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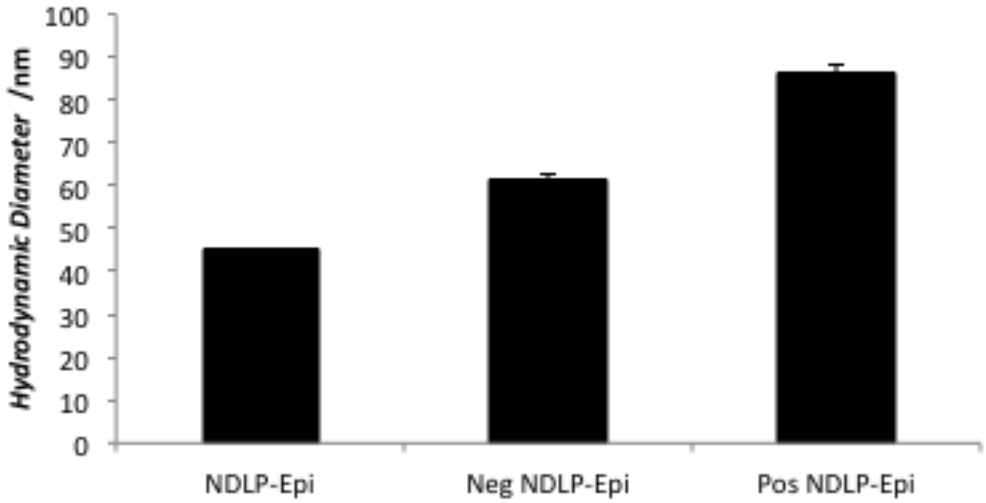
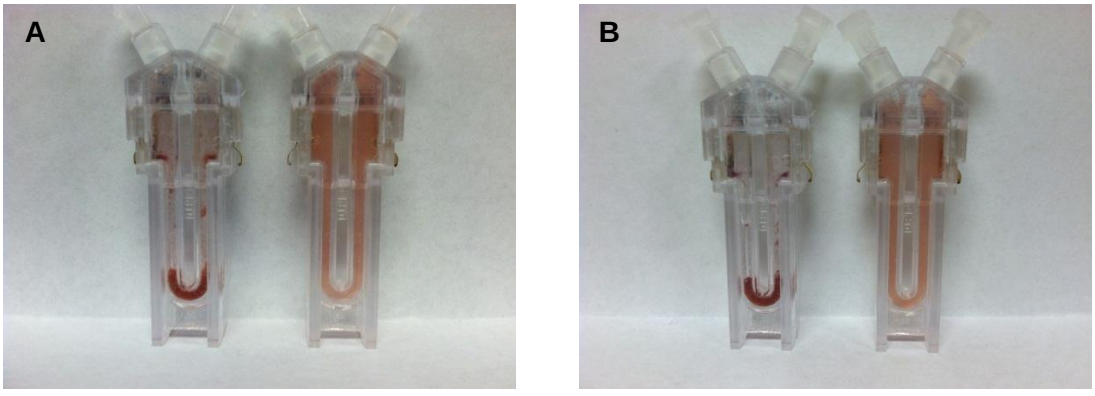
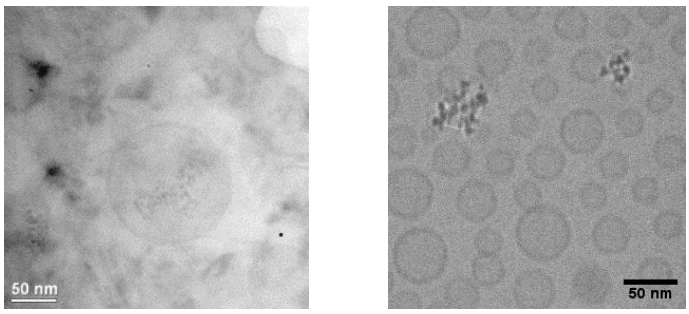
Supporting Information
Supplementary Figures



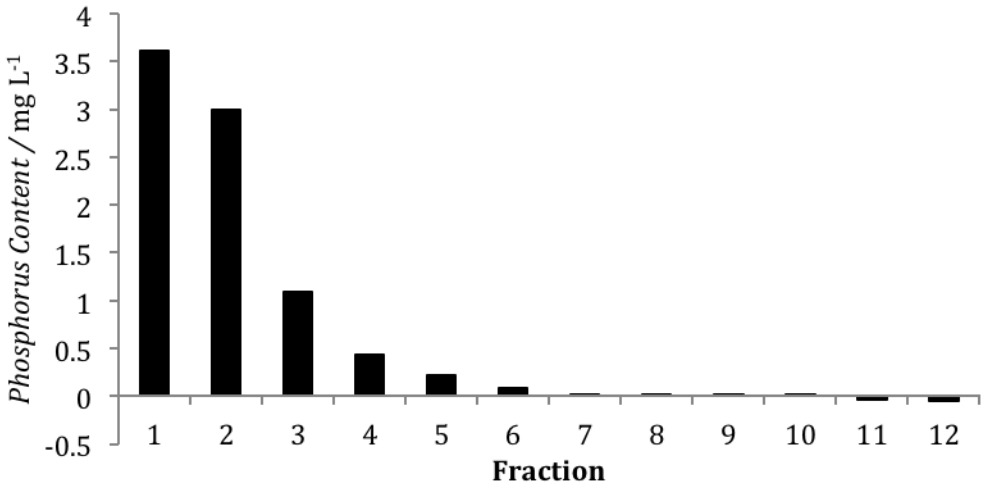
Supplementary Figure S1: NDLP size changes with targeting. NDLP-Epirubin increases in size with the addition of streptavidin (Neg NDLP-epirubicin) and then the targeting antibody (Pos NDLP-Epi). The size increases with the addition of streptavidin and antibody are commensurate with the molecules added. The radius of streptavidin has been reported to be 4nm[1] while the dimensions of IgG are 14 x 8.5 x 4.0nm[2]. Here we observed an increase of 16nm in particle diameter with the addition of streptavidin, which would correspond to the addition of two streptavidin molecules. With the addition of the targeting antibody the diameter increased by 25 nm, which would correspond to the addition of 2 IgGs that are not oriented precisely on their long axis.



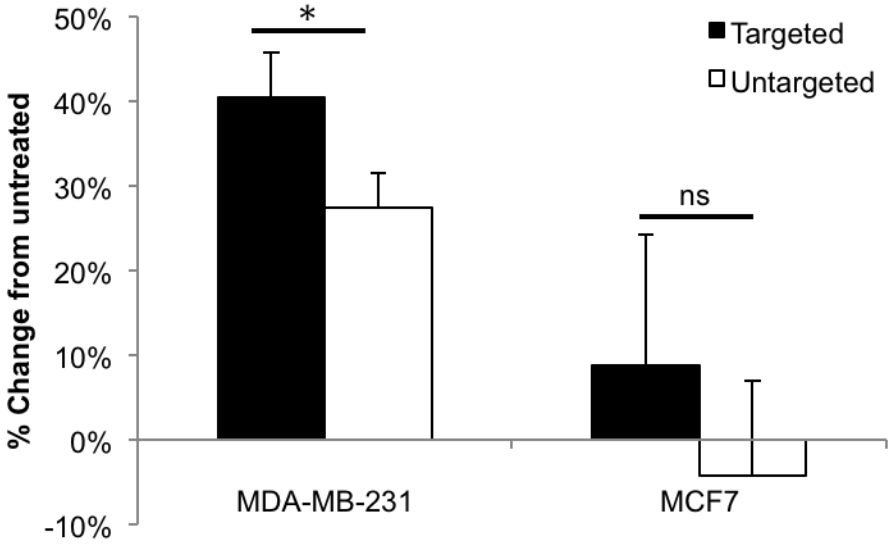
Supplemental Figure S2: Stability of NDLP-epirubicin in phosphate buffered saline (PBS). ND-Epi (left) and NDLP-Epi (right) suspended at 0.23 mg mL^{-1} (ND) in 0.9X PBS. After suspension in PBS, ND-Epi precipitated within 15 minutes (A), however NDLP-Epi remained stable in solution for more than 72 hours (B).



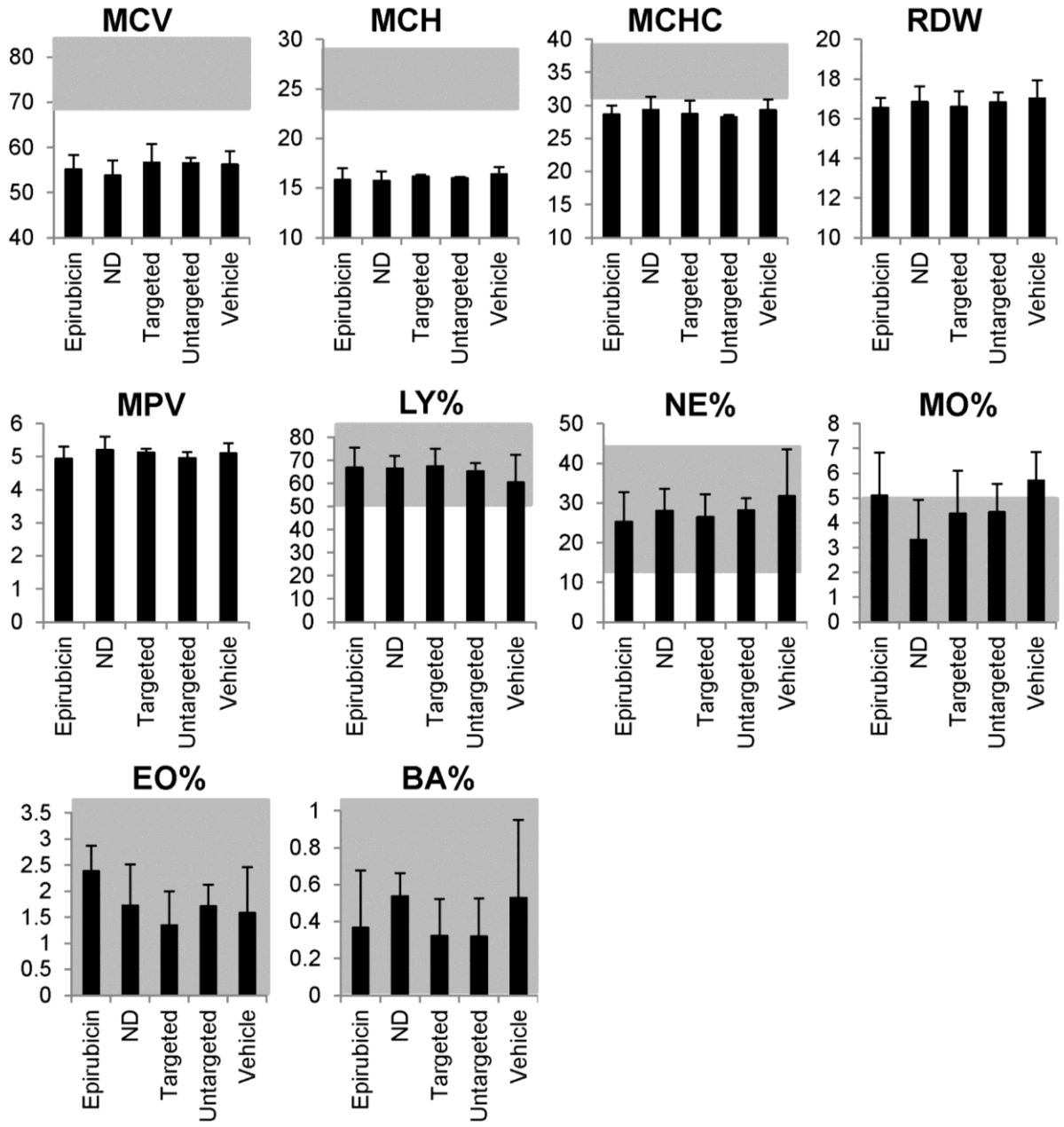
Supplemental Figure S3: CryoTEM of NDLPs shows two subpopulations of NDLPs in solution. (Left) NDs encapsulated within a lipid vesicle. (Right) Formation of ND-lipid clusters.



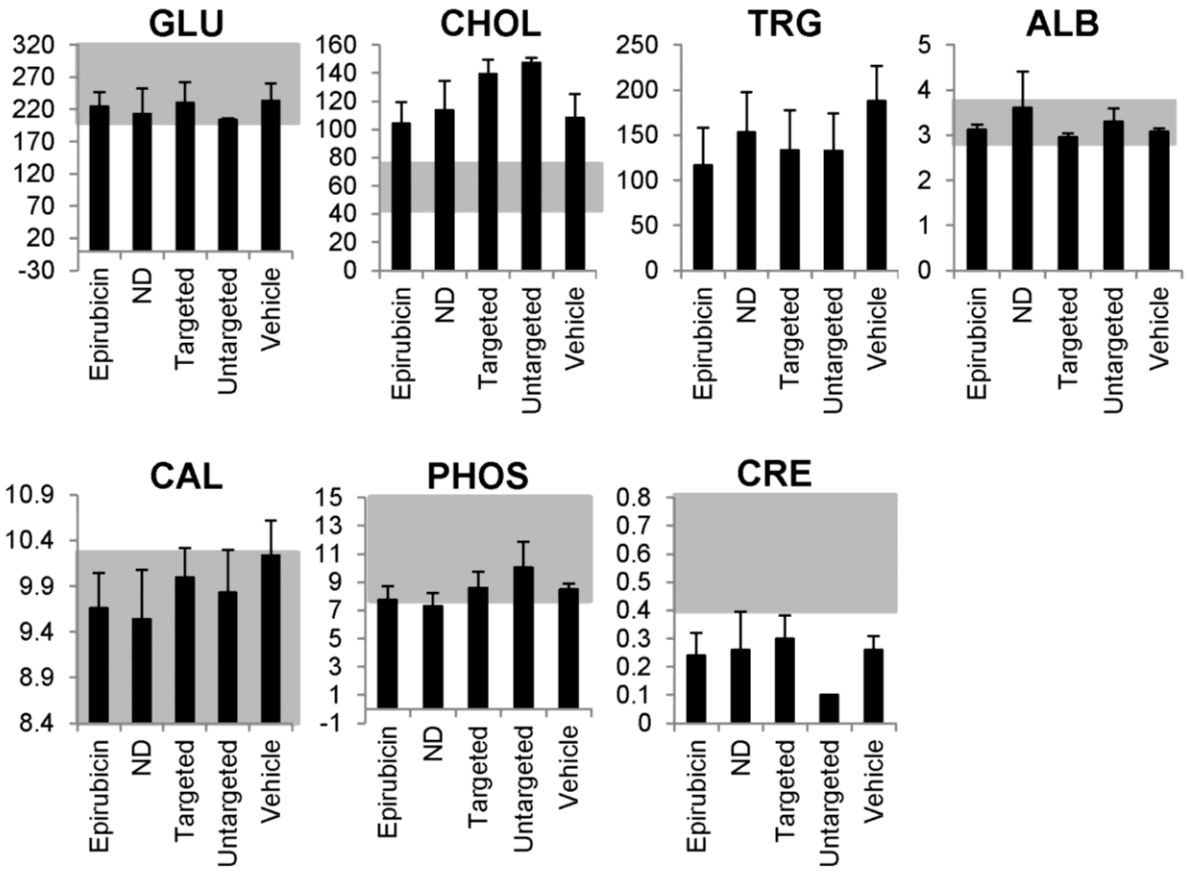
Supplemental Figure S4: ICP-AES of size exclusion chromatography fractions. Phosphorus was detected in fractions 1-10, which is consistent with DID fluorescence in figure 3c. Phosphorus was detected at 213.618nm with an In (230.606nm) internal standard.



Supplemental Figure S5: NDLPs made from ND-Xenolights™ CF750 target EGFR overexpressing MDA-MB-231 cells compared to untargeted NDLPs and MCF7 cells, which do not overexpress EGFR (*p=0.004).



Supplemental Figure S6: CD-1 mice were treated with 150μg epirubicin (n=5), 833μg of ND (n=5), equivalent of untargeted NDLP (n=3), targeted NDLP (n=4) or vehicle control (n=5) for 24 hours prior to hematological and serum chemistry analysis. No significant differences between treatment and vehicle control groups were observed. Gray region indicates expected normal range for 6-8 week-old, female, CD-1 mice. MCV- mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDW – red cell distribution width, MPV – mean platelet volume, LY% - lymphocyte percent, NE% - neutrophil percent, MO% - monocyte percent, EO% - eosinophil percent, BA% - basophil percent.



Supplemental Figure S7: CD-1 mice were treated with 150μg epirubicin (n=5), 833μg of ND (n=5), equivalent of untargeted NDLP (n=3), targeted NDLP (n=4) or vehicle control (n=5) for 24 hours prior to hematological and serum chemistry analysis. No significant differences between treatment and vehicle control groups were observed. Gray region indicates expected normal range for 6-8 week-old, female, CD-1 mice. GLU- glucose, CHOL – cholesterol, TRG – triglycerides, ALB – albumin, CAL – calcium, PHOS – phosphate, CRE – creatinine.

Experimental Methods

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, Missouri) unless otherwise noted. Nanodiamonds were acquired from the Nanocarbon Research Institute (Nagano, Japan). PEG-ylated lipids were acquired from Avanti Polar Lipids (Alabaster, Alabama).

ND Synthesis

Amine modified NDs were produced according to the protocols from Kruger *et a*l [3], Zhang *et al* [4] and Chow *et al* [5]. Briefly, after reduction of the ND surface (2.5g) with BH_3•THF (25mL, 1M) for 3 days, the ND surface (1g) was functionalized with (3-aminopropyl)trimethoxysilane (100mL, 5%). After purification by centrifugation, amine functionalized NDs (10mg) were then reacted with the succinimidyl esters of AlexaFluor 488, AlexaFluor 555 or Xenolights CF750 (1mg, AlexaFluor from Life Technologies and Xenlolights from Caliper Life Sciences) according to the protocol described by Chow *et al* [5]. Epirubicin was loaded onto unmodified ND clusters using NaOH (final concentration 2.5mM), as was previously described by Chow *et al* for doxorubicin [5]. Epirubicin loading was quantified by absorbance measurements of the supernatant at 485nm and compared to a standard curve.

Nanodiamond-Liposome Hybrid Particle (NDLP) Synthesis

ND-liposome hybrid particles were synthesized by hydration of lipid thin films with concentrated ND-conjugate solutions. Egg phosphatidylcholine (EPC), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG-biotin) in chloroform were combined in a round bottom flask (weight ratio 20:2:1 EPC: cholesterol: Biotin-PEG-DSPE). For imaging studies 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DID, 1% wt) was added to the lipid mixture. Chloroform was then removed by rotary evaporation and lyophilization, leaving a lipid thin film. The thin film was rehydrated with ND-complexes (5 mg mL^{-1} in water, 5:1 EPC:ND by weight). NDLPs were sized by probe sonication for 4 cycles of 30 seconds on, 30 seconds off in a cold-water bath and then purified by sterile filtration through hydrophilic polyethersulfone membranes (0.2 μm, Pall Corporation, Port Washington, NY). NDLPs were then targeted by

incubation with streptavidin-AlexaFluor 555 (Life Technologies, Grand Island, NY) and biotinylated anti-EGFR or non-specific antibody (R&D Systems, Minneapolis, MN) for a minimum of 30 minutes (molar ratio 4:1:1 of DSPE-PEG-Biotin : Streptavidin : Antibody). Untargeted complexes contained streptavidin without antibody.

NDLP Characterization

Size and zeta potential of NDLP solutions was determined by analysis of ND suspensions (0.2-0.3 mg mL^{-1}) using a Zetasizer Nano (Malvern Instruments, Worcestershire, UK). Data represents the average of at least 3 runs. Size is the z-average and standard deviation from those 3 runs while zeta-potential and zeta-deviation are plotted on the zeta potential graphs. NDLP-555 labeled with DID was imaged by confocal microscopy (Quantitative Bioelemental Imaging Center, Northwestern University) prior to sizing and after allowing particles to settle for 1 hour. Flow cytometry analysis was performed on suspensions NDLP-488 labeled with DID (2.3 μg ND mL^{-1}) before and after sterile filtration. NDLPs were compared to DID labeled empty liposomes and free ND-488. Cryo-TEM was performed on sized and filtered NDLP-488 suspensions. Solutions were loaded onto 300 mesh lacy carbon grids (5μL) and plunge frozen in liquid ethane using a Vitrobot (FEI Company, Hillsboro, Oregon, USA). Grids were then imaged using a JEOL 1230 transmission electron microscope equipped with a Gatan 831 CCD (100 kV). Size exclusion chromatography was performed on a DID-labeled NDLP-488 suspension after filtration using a Sepharose CL-4B column equilibrated with phosphate buffered saline. NDLP solution (1.0 mL, 2.3 mg ND mL^{-1}) was loaded onto the column and separated into approximately 1mL fractions. The column was run twice and fractions from the second run were analyzed for fluorescence using a plate reader. Lipid content of fractions was also verified by

ICP-AES for phosphorus (Integrated Molecular Structure Education and Research Center, Northwestern University).

Antibody Loading

Antibody attachment was verified using a modified indirect-ELISA protocol. Human EGFR (R&D Systems, Minneapolis, MN) was adsorbed to 96-well plates and then incubated with PBS, biotinylated antibody alone, targeted or untargeted NDLPs. Streptavidin-AlexaFluor 555 fluorescence indicated successful protein adsorption and antibody detection while AlexaFluor 488 fluorescence demonstrated NDLP presence after plate washing. Antibody loading was quantified by gel electrophoresis of the tangential flow filtration (MicroKros Hollow Fiber Modules, 500kDa pore) filtrate of targeted and untargeted NDLPs. The 4-12% bis-tris gel was run in MES buffer for 37 minutes and stained with SimplyBlue SafeStain (Life Technologies, Grand Island, NY). After imaging protein bands were photographed and quantified using ImageJ (NIH, Bethesda, MD).

In vitro Imaging and Therapy

For confocal microscopy MDA-MB-231 or MCF7 cells ($4.0x10^4$) were plated in chamber slides and incubated with targeted or untargeted NDLP-488 (58μg ND) for 24 hours. Cells were washed, stained with Hoechst 33258 (Life Technologies, Grand Island, NY) and imaged using Zeiss Axio Observer Z1 confocal microscope. For quantitation of NDLP uptake MDA-MB-231 and MCF7 cells were plated in black-wall, clear-bottom 96-well plates with the media without phenol red ($1x10^4$ cells per well). Both cell lines were incubated with varying volumes of targeted or untargeted NDLP-488 for 12 hours prior to washing and then incubated an additional 12 hours prior to imaging with a BioTek Synergy 4 plate reader. Data represents the mean and standard deviation from 4 wells. Cells were pretreated with EGF for 1hr prior to incubation with

23μg of targeted or untargeted NDLP-488 for 24 hours, followed by washing and analysis by plate reader. Data is the mean and standard deviation from 4 wells. Cell viability was assessed by formazan-based *in vitro* Toxicology Assay (TOX1 assay) using the manufacturer's protocol after incubation with NDLPs for 48hours.

Murine Mammary Carcinoma Model and Treatment

$1x10^6$ MDA-MB-231-luc-D3H2LN mammary carcinoma cells (Caliper Life Sciences) were injected into the fifth mammary fat pad of immunodeficient NOD/SCID female mice. For NIR localization experiments, tumor-bearing mice were treated with 100 μL PBS, NDLP-750 (1mg ml^{-1} ND) or anti-EGFR-NDLP-750 (1mg ml^{-1} ND) two weeks after tumor cell implantation. Following treatment, mice were imaged for fluorescent signal intensity by the IVIS Spectrum imaging system (Caliper Life Sciences) one hour post-injection and every 24 hours after that for one week. For long-term tumor treatment experiments, tumor-bearing mice were treated with 300 μl PBS, 150 μg epirubicin (Epi) or 150μg Epi equivalent of NDLP-Epi (1.4 mg ml^{-1} ND) or anti-EGFR-NDLP-Epi (1.4 mg ml^{-1} ND) every week over a seven week time-course. Following injection, luciferase signal intensity was determined by the IVIS Spectrum imaging system.

ND & NDLP Toxicity Study

22-24g female CD-1 mice (Charles River Laboratories, Wilmington, MA) were administered 150μg of epirubicin (n=5), 150μg equivalent of targeted (n=4) or untargeted NDLP solutions (n=3), 833μg plain NDs (equivalent to NDLP solution, n=5) or vehicle control (tris-buffered saline containing 0.1% BSA, n=5) by tail vein injection. Mice were sacrificed and blood extracted after 24 hours. Whole blood and serum samples were sent to Charles River Laboratories for complete hematological and serum chemistry analysis (Wilmington, MA).

Statistical Methods

All statistics were performed in Microsoft Excel. All testing was two-tailed, assumed equal variances and used a significance level of 0.05.

References

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