Electronic Supplementary Material

Vascular-confined multi-passage discoidal nanoconstructs for the low-dose docetaxel inhibition of triple-negative breast cancer growth

Alessia Felici[1,2], Daniele Di Mascolo[1], Miguel Ferreira[1], Simone Lauciello[3], Luca Bono[4], Andrea Armirotti[4], Arunkumar Pitchaimani[1], Anna Lisa Palange[1,§], and Paolo Decuzzi[1,§] (✉)

[1] *Laboratory of Nanotechnology for Precision Medicine, Fondazione Istituto Italiano di Tecnologia, Genoa 16163, Italy*
[2] *Department of Informatics, Bioengineering, Robotics and System Engineering, University of Genoa, Genoa 16126, Italy*
[3] *Nanochemistry Laboratory, Fondazione Istituto Italiano di Tecnologia, Genoa 16163, Italy*
[4] *Analytical Chemistry Laboratory, Fondazione Istituto Italiano di Tecnologia, Genoa 16163, Italy*
[§] *Anna Lisa Palange and Paolo Decuzzi contributed equally to this work.*

Supporting information to https://doi.org/10.1007/s12274-021-3507-8

Supplementary Methods

Synthesis of DSPE-Cy5. DSPE-Cy5 were synthesized as reported by Lee and coworkers with some modifications [1]. 15 mg of DSPE-NH_2 were dissolved in 3 mL of dichloromethane (DCM) and 1.5 mL of MeOH. 0.98 eq of Cyanine-5 NHS ester was dissolved in 200 ml of dimethylformamide (DMF) and added to the previous solution. A catalytic amount of triethylamine (TEA) was added to the reaction and left to stir for 16 h. The resulting product was precipitated with cold diethyl ether, then washed three times with cold diethyl ether obtaining the final compound with a yield of 90%.

Synthesis of Gd(DOTA)-DSPE. 15 mg of DSPE-NH2 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine) were dissolved in 3 mL of DCM and 1.5 mL of MeOH. 0.98 eq of DOTA-NHS ester were dissolved in 200 ml of H_2O and added to the previous solution. A catalytic amount of TEA was added to the solution and left to stir for 16 hours. The intended product was precipitated with cold diethyl ether. The product was washed for three times with cold diethyl ether getting a final yield of 90%. MS (ESI): m/z – calculated for $C_{57}H_{108}N_5O_{15}P$, 1133,73; found for 1133,75. The solution of the Gd(DOTA)-DSPE complex was prepared through the mixing of the DOTA-DSPE prepared before with 95% equivalents of $GdCl_3$. The solution was adjusted to pH ~5.8 with aqueous NaOH and was allowed to react for 24 h at room temperature. The absence of free metal was checked in each sample by testing with xylenol orange.

Supplementary Results

Morphology of 'single-passage' vs 'multiple-passage' Discoidal Polymeric Nanoconstructs (DPN). As compared to the 'single-passage' method, the electron and optical density of the 'multi-passage' DPN were higher suggesting a larger mass of polymers and fluorescent imaging probes trapped within the matrix, as can be inferred by comparing **Figure S1A-B**.

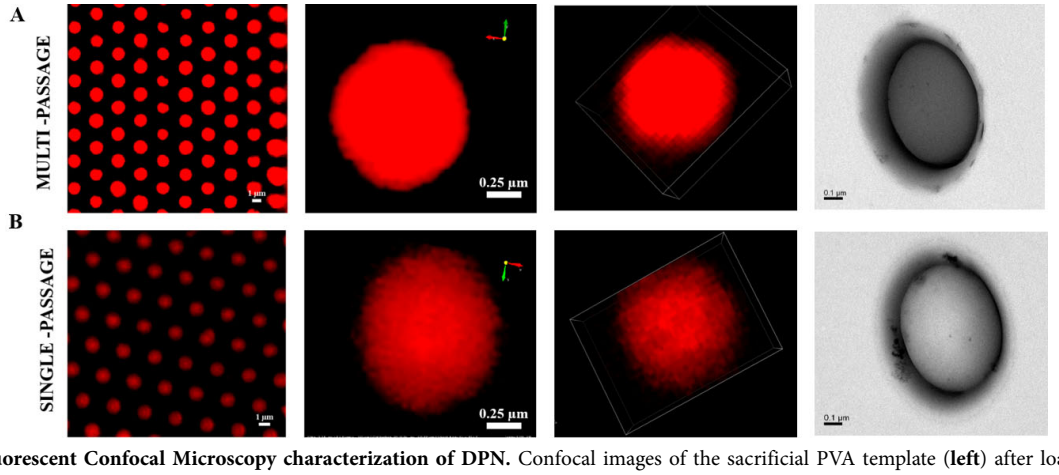

Figure S1 Fluorescent Confocal Microscopy characterization of DPN. Confocal images of the sacrificial PVA template (**left**) after loading with the polymeric paste containing lipid-Rhodamine B. Confocal microscopy images of individual DPN (**center**). Electron Microscopy images of DPN (**right**). Top and bottom rows are for 'multi-passage' **(A)** and the 'single-passage' **(B)** DPN, respectively.

Address correspondence to paolo.decuzzi@iit.it

In vitro Cytotoxicity.

To evaluate any possible toxic effect associated with the nanoconstructs, MTT test were performed up to 72h upon incubation of MDA-MB231 cells with increasing concentrations of empty DPN. Briefly, different number of cells were seeded in 96-well plates for each time point – 10^4 cells/well for 24h, 7.5×10^3 for 48h, 5×10^3 for 72h – and incubated at 37°C, 5% CO_2. Then, cells were treated with EMEM containing increasing number of particles (0 – 800 DPN/cell) replicating the same conditions used for the DTXL-DPN toxicity assays. After 24, 48 and 72h, the treatment solution was removed and replaced with MTT solutions, according to the manufacturer's instructions. The resulting formazan crystals were then dissolved in ethanol (200 µL/well) and the absorbance read at 570 nm using a microplate reader (Tecan, CH). Six replicates were considered for each DPN concentration. Data were collected when the absorbance ranged between 0.8 and 1.2. Cell viability was normalized to that of untreated cells. As reported in **Figure S2**, empty DPN cause negligible toxicity even at the highest tested concentration (800 DPN/ cell) up to 72h.

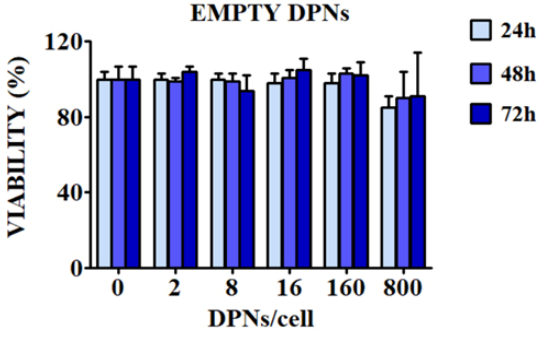

Figure S2 Cell viability on MDA-MB 231 cells upon incubation with empty DPN.

DTXL-DPN in vivo therapeutic efficacy.

Tumor growth was followed by caliper measurements, assessing the volume in cm^3, and optical imaging, assessing the radiance efficiency, as documented in the left and right columns of **Figure S3**, respectively**.** Both measurements present a continuous tumor growth for the untreated mice, a delayed growth for free-DTXL treated mice and an arrest in growth for the mice treated with DTXL-DPN.

Representative bioluminescent images of untreated, free-DTXL treated and DTXL-DPN treated mice are reported in **Figure S4A**, at the end of the treatment. **Figure S4B** quantities the difference in bioluminescence intensity for the explanted tumors. Again, the bioluminescence data confirm the therapeutic benefits associated with DTXL-DPN treatment over saline control and free DTXL therapeutic cases.

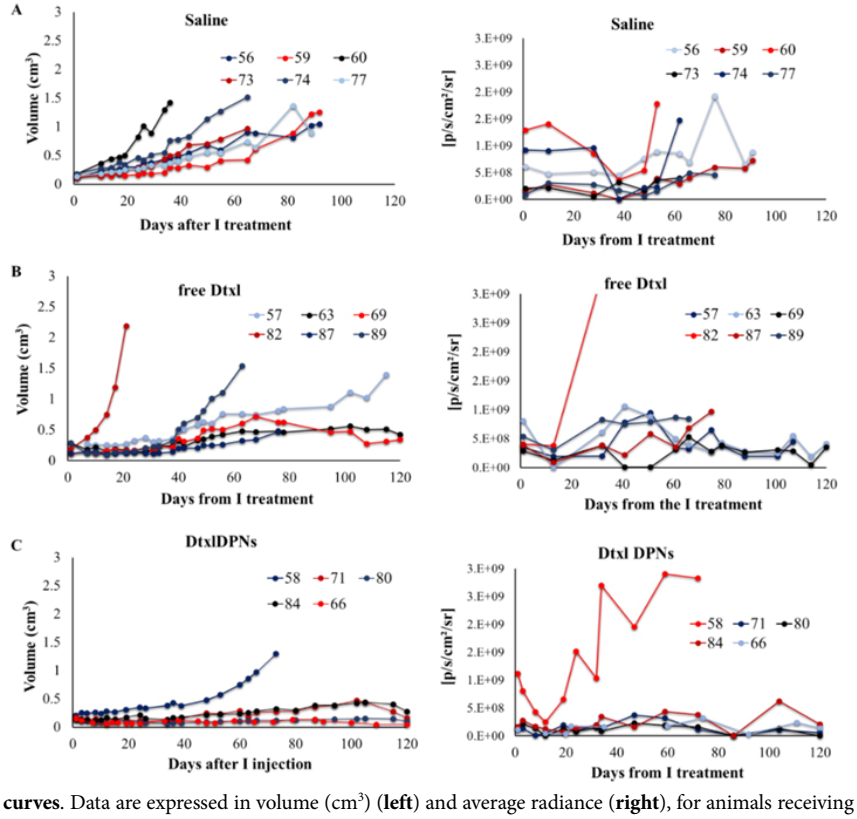

Figure S3 **Tumor growth curves**. Data are expressed in volume (cm^3) (**left**) and average radiance (**right**), for animals receiving a **A.** saline; **B.** free-DTXL; and **C.** DTXL-DPN injection.

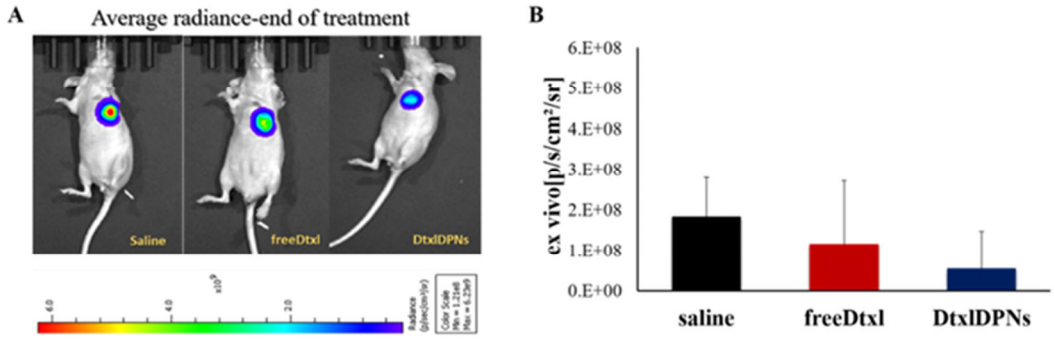

Figure S4 **Tumor size at the end of the treatment. A.** Representative images of average radiance at the end of the treatments for the three different experimental groups. **B.** Average radiance of the explanted tumors. (Data are plotted as mean radiance ± SD).

Cy5-DPN in vivo biodistribution.

The Cy5-DPN biodistribution was assessed upon intravenous injection using a whole animal optical imaging system (IVIS). Mice bearing orthotopic breast cancers were systemically administered with fluorescent DPN and monitored longitudinally by acquiring images at 30 min, 1, 3, 6 and 24h post injection. IVIS images at all time points for mice in supine positions are collected in **Figure S5**. Within the first few hours, fluorescence is mostly detected in the abdominal cavity where the liver processes most of the blood flow. The radiance associated with this portion of the animal body was observed to continuously reduce in favor of a stable tumor accumulation. The ex-vivo images of explanted organs, assembled in **Figure S6**, confirmed a significant particle accumulation in the tumors.

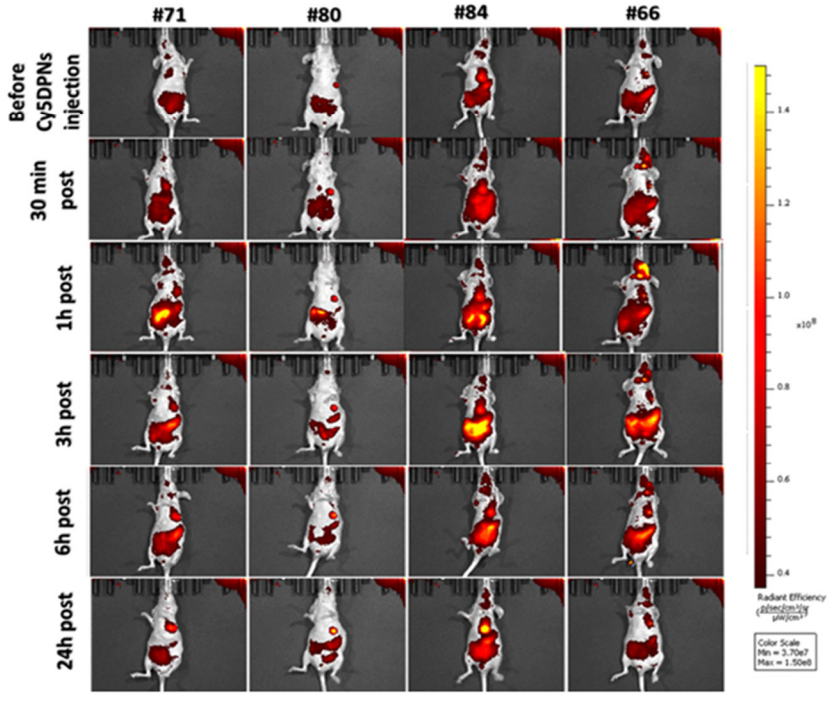

Figure S5 **Biodistribution studies with Cy5-DPN**. Fluorescent imaging of animals in supine position at different time points post Cy5-DPN systemic injection.

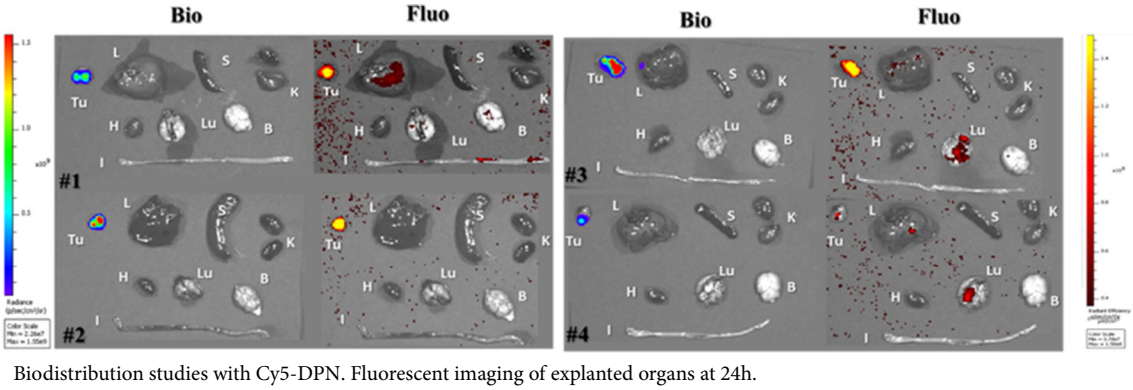

Figure S6 Biodistribution studies with Cy5-DPN. Fluorescent imaging of explanted organs at 24h.

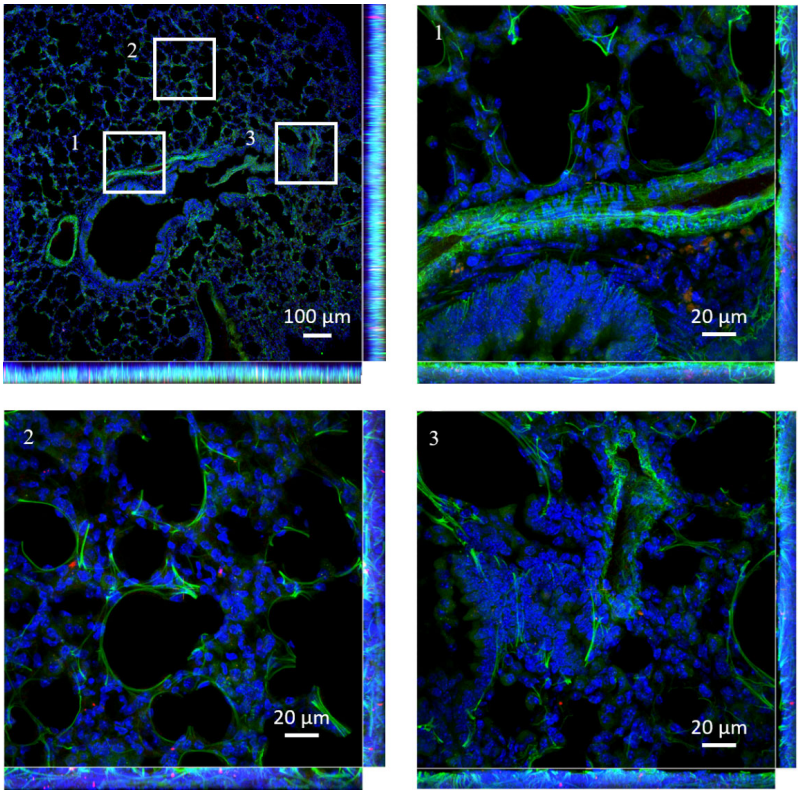

Figure S7 Immunohistochemistry and DPN localization in the pulmonary vasculature. DPN loaded with DSPE-Cy5 (red) are confined in the lungs vasculature stained with CD31 antibody (green). Cell nuclei are stained with DAPI (blue).

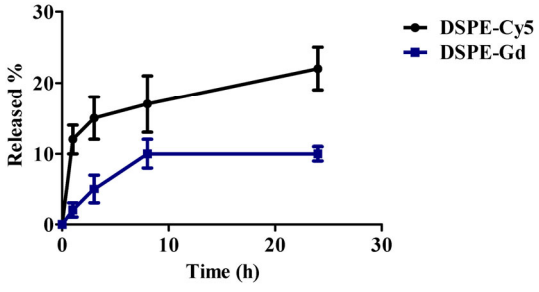

Figure S8 Cy5-DSPE and DSPE-Gd release from DPN. Gd(DOTA) appears to be more stably associated with DPN (blue curve) rather than DSPE-Cy5 DPN (black curve) within 24h. Particles were incubated under continous steering in a 4l aqueous solution.

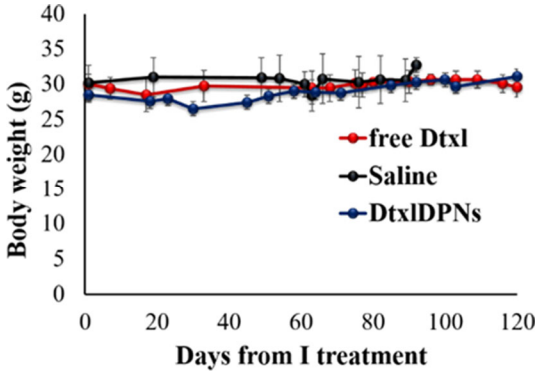

Figure S9 Body weight curves of the three different experimental groups. (Data are presented as the mean weights ± SD).

Gd and DTXL organ specific accumulation.

The accumulation of the specific particles in different tissues has been investigated using DPN containing Gd(DOTA)-DSPE as contrast agent directly mixed with the polymeric mixture of PLGA and PEG-DA, and quantified using ICP-MS. As documented by the stability studies **(Figure S9)**, DOTA-Gd was stably associated with DPN, hence its biodistribution would represent the biodsitribution of DPN. The DTXL accumulation in various tissues was assessed via LC-MS. The tables below list the absolute and normalized by the organ mass percentage of Gd and DTXL accumulation. Notice that the total amount of Gd recovered is about 75%, whereas the toal amount of DTXL recovered was about 80%.

Table S1 Quantification of the Gd and DTXL tissue accumulation expressed as absolute percentage of the injected dose normalized by the organ mass (ID%/g) at 24h post systemic injection, as determined via ICP-MS and LC-MS analyses, respectively.

Organs	%ID/g DTXL-DPN	SD	%ID/g Gd-DPN	SD	%ID/g DTXL	SD
Lungs	55.5	3.02	46.44	1.50	5.18	0.14
Liver	50.5	3.61	36.00	9.25	1.20	0.34
Spleen	30.4	1.00	35.90	1.01	1.37	0.11
Kidney	2.0	0.17	2.63	0.26	0.40	0.03
Tumor	1.4	0.03	0.57	0.02	0.48	0.03

Table S2 Quantification of the Gd and DTXL tissue accumulation expressed as absolute percentage of the injected dose (ID%) at 24h post systemic injection, as determined via ICP-MS and LC-MS analyses, respectively.

Organs	%ID DTXL-DPN	Sd	%ID Gd-DPN	Sd	%ID DTXL	Sd
Lungs	16.77	3.02	10.43	1.50	0.80	0.14
Liver	59.00	3.61	57.50	9.25	1.96	0.34
Spleen	3.50	1.00	4.20	1.01	0.28	0.11
Kidney	0.90	0.17	1.13	0.26	0.18	0.03
Tumor	0.08	0.03	0.08	0.02	0.03	0.03
TOTAL	81.3		73.35		3.26	

References

[1] Lee A, De Mei C, Fereira M, Marotta R, Yoon HY, Kim K, et al. Dexamethasone-loaded polymeric nanoconstructs for monitoring and treating inflammatory bowel disease. Theranostics. 2017; 7: 3653.