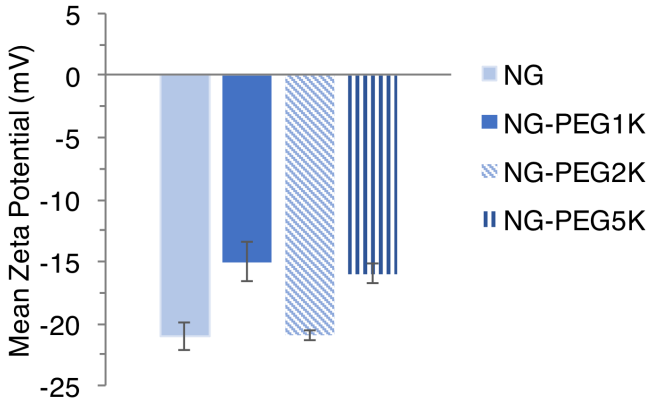


Figure S3. Mean zeta potential of NG prepared from polymer p(OEGMA-*co*-PDSMA-*co*-Cy7), NG-PEG1K following PEG M_n 1000 modification, NG-PEG2K following PEG M_n 2000 modification, and NG-PEG5K following PEG M_n 5000 modification obtained by DLS (avg. ± std. dev, n=4 measurements).

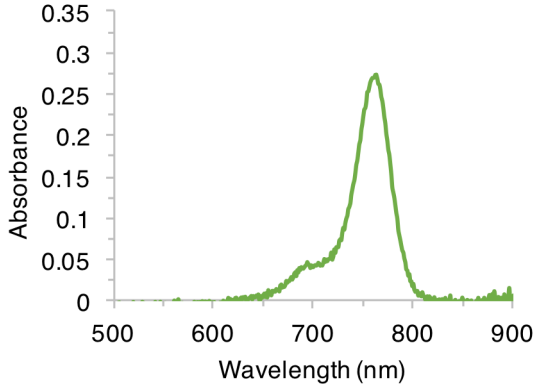


Figure S4. UV-vis absorption Spectra of Cy7-conjugated nanogel obtained using a NanoDrop spectrophotometer.

Table S2. Nanogel final polymer and Cy7 probe concentrations for 100 μL *in vivo* injection.

Series	Name	Polymer (mg/mL)	Cy7 (μM)
Size	28 nm	5.1	38
Size	50 nm	4	18
Size	80 nm	4.5	13
Size	135 nm	2.2	27
Length PEG	36 nm-PEG1K	0.69	45
Length PEG	56 nm-PEG2K	0.69	23
Length PEG	58 nm-PEG5K	0.69	16.8
Length PEG	78 nm-PEG1K	0.69	127
Length PEG	78 nm-PEG2K	0.69	50
Length PEG	79 nm-PEG5K	0.69	26.9
Percent PEG	49 nm 29% PEG	2.5	88
Percent PEG	44 nm 24% PEG	2.5	100
Percent PEG	42 nm 18% PEG	2.5	96
Percent PEG	34 nm 6% PEG	2.5	100
Percent PEG	31 nm 0% PEG	2.5	150
Small Size High PEG	36 nm 46% PEG	2.2	112
Small Size High PEG	35 nm 43% PEG	2.2	727

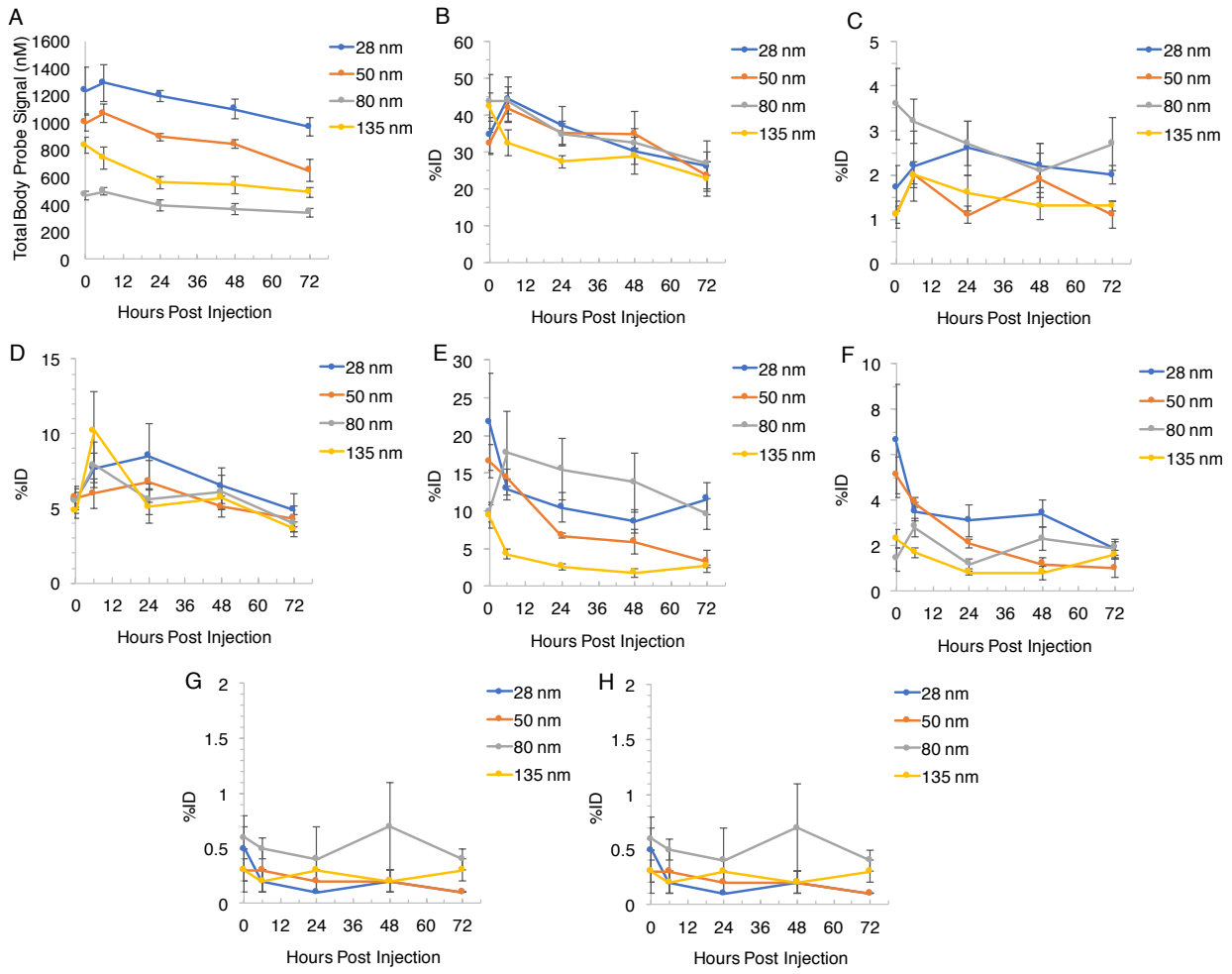


Figure S5. Size series quantitative *in vivo* (A) whole body probe signal, (B) %ID liver, (C) %ID spleen, (D) %ID intestine, (E) %ID lungs, (F) %ID heart, (G) %ID left kidney, and (H) %ID right kidney over 72 hours following intravenous administration obtained by FMT imaging. Data are given as mean ± standard deviation (n = 5).

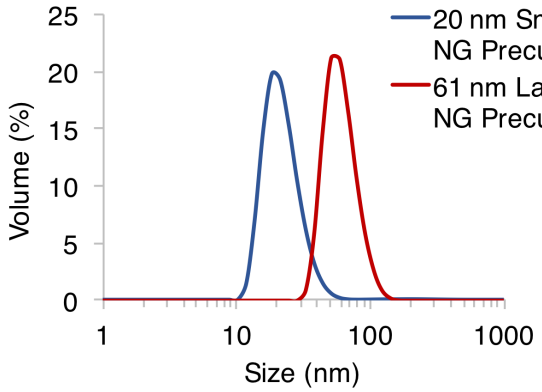


Figure S6. Size distribution of 20 nm nanogel (precursor to 36 nm-PEG1K, 56 NM-PEG2K, and 58 nm-PEG5K) and 61 nm nanogel (precursor to 78 nm-PEG1K, 78 nm-PEG2K, and 79 nm-

PEG5K) for the Length PEG Series prior to PEG modification obtained by DLS measurements in water.

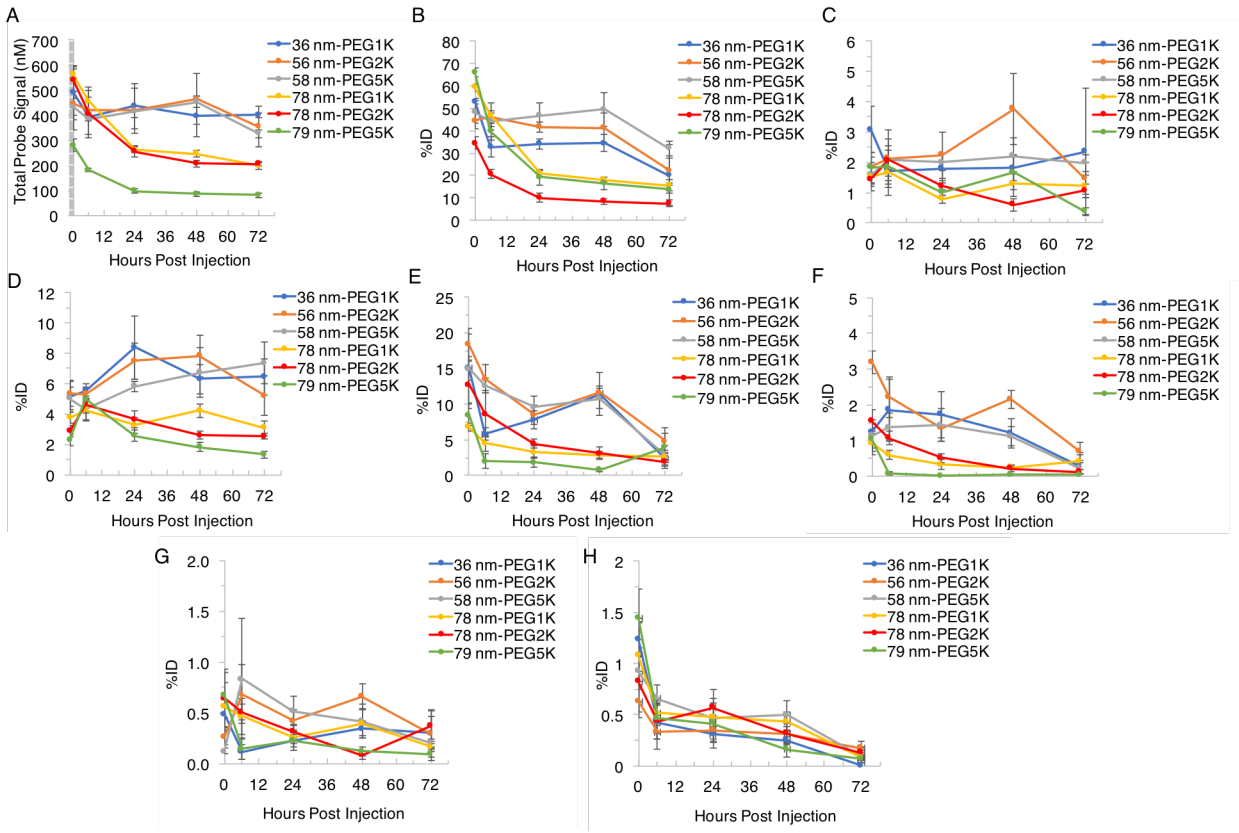


Figure S7. Length PEG series quantitative in vivo (A) whole body probe signal, (B) %ID liver, (C) %ID spleen, (D) %ID intestine, (E) %ID lungs, (F) %ID heart, (G) %ID left kidney, and (H) %ID right kidney over 72 hours following intravenous administration obtained by FMT imaging. Data are given as mean ± standard deviation (n = 5, except n = 4 for 72 h measurement of 36 nm-PEG1K nanogel).

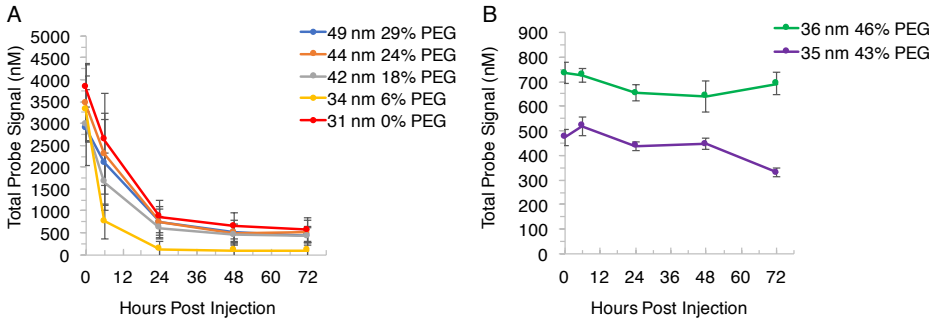


Figure S8. FMT imaging *in vivo* whole body probe signal of (A) Percent PEG series nanogels and (B) Small Size High PEG series nanogels 72 hours following intravenous administration. Data are given as mean ± standard deviation (n = 5).

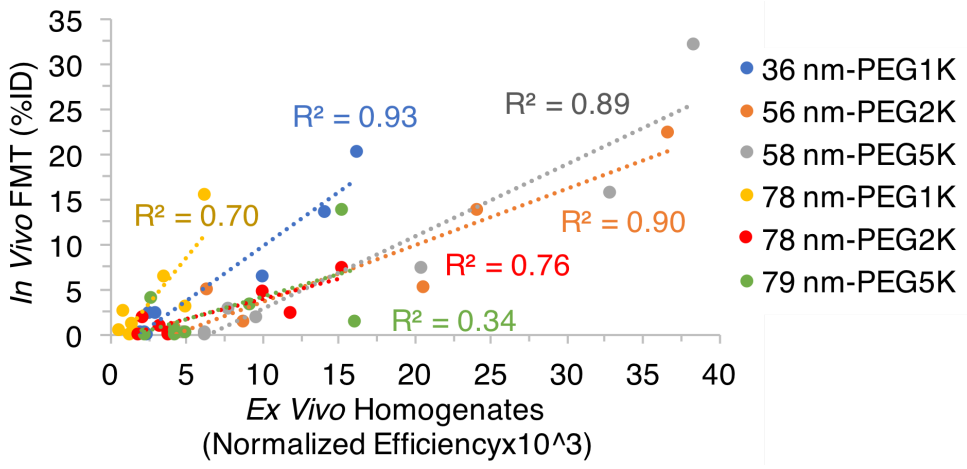


Figure S9. Comparison between *in vivo* FMT and *ex vivo* homogenate results of biodistribution values for tumor, liver, lung, spleen, intestine, heart, left kidney, and right kidney tissues using mean value of nanogels 36 nm-PEG1K, 56 nm-PEG2K, 58 nm-PEG5K, 78 nm-PEG1K, 78 nm-PEG2K, and 79 nm-PEG5K. Data are given as mean ± standard deviation (n = 5, except n = 4 for *in vivo* 72 h measurement of 36 nm-PEG1K nanogel).

Statistics: Data are expressed as mean ± standard deviation of the five mice tested per sample. Significance of data was analyzed using one-way analysis of variance (ANOVA), and post-hoc analysis by the method of Holm-Sidak. A Kruskall-Wallace ANOVA by ranks with post-hoc analysis was performed if the data did not pass normality or equal variance testing, by the method of Tukey. Statistical comparisons were performed for the total body probe between time points within each group, and between groups at each time point. Statistical comparisons of %ID/g tumor were performed between time points within each group, and between groups at each time point. Likewise, statistical comparisons of %ID of individual tissues were performed between time points within each group, and between groups at each time point. Statistically significant differences were set at p values ≤ 0.05, and calculated using SigmaPlot 12.0 software.