SUPPORTING INFORMATION: Targeted PRINT Hydrogels: The Role of Nanoparticle Size and Ligand Density on Cell Association, Biodistribution, and Tumor Accumulation

Kevin G. Reuter,[†] *Jillian L. Perry,*[¶] *Dongwook Kim,*[‡, §] *J. Christopher Luft,*[‡] *Rihe Liu,*[‡, §] *Joseph M. DeSimone**[, †, ¶, ¥, □, ○]

[†]Department of Chemistry, [¶]Lineberger Comprehensive Cancer Center, [‡]Eshelman School of Pharmacy, [§]Carolina Center for Genome Sciences, [¥]Institute for Nanomedicine, University of North Carolina, Chapel Hill, North Carolina 27599, United States

[□]Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, North Carolina 27695, United States

[○]Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, United States

Corresponding Author

*E-mail: desimone@unc.edu

Materials

2-aminoethyl methacrylate hydrochloride (AEM), diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide (TPO), poly (ethylene glycol) diacrylate (Mn 700) ($PEG_{700}DA$), trypsin, ethylenediaminetetraacetic acid (EDTA), and sucrose were obtained from Sigma-Aldrich. PTFE syringe filters (13 mm membrane, 0.2 μm pore size), Thermo Scientific Dylight 488, 650, and 680, Thermo Scientific immobilized (tris(2-carboxyethyl)phosphine)disulfide reducing gel (TCEP), Thermo Scientific HyClone fetal bovine serum (FBS), triethylamine (TEA), acetic anhydride, pyridine, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (1M HEPES buffer), methanol, sterile water, dimethylformamide (DMF), Dulbecco's phosphate buffered saline (pH 7.4) (DPBS), 1X phosphate buffered saline (pH 7.4) (PBS), and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Fisher Scientific. Maleimide-PEG_{5k}-succinimidyl carboxy methyl ester (Mal-PEG_{5k}-SCM), methoxy-PEG_{5k}- succinimidyl carboxy methyl ester ($mPEG_{5k}$-SCM), and methoxy-PEG_{1k}-thiol ($mPEG_{1k}$-SH) were all obtained from Creative PEGWorks (Winston Salem, NC). Polyvinyl alcohol (M_w 2000) (PVOH) was bought from Acros Organics and 2 μm conventional filters were purchased from Agilent Technologies. Borate buffer (pH 9.5) was acquired from RICCA Chemical Company. Murine alveolar macrophage (MH-S) cells and human epidermoid carcinoma (A431) cells were purchased from American Type Culture Collection. PRINT molds (80 nm x 80 nm x 320 nm and 55 nm x 55nm x 60 nm) were acquired from Liquidia Technologies. Tetraethylene glycol monoacrylate (HP_4A) was synthesized in-house by previously described methods.[1] Monomeric EGFR targeting affibody ($Z^{EGFR1907}$), fluorescein-labelled analog, and the wild-type affibody control were prepared with methods previously described.[2]

Methods

PRINT Particle Fabrication

The nano fabrication technique, Particle Replication in Non-wetting Templates (PRINT), has previously been described in greater detail.[3-5] In this account, our general pre-particle solution (PPS) consisted of a 3.5 wt% of cure-site monomer (CSM) in methanol. For studies quantifying density of affibody a CSM composition of 69 wt% HP_4A (main monomer), 20 wt% AEM (functional monomer), 10 wt% $PEG_{700}DA$ (cross-linker), and 1 wt% TPO (photoinitiator) was used. For all *in vitro* and *in vivo* studies a CSM composition of 68 wt% HP_4A, 20 wt% AEM, 10 wt% $PEG_{700}DA$, 1 wt% TPO, and 1 wt% Dylight maleimide (488, 650, or 680; imaging agent) was used. In our studies, two different NP types (55 nm x 55 nm x 60 nm and 80 nm x 80 nm x 320 nm) were fabricated. Using a Mayer rod (#3 for 80 x 320 nm; #2 for 55 x 60 nm) and a standard syringe pump (pump rate: 90 mL/h for 80 x 320 nm, 60 mL/h for 55 x 60 nm), a thin film of PPS was drawn onto corona treated PET with the aid of a custom large scale roll-to-roll PRINT nano-fabrication system at 12 ft/min. The methanol was evaporated from the PET delivery sheet using two heat guns resulting in a thin monomer film. The delivery sheet was laminated to the nano-patterned side of mold (either 80 nm x 80 nm x 320 nm or 55 nm x 55 nm x 60 nm Teflon-like mold) using a pressurized nip (80 PSI), filling the patterned nano-cavities via capillary action, and then delaminated. The acrylate monomer mixture was cured in the filled mold upon passing through a UV-LED (Phoseon, $\lambda_{\text{spectral output}}$ 395 nm, 3 SCFM N_2) yielding cured particles in the mold. A PVOH harvesting sheet was laminated to the filled mold, passed through a heated/pressurized nip (80 PSI, 160°C, 3 ft/min), cooled to room temperature, and then the harvesting sheet was split from the empty mold. The nanoparticle array was collected from the harvesting sheet by passing it through a bead of sterile water (4 ft/min; 2 mL water per 10 foot section), in turn, dissolving the PVOH and yielding a particle suspension. The NP

suspension was passed through a 2 μm Agilent filter to remove residual scum layer or particulates. The suspension was further purified by centrifugation with an Eppendorf 5417R centrifuge at 7,000 RPM for 20-30 min and the pelleted impurities were discarded by decantation from the purified supernatant. Excess PVOH was removed by centrifugation at 14,000 RPM (20-30 minutes for 80 x 320 nm, 1-3 hours for 55 x 60 nm) followed by supernatant removal and resuspension of the NP pellet in sterile water. The removal of excess PVOH was repeated three times.

Nanoparticle Characterization

The particle concentration was assessed by thermogravimetric analysis (TGA) on a TA Instruments Q5000 TGA. In short, 10 μL of the nanoparticle suspension was pipette into a tared aluminum sample pan. The sample was heated to 130°C (30°C/min) and held at isotherm for 10 minutes. The sample was then cooled to 30°C (30°C/min) and held for a 2 minute isotherm. The process was repeated with the supernatant from a centrifuged nanoparticle sample in order to account for remaining mass of PVOH in the solution. The particle concentration was determined by subtracting the concentration of remaining stabilizer from the original particle solution yielding the actual NP concentration of the stock solution. The particles were imaged by scanning electron microscopy (SEM) with a Hitachi S-4700 SEM. Before imaging, the particle stock solution was diluted (50 μg/mL) in methanol and 2 μL was pipette onto a silicon wafer. Using a Cressington 108 auto sputter coater, a 1.5 nm palladium-gold coating was sputtered onto the Si wafer and the sample was analyzed. Particle size and zeta potential was determined by dynamic light scattering (DLS) after dilution (0.1 mg/mL in water) using a Zetasizer Nano ZS (Malvern Instruments, Ltd.).

The hydrated dimensions of both particle types were measured by fluid atomic force microscopy (AFM) using an Asylum Research MFP-3D atomic force microscope. Height and phase retraces were measured in water, in AC mode using a silicon nitride cantilever (Budget Sensors, k = 0.06 N/m) at a 1 Hz scan rate and 2 μm scan size. The AFM sample was prepared by placing a droplet of suspended nanoparticles on a glass slide and the solvent was allowed to evaporate from the sample. With the particles settled on the slide, a droplet of water was placed on the sample to rehydrate the NPs and another droplet of water was placed on the cantilever tip. The hydrated AFM tip and glass slide were married and the images were collected. The dimensions and subsequent surface area of both particle types (both 80 x 320 nm and 55 x 60 nm) were measured by using either the phase or height retrace.

Surface Conjugation of NPs for Non-targeted Biodistribution Study

After particle purification, the suspension was washed into DMF (3x) using the centrifugation method (14,000 RPM; 0.5 to 1 h) and concentration was assessed by TGA analysis. The particle solution (1 mg; 0.865 mg/mL) was reacted with TEA (100 μL) and shaken for ten minutes on a shaker plate at room temperature (Eppendorf; 1400 rpm). Methoxy-PEG_{5k}-SCM was dissolved in DMF (14 mg; 100 mg/mL) added to the reaction mixture and left to react overnight. The reaction mixture was washed in DMF (2x) by centrifugation and resuspended in DMF (1 mg; 1 mg/mL). Post-PEGylation, the unreacted amine groups on the nanoparticle were quenched upon addition of pyridine (10 μL) and acetic anhydride (14 μL) and the mixture was shaken at room temperature for 30 minutes. The reaction mixture was washed in DMF (1x), pH 9.5 borate buffer (1x), and sterile water (3x). Following surface modification of both nanoparticle types, the particles were analyzed by DLS, TGA, and SEM.

Affibody Quantification

For affibody quantification studies, the same conjugation methods were used for both PEGylation and acetylation procedures as described above. However, in place of the inert methoxy-terminated PEG_{5k}-SCM, a maleimide-terminated PEG_{5k}-SCM was conjugated in order to functionalize the cysteine-terminated Z^{EGFR} affibody to the NP surface. Following surface modification, the functionalized NPs were characterized by TGA and DLS analysis. After characterization, the NP suspension (1 mg) was centrifuged and swapped to a 20 mM HEPES / 10 mM EDTA solution at ~1 mg/mL. Immobilized TCEP disulfide reducing gel (1.5x volume of affibody solution) was washed three times by spinning down the gel beads, removing the supernatant, and resuspending in HEPES/EDTA solution (1 min; 600 rpm). FITC-labelled Z^{EGFR} affibody (0.5 mg/mL) modified with a non-structural terminal cysteine group was added to the pelleted TCEP gel and the slurry was mixed on a shaker plate (1200 rpm) at room temperature for 45 minutes. After disulfide reduction, the slurry was centrifuged (2 min; 600 rpm) and the affibody supernatant was removed from the reducing gel. The activated affibody was immediately added to the modified-nanoparticle solution (1 mg; 1 mL total volume) and shaken overnight (1400 rpm). The mass of affibody charged per mg of NPs varied depending on the nanoparticle type being functionalized (80 x 320 nm v. 55 x 60 nm). When modifying 80 x 320 nm hydrogel particles, affibody was charged at 2.5, 5, 10, 25, or 50 μg per mg modified-NPs; however, with 55 x 60 nm NPs, affibody was charged at 10, 20, or 40 μg per mg NP. Thiol-PEG_{1k}-SH was dissolved in the HEPES buffer solution at 100 mg/mL and added (27 μL) to the NP reaction which continued to shake for three hours. The NP reaction was then centrifuged and

washed in water (4x). The FITC-tagged affibody NPs were then characterized by DLS, SEM, and TGA.

After conjugating fluorescently-tagged affibody to the nanoparticle surface, the amount of affibody conjugated to the particle was assessed by fluorescent analysis. The FITC tagged affibody-PEG-NP (1 mg/mL) solution was diluted 1:10 in borate buffer and transferred (200 μL) into a Corning black well plate in triplicate. The supernatant from the same nanoparticle solution was added to the black 96-well plate using the same method. A serial dilution of unconjugated FITC-affibody was utilized to create a standard curve (10 μg/mL - 0.01 μg/mL) and fluorescence measurements (λ_{ex} = 494 nm; λ_{em} = 521 nm) of the 96-well plate containing the (1) targeted nanoparticle (2) standard curve (3) and supernatant were taken using a SpectraMax M5 plate-reader. The fluorescence in the supernatant was subtracted from the fluorescence observed in the nanoparticle suspension and the final fluorescence correlated to FITC-tagged affibody concentration via standard curve.

Surface Conjugation of NPs for Targeted In Vitro and In Vivo Studies

For *in vitro* and *in vivo* studies with targeted nanoparticles, the conjugation methodology outlined above was followed. However, instead of FITC-labelled affibody, a non-tagged analog was used. A wild-type affibody was utilized as a negative control and it was conjugated to the functionalized NPs with identical methods. Additionally, a non-targeted PEG control was used as a second negative control and it was fabricated by following all steps outlined above except for the addition of targeting ligand. A completely non-functionalized positively charged particle was used as a positive control for *in vitro* experimentation.

A431 Cell Association and Competition Assay

For cellular association experiments, A431 cells were plated in complete medium (DMEM) at 20,000 cells per well in a 24-well plate and incubated overnight (37°C, 5% CO_2) to 50% confluence. Dye-labelled particles were then dosed at three different concentrations (5, 15, 30 μg / mL) onto the A431 cells and incubated for 4 h. At these time points, cells were washed (3x) with 0.5 mL 1x PBS and the cells were detached from the plate with 1x trypsin/EDTA (300 μL / well). Cells were resuspended in 500 μL of a 0.2% TB solution (1:1 1x PBS with 10% FBS) for a total sample volume of 800 μL. The A431 cells were transferred to a polypropylene tube and analyzed using a flow. For each sample 10,000 cells were measured.

Cell uptake competition studies were carried out with the exact same cellular preparation procedure. However, epidermoid carcinoma cells were first incubated with the free unlabeled monomeric Z^{EGFR} affibody for 30 minutes at five different concentrations (0, 3.5, 35 350, 3000 ng / mL). Following this, EGFR-targeted PRINT particles (15 μg/mL) were added and incubated for an additional 4 h, as previously described. Finally, particle with the pre-dosed A431 cells was analyzed via flow cytometry studies, as outlined above.

Macrophage Association Assay

Murine alveolar macrophage cells (MH-S) were used to determine how nanoparticle association and internalization varies as a function of both targeting ligand density and nanoparticle size. In these studies, MH-S cells were plated (40,000 cells per well) in a 24-well plate and incubated at 37°C for 48 h. Following the time period, dye-labelled (Dylight 488) nanoparticles (20 μg / mL)

were incubated with the macrophage cells for 4 and 24 h. At the time points, cells were washed three times with 0.5 mL 1x PBS and 1x trypsin/EDTA (300 μL / well) was added to detach the cells from the plate. The cell solution was transferred to a polypropylene tube and analyzed using a flow cytometer. For each sample 10,000 cells were measured.

In Vivo Studies

All animal studies were carried out with the approved protocol by The University of North Carolina Animal Care and Use Committee. Female athymic nude mice (*Foxn1*^{nu}; 20- 30 g) were dosed via tail vein injection from an 8 mg /mL nanoparticle suspension in an isotonic 9.25 wt% sucrose solution.

For all tumor studies, mice aged 4-6 weeks and 20-25 grams in body weight were purchased from UNC-Chapel Hill's animal core. A431 cells were administered (2 x 10^6 cells in 150 μL of DMEM) subcutaneously into the right rear flank of each mouse and tumor volume was estimated by the formula: $mm^3 = (w^2 \times l) / 2$, where w = width and l = length of the tumor. After approximately 4 weeks, tumors were 300-500 mm^3 and mice were randomized for each study arm.

To determine nanoparticle size dependence on passive tumor accumulation, PEGylated/non-targeted hydrogels of two distinct sizes were dosed into tumor-bearing mice. Nanoparticle injections and harvesting of organs were performed with aid from the Animal Studies Core at UNC-Chapel Hill. Mice were dosed (60 mg / kg) with 80 x 320 nm particles, 55 x 60 nm particles, or sucrose (N = 4 per arm). After 24 h, mice were anesthetized with a ketamine/ dexmedetomidine blend. Mice were then euthanized via cardiac puncture for blood collection (stored in heparinized Eppendorf tubes) and cervical dislocation for a secondary

conformation of death. Tissues were harvested (liver, spleen, lung, kidney, and tumor), weighed, and transferred to 12-well plates for fluorescence analysis on an IVIS Lumina instrument (Caliper Life Sciences) and fluorescence filters were set (λ_{ex} = 675 nm / λ_{em} = 720 nm). Harvested blood (100 µL) from all samples was transferred into a black 96-well plate and a small aliquot of each particle type was pipette into blood from a control mouse (5 µL NP stock in 195 µL blood) in order to normalize fluorescence between NPs.

In an effort to determine how targeting ligand density affects blood retention over time, 80 x 320 nm particles were administered and harvested at several time points. These studies were conducted with five different nanoparticle study groups: 6.5 $x10^{-4}$ LG / nm^2, 1.8 $x10^{-3}$ LG / nm^2, 4.5 $x10^{-3}$ LG / nm^2, PEG control, and a wild-type negative control (12.5 mg / kg). Mice were harvested at five different time points: 0.083, 0.5, 1, 3, and 24 h (N = 4 per time point for each NP arm). Tissue harvests and fluorescence analysis were both conducted in the same manner as methods previously described. PK analysis of blood retention studies were conducted with MS Excel PK Solver 2.0.[6] Data was fit to a non-compartmental model for all nanoparticle type.

Targeted tumor accumulation and biodistribution studies with targeted 80 x 320 nm hydrogels in A431 tumor-bearing mice were conducted as a function of targeting ligand density. The general procedure outlined in the non-targeted particle biodistribution section above was followed, except targeting ligand was utilized. For this study, five different particles types were analyzed: 6.5 $x10^{-4}$ LG / nm^2, 1.1 $x10^{-3}$ LG / nm^2, 1.8 $x10^{-3}$ LG / nm^2, PEG control, and a wild-type negative control (12.5 mg / kg). It should be noted that the NP ligand density at 4.5 $x10^{-3}$ LG / nm^2 was not conducted due to poor blood retention observed in the previous pharmacokinetic experiment. Additionally, Dylight 650 was used as the nanoparticle imaging agent instead of Dylight 680.

In order to determine how NP size and targeting density affect tumor accumulation and biodistribution, *in vivo* trials were conducted with 55 x 60 nm particles in tumor-bearing mice. In this experiment five different nanoparticle types were investigated: 1.2 $x10^{-3}$ LG / nm^2, 2.0 $x10^{-3}$ LG / nm^2, 3.0 $x10^{-3}$ LG / nm^2, PEG control, and negative control. Notably, ligand loadings were designed to closely match that of the 80 x 320 nm tumor accumulation study at the two highest ligand densities. As well, a third ligand density was appended onto the study to potentially enhance nanoparticle uptake in the tumor even further.

Table S1. Targeted nanoparticle characterization via dynamic light scattering

Type (nm)	Surface Modification	D_h (nm)[a]	PdI[a]	ζ (mV)[a]	Ligand Density (LG / nm^2) [b]
80 x 320	Mal-PEG_{5k} /Acetylation	270.1 ± 1.4	0.063 ± 0.022	-24.6 ± 0.4	-
	Methoxy-PEG_{1k}	271.2 ± 0.7	0.017 ± 0.019	-27.4 ± 0.1	-
	2.5 µg LG Charge	270.1 ± 1.7	0.020 ± 0.017	-25.7 ± 0.9	$6.5 x10^{-4} \pm 1.2 x10^{-4}$
	5 µg LG Charge	276.1 ± 2.5	0.017 ± 0.012	-25.5 ± 0.6	$1.1 x10^{-3} \pm 1.5 x10^{-4}$
	10 µg LG Charge	277.6 ± 2.3	0.015 ± 0.014	-22.0 ± 0.3	$1.8 x10^{-3} \pm 2.1 x10^{-4}$
	25 µg LG Charge	290.2 ± 3.7	0.049 ± 0.042	-17.7 ± 0.7	$3.3 x10^{-3} \pm 1.6 x10^{-4}$
	50 µg LG Charge	295.5 ± 3.8	0.109 ± 0.032	-13.0 ± 1.1	$4.5 x10^{-3} \pm 7.5 x10^{-4}$
55 x 60	Mal-PEG_{5k} /Acetylation	172.7 ± 2.5	0.13 ± 0.019	-17.2 ± 0.3	-
	Methoxy-PEG_{1k}	174.0 ± 1.1	0.15 ± 0.019	-13.6 ± 0.5	-
	10 µg LG Charge	163.2 ± 4.8	0.11 ± 0.017	-14.3 ± 0.2	$1.2 x10^{-3} \pm 2.2 x10^{-4}$
	25 µg LG Charge	170.5 ± 0.8	0.16 ± 0.012	-13.9 ± 0.6	$2.0 x10^{-3} \pm 3.6 x10^{-4}$
	40 µg LG Charge	165.9 ± 1.6	0.082 ± 0.017	-15.2 ± 0.9	$3.0 x10^{-3} \pm 5.8 x10^{-4}$

[a] Measured by dynamic light scattering
[b] Calculated from fluorescence analysis

Table S2. Pharmacokinetic parameters of targeted 80 x 320 nm particles

Ligand Density (LG / nm^2)	$t_{1/2}$ (h)	**CL** (mL/h)	AUC_{0-t} (h*mg/mL)
0.65 x 10^{-3}	3.91	0.81	0.38
1.8 x 10^{-3}	3.32	0.92	0.34
4.5 x 10^{-3}	0.68	4.66	0.06
WT Control	8.19	0.42	0.65
PEG Control	11.17	0.14	1.70

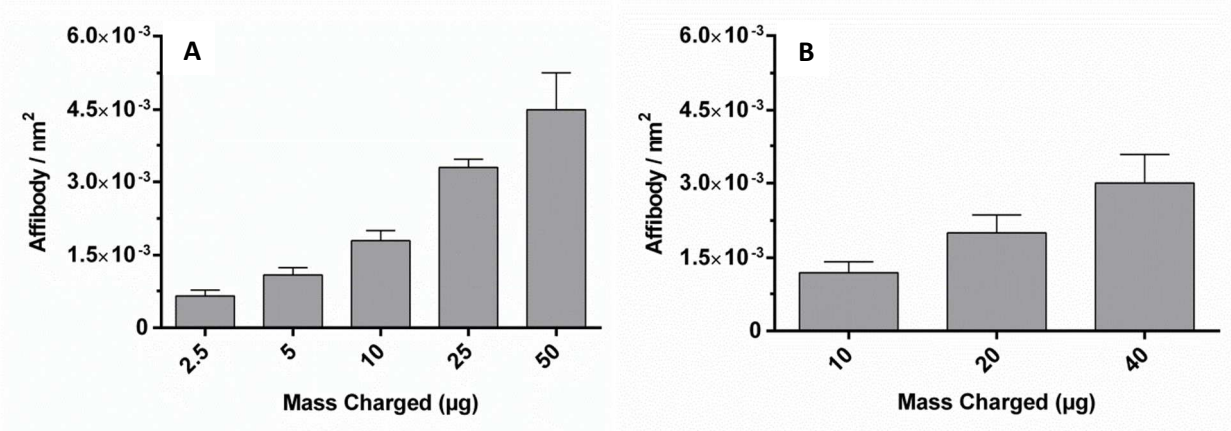

Figure S1. Quantification of targeting ligand on 80 x 320 nm (A) and 55 x 60 nm (B) NPs based upon various amounts of fluorescein-labeled Z^{EGFR} affibody charged (N = 4).

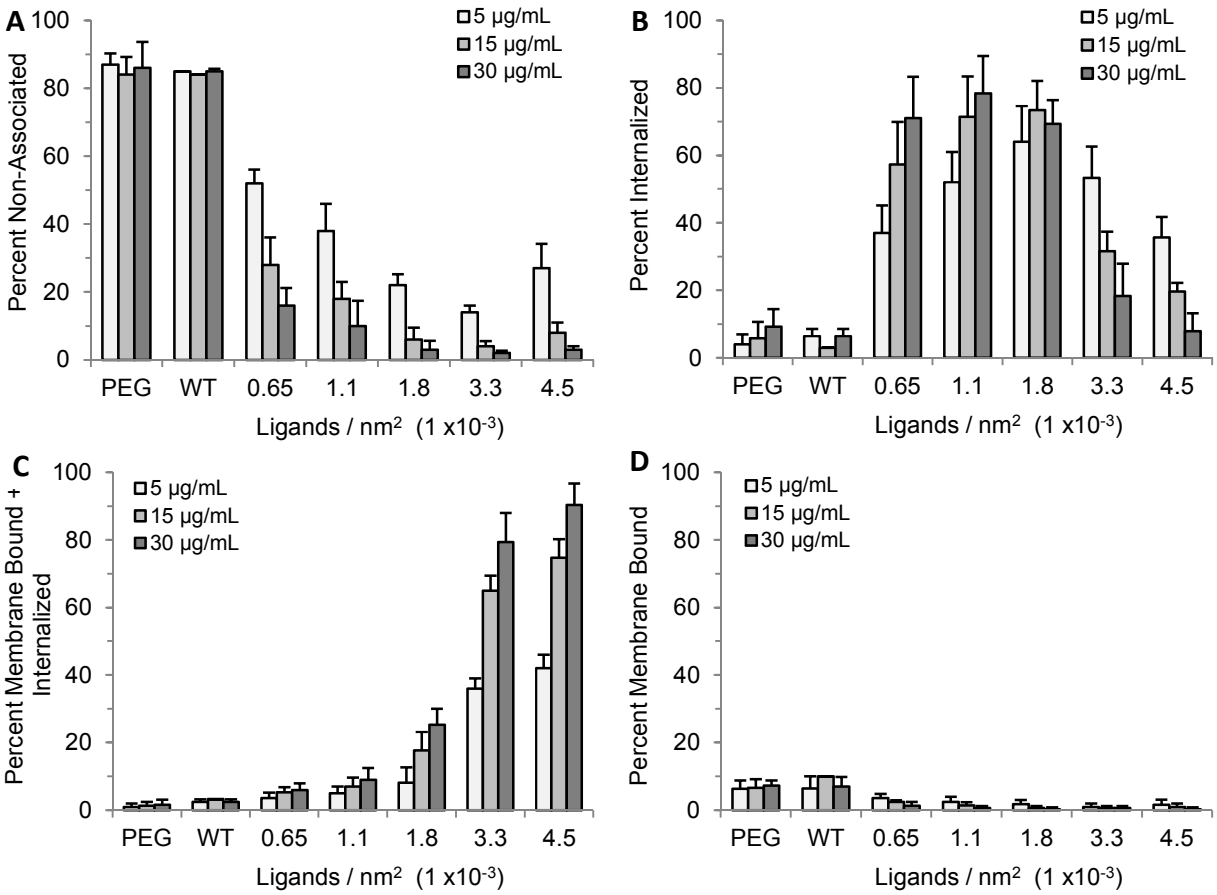

Figure S2. Sub-populations A431 cell interactions with 80 x 320 nm particles at five different targeting ligand densities and various dosages (N = 3).

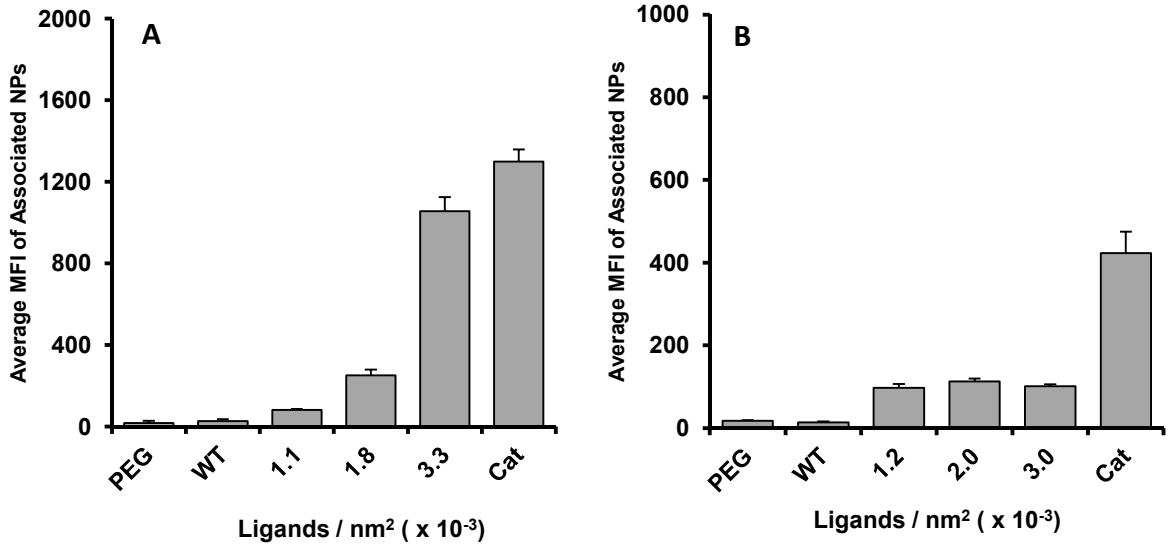

Figure S3. Average mean fluorescence intensity of epidermoid carcinoma cells (A431) associated with targeted 80 x 320 nm (A) and 55 x 60 nm (B) at three distinct ligand densities (N = 3).

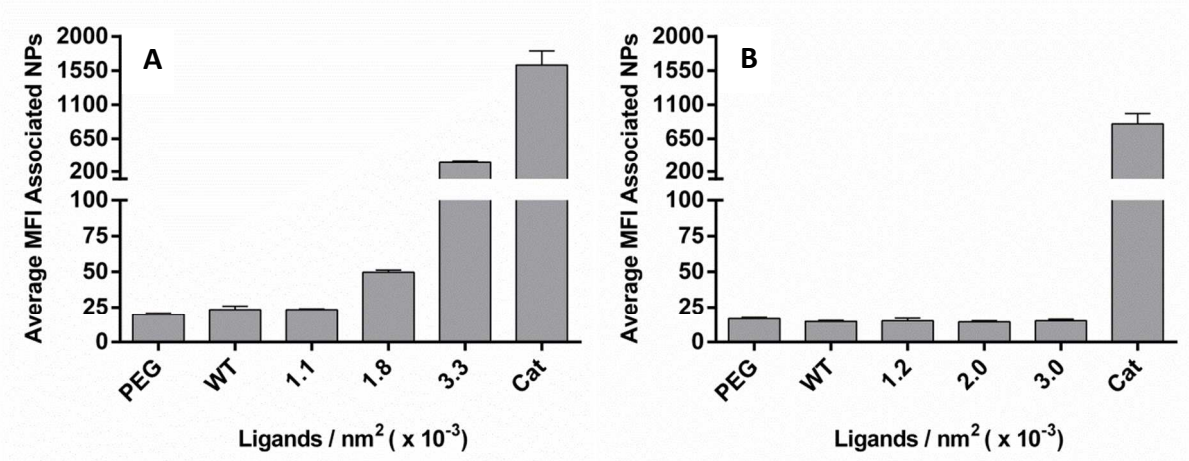

Figure S4. Average mean fluorescence intensity of alveolar macrophage cells (MH-S) associated with targeted 80 x 320 nm (A) and 55 x 60 nm (B) at three distinct ligand densities (N = 3).

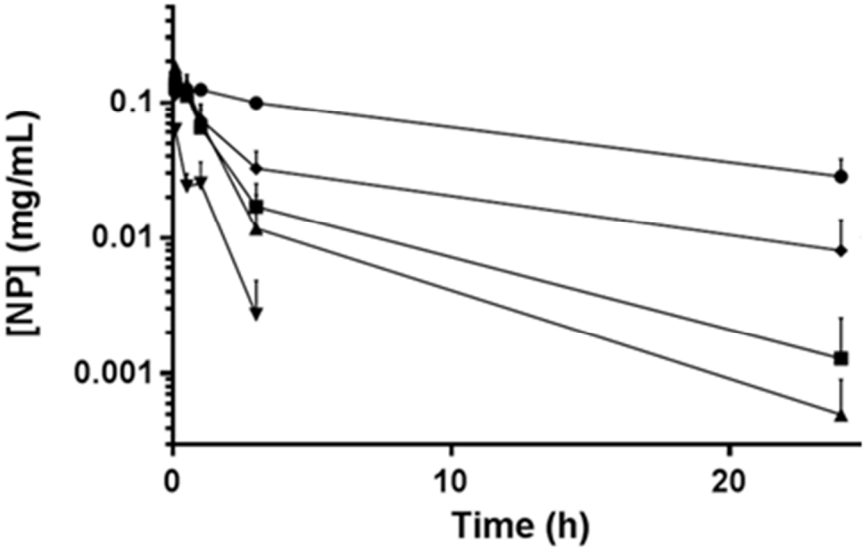

Figure S5. Blood pharmacokinetics of 80 x 320 nm NPs at various targeting densities [square = 6.5 x10^{-4} LG / nm^2; triangle = 1.8 x10^{-3} LG / nm^2; inverted triangle = 4.5 x10^{-3} LG / nm^2] at five time points (0.083, 0.5, 1, 3, and 24 h). PEGylated [circle] and wild-type [diamond] used as controls (N = 4; per point).

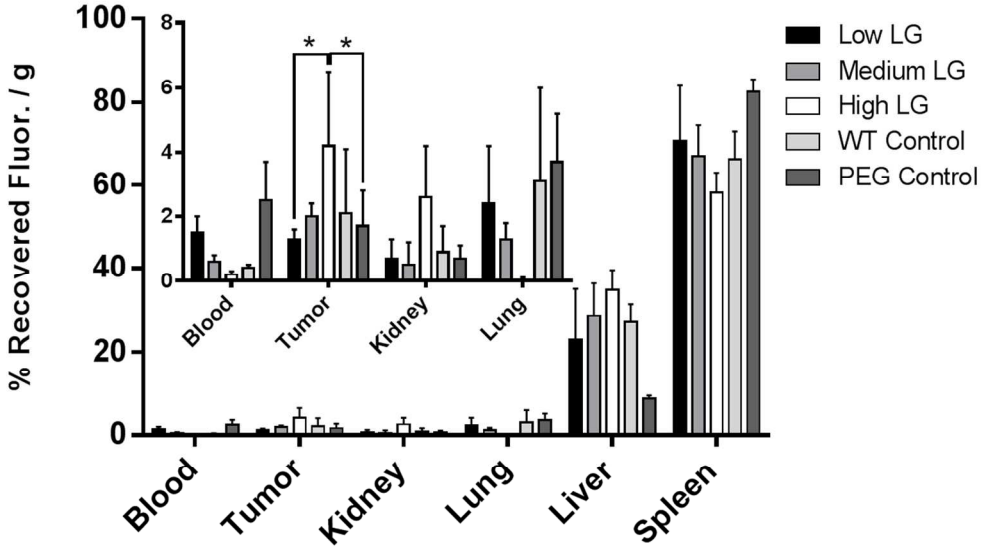

Figure S6. Biodistribution of 80 x 320 nm particles at 24 hours (N = 4). PEGylated and wild-type affibody particles used as controls [Low LG = 6.5 x10^{-4} LG / nm^2; Medium LG = 1.1 x10^{-3} LG / nm^2; High LG = 1.8 x10^{-3} LG / nm^2]. *, $P < 0.05$.

REFERENCES

1. Guzman, J.; Iglesias, M. T.; Compan, V. and Andrio, A. *Polymer* **1997**, 38, 5227-5232.
2. Kim, D.; Yan, Y.; Valencia, C. A. and Liu, Rihe. *PLoS One* **2012**, 7, 1-13.
3. Gratton, S. E. A.; Napier, M. E.; Ropp; P. A.; Tian, S. and DeSimone J. M. *Pharm. Res.* **2008**, 25, 2845-2852.
4. Rolland, J. P.; Maynor, B. W.; Euliss, L. E.; Exner, A. E.; Denison, G. M. and DeSimone, J. M. *J. Am. Chem. Soc.* **2005**, 127, 10096-10100.
5. Perry, J. L. and Reuter, K. G.; Kai, M. P.; Herlihy, K. P.; Jones, S. W.; Luft, J. L.; Napier, M.; Bear, J. E. and DeSimone, J. M. *Nano Lett.* **2012**, 12, 5304-5310.
6. Zhang, Y.; Huo, M.; Zhou, J. and Xie, S. *Comput. Methods Programs Biomed.* **2010**, 99, 306-314.