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Supplementary Materials for

Nanodiamond Therapeutic Delivery Agents Mediate Enhanced Chemoresistant Tumor Treatment

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Materials and Methods

Materials

The acid-oxidated ND gel (15% w/v in water) was obtained from the NanoCarbon Research Institute Ltd. Lipopolysaccharides (LPS), D-Galactosamine (D-GalN), Doxorubicin-HCl (Dox), Borane-tetrahydrofuran complex (BH3·THF, 1M), (3-aminopropyl) trimethoxysilane and tetrahydrofuran (THF) were purchased from Sigma-Aldrich. XenoFluor^{tm} 750 succinimidyl ester was obtained from Caliper LifeSciences. The succinimidyl ester of Alexa Fluor® 488 carboxylic acid was purchased from Invitrogen. All commercial reagents were used without further purification.

Elemental analyses were performed by Columbia Analytical Services, Inc. Fourier Transform Infrared Spectroscopy (FTIR) experiments were performed using a Nexus 870 spectrometer (Thermo Nicolet, Keck-II, Northwestern University) based on OMNIC^{TM} software.

Preparation of tumor cell lines

LT2M cells were derived from a primary MYC-driven liver tumor. Briefly, a tumor-bearing LT2-Myc mouse was sacrificed and tumor tissue was dissected. Tumor cells were disaggregated with a scalpel and treated with collagenase/dispase (1 mg/ml in PBS) to generate a single-cell suspension. Primary tumors cells were grown in DMEM + 10% FCS with non-essential amino-acids on collagen-coated plates. Cells were subsequently passaged in the similar media but did not require collagen treated plates. The 4T1, Huh7 and MDA-MB-231 cell lines were obtained from the UCSF Cell Culture Facility. LT2M cells and 4T1 cells were grown in RPMI 1640 supplemented with 10% FBS and penicillin (100 IU/ml)/streptomycin (100 μg/ml).

Particle size and zeta potential measurements

The NDX complexes were freshly prepared by mixing a 10 mg/ml ND solution with a 2 mg/ml DOX solution at 1:1 volume ratio in the presence of NaOH (the final NaOH concentration in solution is 2.5 mM). The mixture was vortexed for 20 seconds and incubated for 30 minutes at room temperature before measurement. Particle size and zeta potential measurements were performed using the Zetasizer Nano ZS (Malvern). Nanoparticle size measurements were performed at 25°C and a 173° scattering angle. The mean hydrodynamic diameter was determined via cumulative analysis. Determination of the zeta potential was based on electrophoretic mobility of the nanoparticles in the aqueous medium, which was performed using folded capillary cells in automatic mode.

Determination of myelosuppression

Myelosuppression was determined by the reduction of white blood cell counts below normal levels (4K/μl). Wildtype FVB/N mice were tail-vein injected with PBS, Dox (400μg), or NDX (400μg Dox equivalent) in 200 μl. Serum was collected just prior to treatment and 24 hours after treatment and analyzed by HEMAVET 950S (Drew Scientific).

ELISA

For the serum ALT and serum IL-6 studies, age and sex-matched wildtype FVB/N mice were intravenously injected with 500µg of unmodified, suspended NDs (n=3) or PBS (n=3). One week after injection, serum was isolated and analyzed by ELISA for serum Il-6 (BD Biosciences) and serum ALT (TECO Diagnostics) according to manufacturer protocols. Positive controls for ELISA were obtained by intraperitoneal injection of age and sex-matched wildtype FVB/N mice with LPS (2.5 µg/kg)/d-GalN(200 mg/kg) (n=2). 6 hours post-injection, serum was obtained and analyzed.

Biodistribution analysis

NDs covalently labeled with the near-infrared XenoFluor^{tm} 750 dye (Caliper Lifesciences) or unlabeled control NDs were injected into age and sex-matched wildtype FVB/N mice at various amounts and whole-body and organs were analyzed at 1 hour, 1 day, 2 days, 3 days, 4 days, 7 days and 10 days post-injection by Xenogen (Caliper Lifesciences) imaging camera (ICG filter, 6 sec., f/s 4, high sensitivity). Organ distribution studies were conducted using NDs covalently labeled with the AlexaFluor® 488 dye (Invitrogen) which were injected into age and sex-matched wildtype FVB/N mice at various amounts and analyzed at 1 day, 4 days, 7 days and 10 days post-injection by fluorescent microscopy (Zeiss Axiophot).

Doxorubicin extraction analysis

Doxorubicin was extracted as previously described (*33*). Briefly, samples were treated with 90% isopropanol (0.075M HCl) overnight at -20^{o}C. Samples were warmed to room temperature and vortexed for 5 minutes. Samples were then centrifuged at 15,000x*g* for 20 minutes and stored at -80^{o}C until analysis. Samples were measure by spectrophotometer: Excitation 470nm, Emission 590nm. Known concentrations of Doxorubicin were added to PBS treated samples for standard curves. Blood circulation half-life ($t_{1/2}$) was determined by $t_{1/2}=0.693/k_{elim}$ where $k_{elim}=(\ln(Conc_{peak}) - \ln(Conc_{trough}))/t_{interval.}$

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay

LT2M or 4T1 tumor cells were seeded overnight in 96 well plates at $1x10^5$ cells per well. Following treatment with drugs in triplicate, cell growth and viability was analyzed by TACS^{tm} MTT assay according to manufacturer protocol (R&D Systems).

Efflux assay

Cell lines are treated with NDX or Dox. Following 1 hour incubation, cells were washed once with 1x PBS and incubated in regular media. Following 4 hour incubation, cells were washed again and analyzed by plate reader for Dox retention (Tecan Safire2). Retention was confirmed by inverted fluorescent microscopy (Zeiss Axiovert).

Tumor volume and Kaplan-Meier analysis

Relative tumor volume was measured up to 21 days by V/V_0 where V is current volume and V_0 is initial volume. Volume was measured using the equation $V = (axb^2)/2$, where *a* is the length

(mm) and *b* is the width (mm) of the tumor and V is the volume (mm^3). For both tumor models, Kaplan-Meier survival analysis was performed by MedCalc statistical software.

TUNEL analysis

Following treatment, normal tissue and tumor tissue were isolated and fixed for 24 hours with 4% paraformaldehyde and sections were processed by the UCSF Craniofacial Histology Core Facility. In situ fluorescent TUNEL staining was done according to manufacturer protocol (Chemicon). Results were analyzed by fluorescent microscopy (Zeiss Axiophot) and quantified with ImageJ (NIH).

Statistical Analysis

Data were analyzed for significance by student's t-test. Results were considered statistically significant with a P-value <0.05.

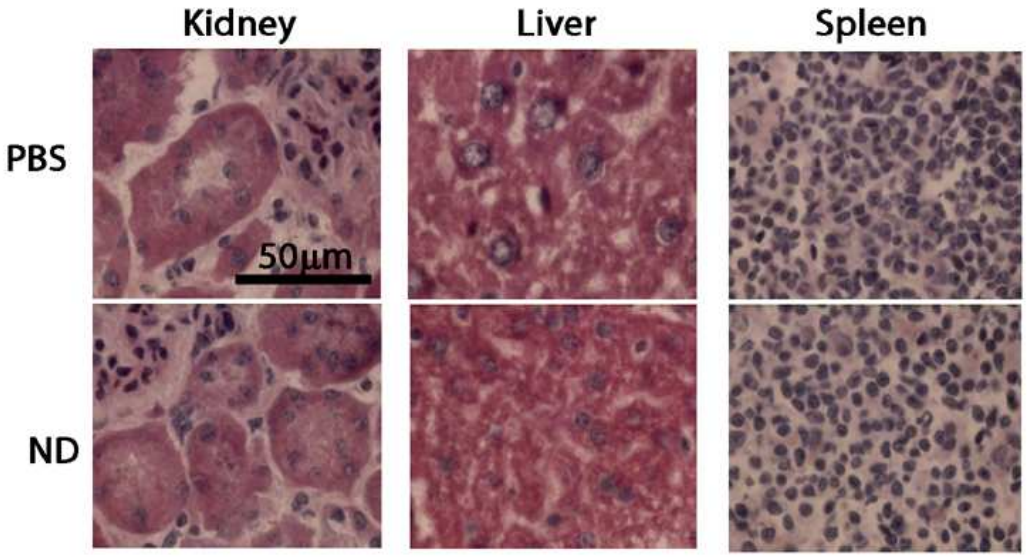

Fig. S1. 100× H&E histopathological analysis. Images are taken of kidney, liver and spleen tissue from treated mice. Age and sex-matched wildtype FVB/N mice tail-vein injected with 500 μg of unmodified nanodiamonds or PBS for one week**,** scale bar: 50 μm

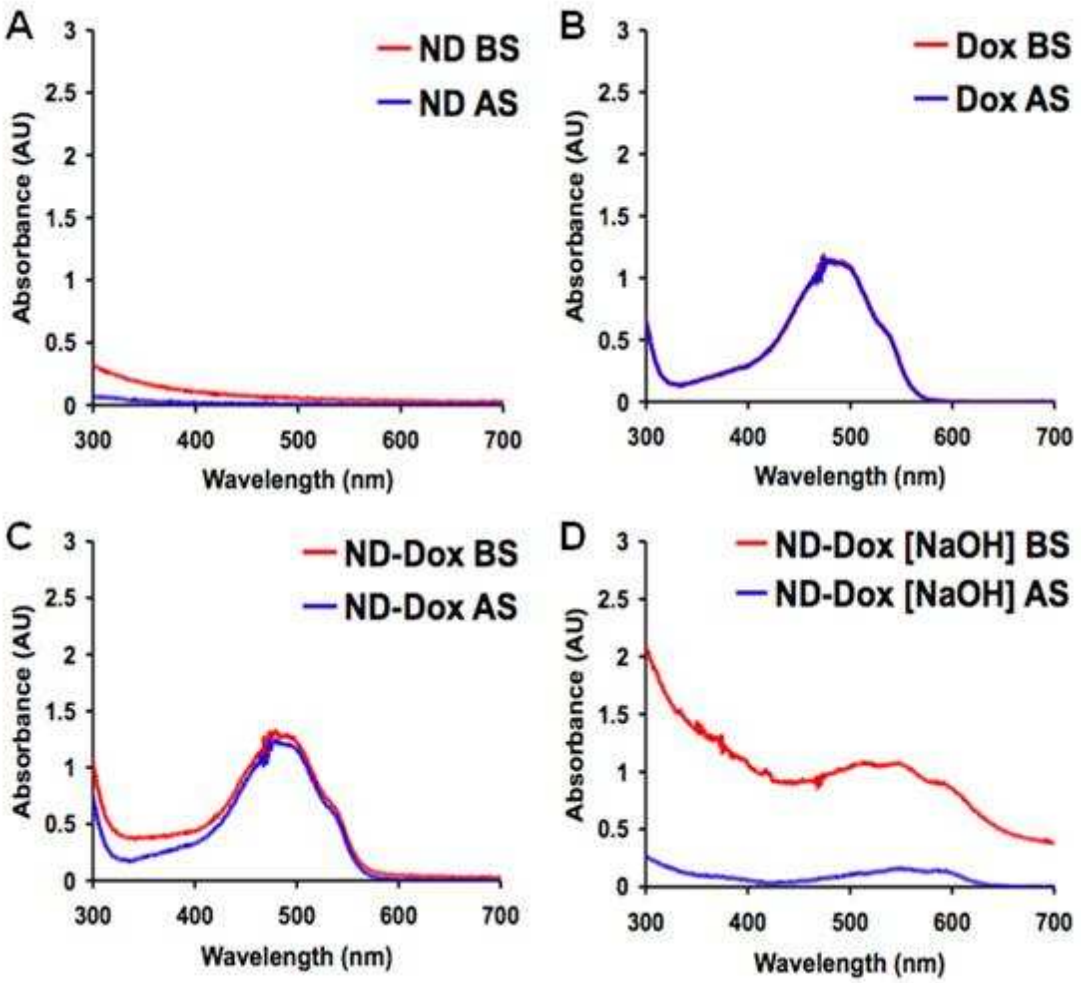

Fig. S2. NDX adsorption spectrophotometry analysis. Absorbance curves were measured before (BS) and after (AS) two hour spins to pellet any NDs or ND-Dox complexes present in each solution. Dox absorption to NDs was assessed by examining ND-mediated Dox-sequestering via centrifugation. Solutions of NDs, Dox, ND-Dox, and ND-Dox [NaOH] were prepared to a concentration of 250 μg/ml and 50 μg/ml for NDs and Dox, respectively, for all solutions. The initial absorbance of each solution was read from 300 - 700 nm and revealed a characteristic Dox peak near 490 nm in solutions containing Dox. The shift in this peak in the ND-Dox [NaOH] solution spectra is due to the Dox complexing with ND clusters.

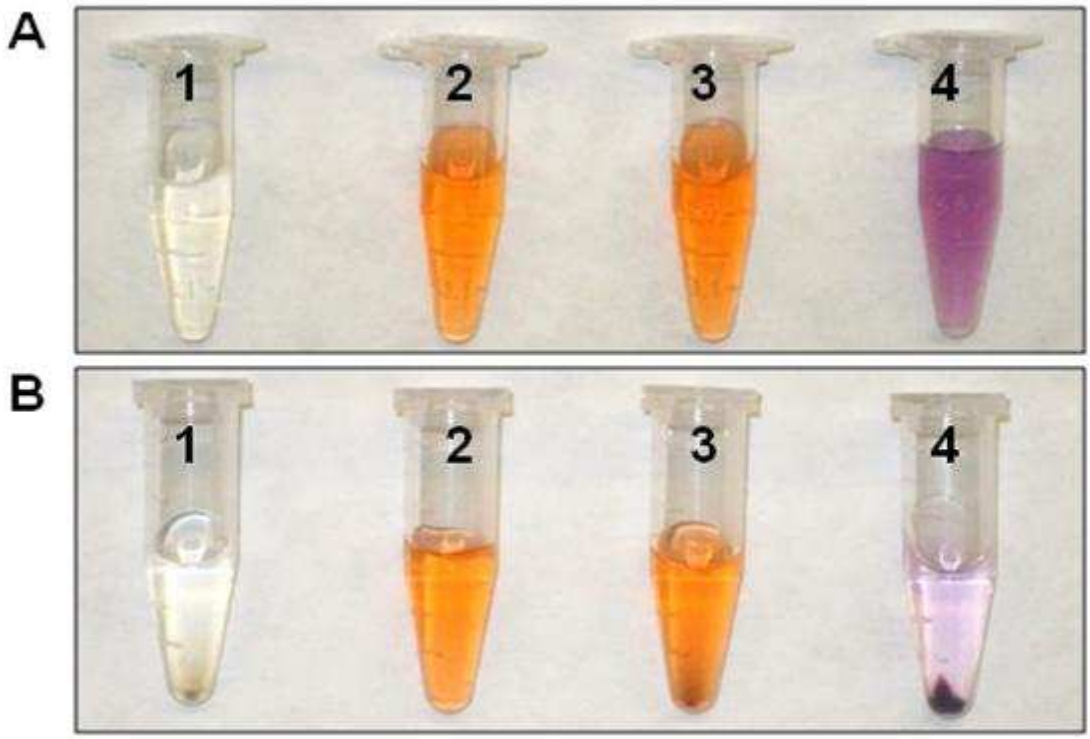

Fig. S3. NDX adsorption comparison. ND (1), Dox (2), and ND-Dox (3) and ND-Dox + NaOH (4) solutions before (A) and after (B) two hour centrifugation at 14000 rpm. To confirm ND-Dox coupling, the samples were centrifuged for two hours at 14000 rpm to pellet any ND clusters in solution and the absorbance of the supernatant from each solution was taken. Successful adsorption and the necessity for NaOH in adsorption is visually evident in the contrast in the color of the solutions before and after centrifugation.

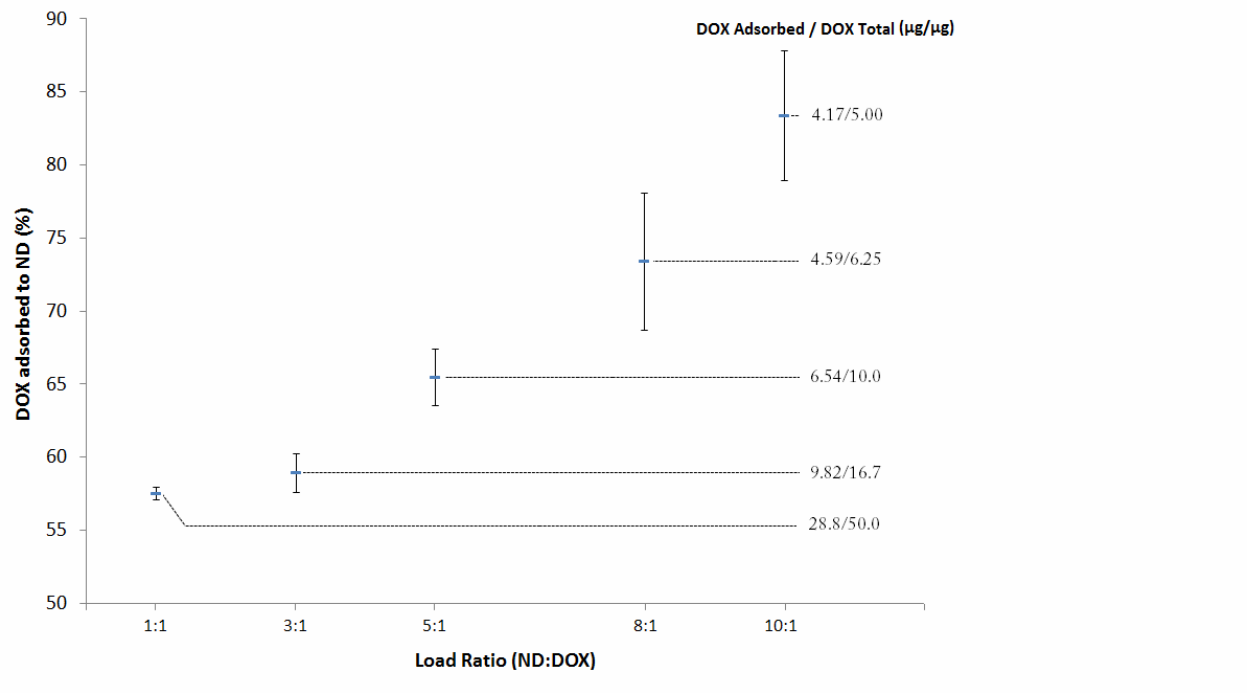

Fig. S4. Dox loading analysis. 50 µg NDs were complexed with varied amounts of DOX in 500 µl of a 2.5 mM NaOH solvent in water, revealing Dox loading efficiency on NDs that varied based upon the initial amount of Dox added. Data represented as mean ± SD (n=3).

Cell Line	IC_{50} (ng/ml)
LT2M cancer cell line	102.7±10.8
4T1 cancer cell line	240.6±1.39

Fig. S5. Cancer cell line Dox resistance. IC50, 50% inhibitory concentration was determined by MTT assay following treatment of cell lines for 48 hours. Data represented as mean ± SD (n=3).

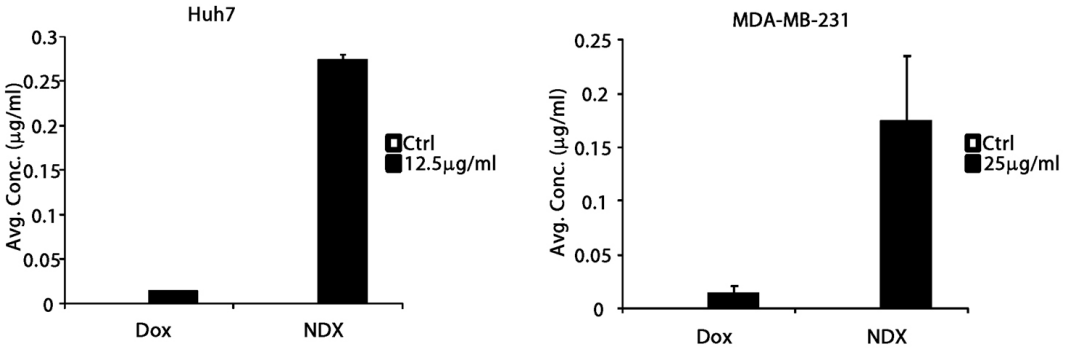

Fig. S6. Dox efflux analysis in human tumor cells. Huh7 and MDA-MB-231 cells were incubated with doxorubicin (Dox) (12.5 or 25 μg/ml) or nanodiamond-conjugated doxorubicin (NDX) (12.5 or 25 μg/ml Dox equivalent). Data represented as mean ± SD.

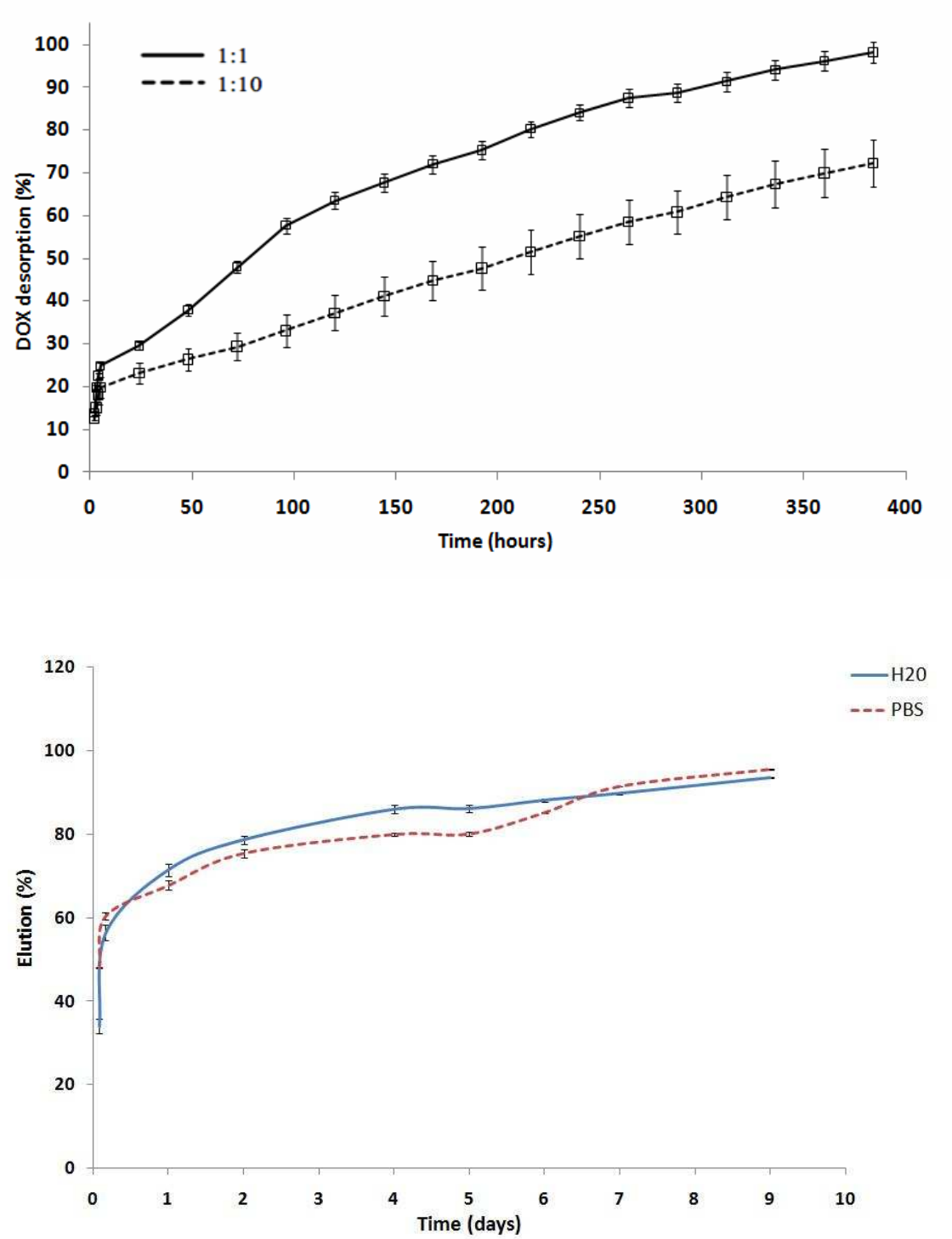

Fig. S7. Dox desorption from ND agglomerates. Dox adsorbs to the ND complexes and can be released in a sustained fashion which is demonstrated in media (top) and water/PBS (bottom). Data represented as mean ± SD (n=3).

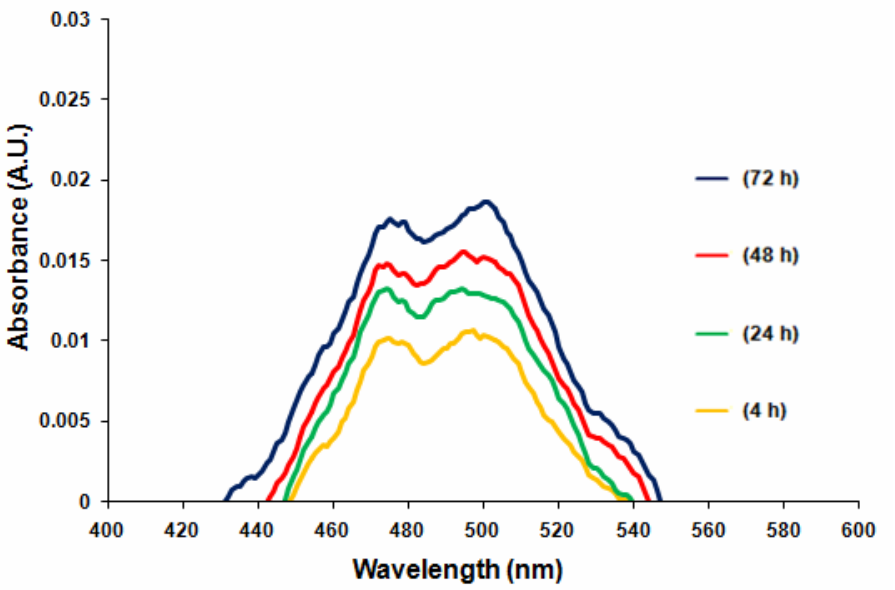

Fig. S8. Dox release spectrophotometry analysis. At various timepoints, the continued release of Dox can be observed from the ND agglomerates in PBS, signifying maintained temporal drug release. Dox desorption from NDs in PBS was measured via UV spectroscopy used to identify a characteristic Dox absorbance peak near 482 nm in the eluate from samples that ranged up to 3 days. The desorption pattern is gradual compared to a direct intravenous injection of free Dox, which results in immediate and complete dispersal of drug known to cause side effects. Unlike free Dox, ND-Dox gradually releases Dox over several days. Moreover, the experiment demonstrates Dox desorption in a solution isotonic to preclinically-relevant environments. Data was plotted as a dynamic average.

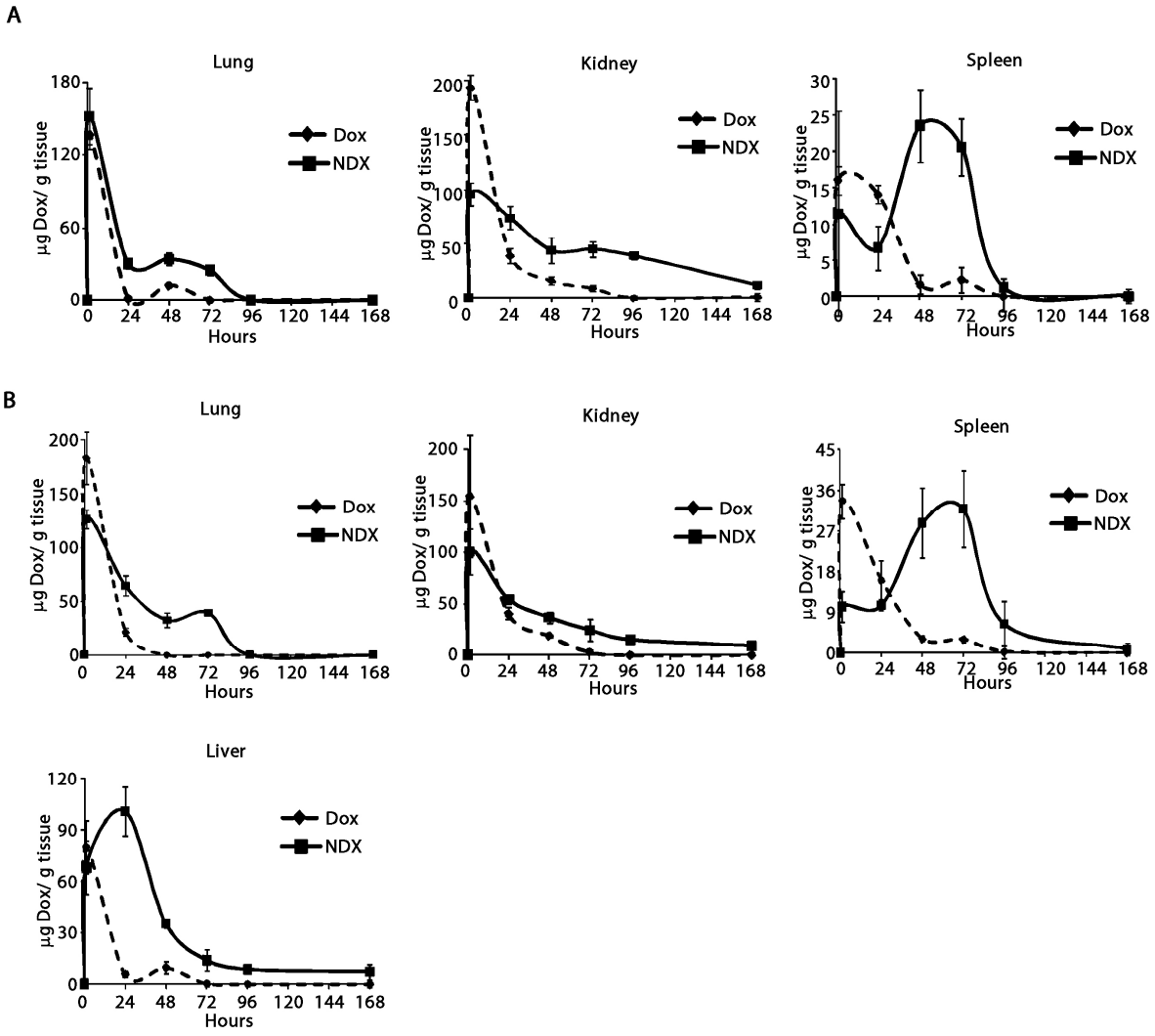

Fig. S9. NDX and Dox tissue retention. (A) 200 µg Dox equivalent of free Dox and NDX were injected into LT2-Myc tumor-bearing mice. Dox was extracted from tissues isolated at 30 min, 24, 48, 72, 96 and 168 hours post-injection. (B) 200 µg Dox equivalent of free Dox and NDX were injected into 4T1 tumor-bearing mice. Dox was extracted from tissues isolated at 30 min, 24, 48, 72, 96 and 168 hours post-injection. Data represented as mean ± SD (n=4).

	Avg. Particle Size (nm)	Zeta Potential (mV)
ND	45.1 ± 0.2	17.7 ± 3.2
ND/Dox 0.1X	80.0 ± 0.7	52.4 ± 2.2
ND/Dox 1X	78.8 ± 0.7	46.0 ± 0.8
XenoFluor™ 750-ND	153.1 ± 1.6	-38.8 ± 0.4
Alexafluor 488®-ND	111.2 ± 1.7	27.1 ± 2.0

Table S1. Size and ζ potential of functionalized NDs. The particles were suspended in deionized water at the following concentrations: ND (50µg/ml, pH 9); ND/DOX 0.1X (0.5mg/ml ND, 0.1mg/ml DOX); ND/DOX 1X (5mg/ml ND, 1mg/ml DOX); ND-XenoFluor^{tm} 750 (100 µg/ml); Alexa Fluor® 488-ND (100 µg/ml). Data are represented as the mean ± SD (n=3).

ND:DOX	DOX Total (µg)	DOX Adsorbed (µg)	DOX Adsorbed per DOX Total (%)	DOX loaded on ND (µg/µg)	DOX loaded on ND (%)
1:1	50.0	28.8 ± 0.2	57.5 ± 0.4	0.575 ± 0.004	57.5 ± 0.4
3:1	16.7	9.82 ± 0.2	58.9 ± 1.3	0.196 ± 0.004	19.6 ± 0.4
5:1	10.0	6.54 ± 0.2	65.4 ± 2.0	0.131 ± 0.004	13.1 ± 0.4
8:1	6.25	4.59 ± 0.3	73.4 ± 4.7	0.092 ± 0.006	9.17 ± 0.6
10:1	5.00	4.17 ± 0.2	83.4 ± 4.4	0.083 ± 0.004	8.34 ± 0.4

Table S2. Dox loading efficiency. 50 µg NDs were complexed with varied amounts of DOX that ranged from 5 to 50µg in 500 µl of a 2.5 mM NaOH solvent in water. Dox that was loaded compared to the added Dox, as well as Dox that was loaded compared to the added NDs were assessed. Data are represented as mean ± SD (n=3).