Supplementary Data

for

Heparosan-Coated Liposomes for Drug Delivery

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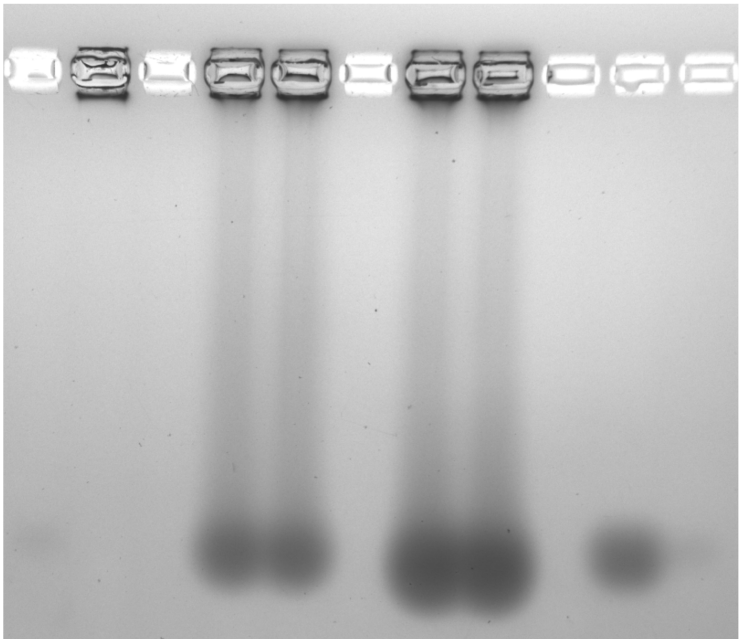
Supplemental Figure 1: Centrifugation-based Testing of HEP-Palm Association with Liposomes

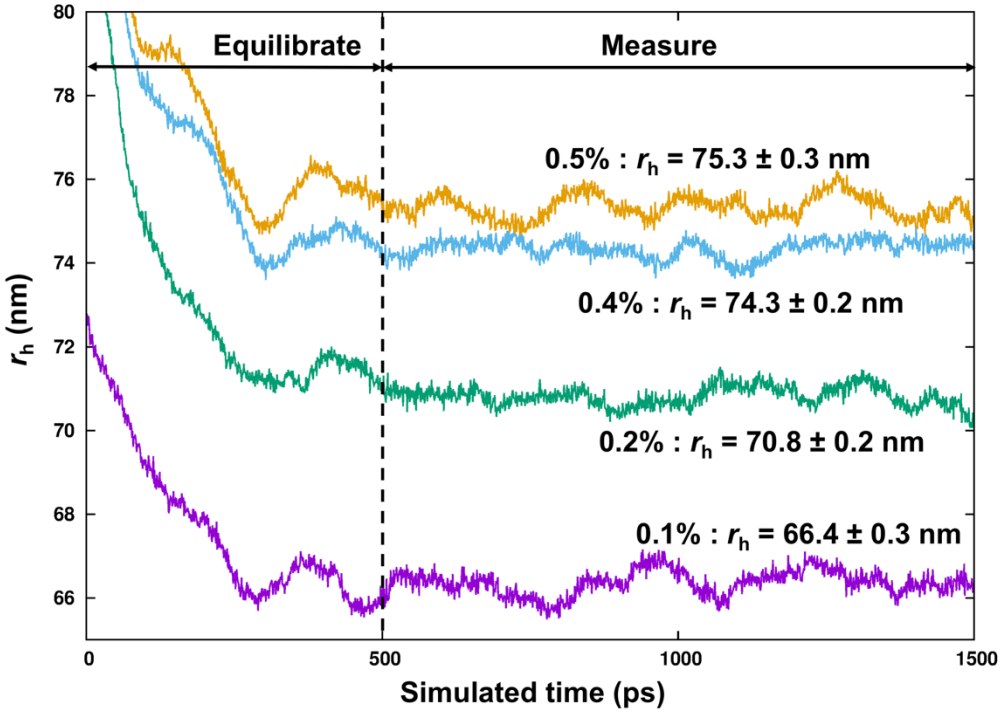
Dox-liposomes were incubated with 0.5 or 1 mol% HEP-Palm in PBS for 2 hours at either room temperature or 37°C. Afterwards, the dox-liposomes were centrifuged, the supernatant removed, and the pellet re-suspended for analysis on a 2% agarose gel run in 1X TAE. Stains-All detection revealed that the amount of dox-liposome-associated HEP was concentration dependent (0.5 versus 1 mol%), but did not improve under elevated temperatures. HEP-lipid and dox-liposome-only lanes (two left-most lanes) confirmed that HEP-lipid did not migrate to the pellet without dox-liposomes and that the dox-liposomes did not mimic HEP staining, respectively. The two right-most lanes are the HEP-Palm loading standards for quantitation.

Hep	1%	-	-	0.5%		-	1%		-	0.5µg	50ng
Dox	-	+	-	+	+	-	+	+	-	-	-
T (°C)	37	37	-	37	22	-	37	22	-	-	-



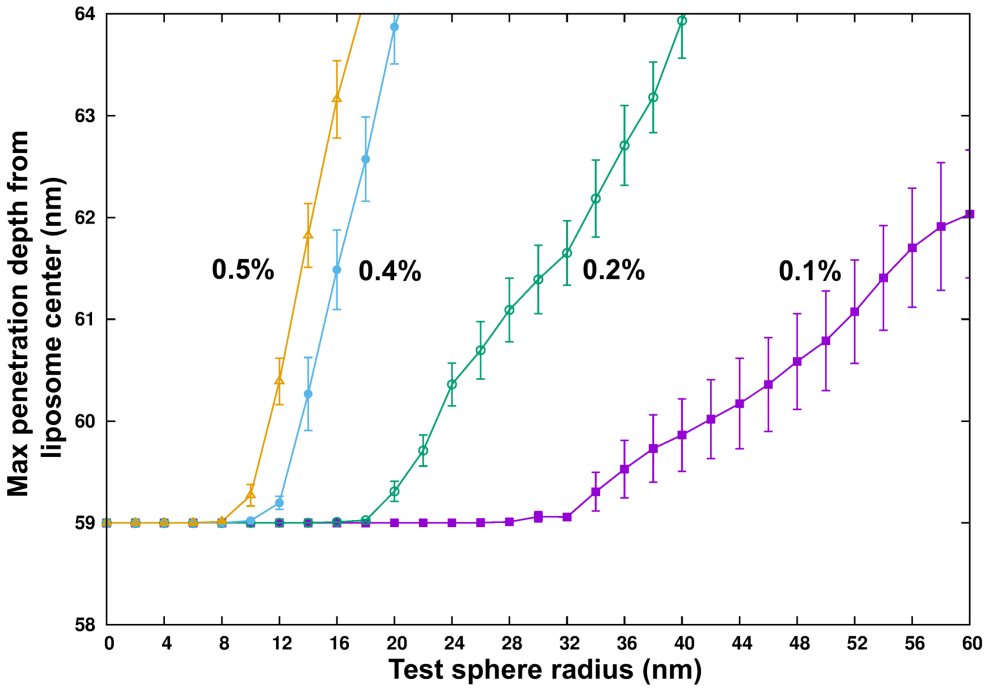
Supplemental Figure 2: Liposome Radius Modeled at Various 13.3 kDa HEP-lipid Concentrations

Simulations of 118 nm liposomes with increasing concentrations of 13.3-kDa HEP-lipid coating (0.1 to 0.5 mol%) showing a collapse during the first 0.5 ns (equillibration), followed by an oscillation around an equilibrium hydrodynamic radius (r_h) for 1 ns where a prediction could be made by averaging. The increase in hydrodynamic radius is less dramatic with higher amounts of HEP-lipid and almost negligible between 0.4 and 0.5 mol%, suggesting that the volume out to ~16 nm from the liposome surface is becoming saturated with HEP chains. (ps, picoseconds)



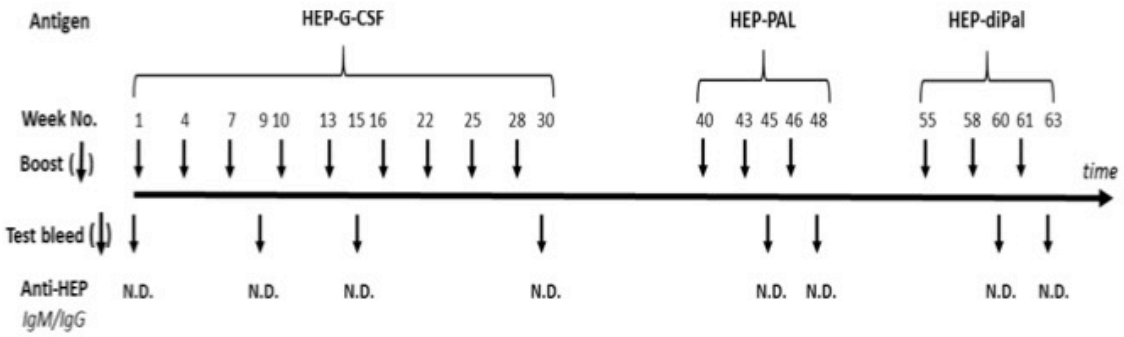
Supplemental Figure 3: Predicted Model Sphere Penetration Depth as a Function of Size for Liposomes with Various 13.3-kDa HEP-lipid Concentrations

To predict the approximate level of protection from the host proteins and the immune system offered to liposomes by 13.3-kDa HEP at various densities (0.1, 0.2, 0.4 or 0.5 mol%), the ability of spheres with defined radius (representing the hydrodynamic radius of potential circulating proteins) to penetrate the HEP chains and reach the liposome surface (located at 59 nm) was tested by computer simulation (ten independent runs averaged and plotted with standard deviation). The test sphere radius is depicted on the *x*-axis. For example, coating a liposome with 0.2 mol% 13.3-kDa HEP-lipid is predicted to allow any protein with a r_h <20 nm to reach the liposome's surface/bilayer. However, 0.5 mol% coating is predicted to bar proteins with a r_h greater than ~12 nm from coming into contact with the liposome surface.



Supplemental Figure 4: Immunization and Bleed Schedule for Various HEP-Conjugates in Rats

Three healthy Sprague Dawley rats were subjected to serial tandem challenges with HEP-granulocyte colony stimulating factor (G-CSF; 9 boosts), HEP-phenylalanine ammonium lyase (HEP-PAL; 3 boosts), then HEP-DiPalm (HEP-DiPal; 3 boosts), followed by periodic test bleeds in this long-term study (~1.2 years).



Supplemental Table 1: Analysis of Blood Chemistry and Histopathology from Mice with MDA-MB-231 Breast Tumor Xenograft

Tumor-bearing NRG mice were treated with saline vehicle alone or with dox-liposomes that were either (i) uncoated or coated with (ii) 5% 2-kDa PEG, (iii) 0.5% 12.5-kDa HEP-Palm, or (iv) 0.5% 13.3-kDa HEP-DiPalm. After animal sacrifice (22 days post tumor implantation for vehicle mice and 34 days for the other treatment groups), blood and organs were collected for analysis. The blood was tested (Antech Diagnostics, Stillwater, OK) for indications of liver (alanine aminotransferase, "ALT;" alkaline phosphatase, "ALP") and renal (blood urea nitrogen, "BUN") function. Tumors were assessed for necrosis and organs (i.e. tumor, heart, lung, kidney, liver, and spleen) were inspected for signs of toxicity, pathogenesis, and micrometastasis by a board-certified veterinary pathologist. *Ordinary One-Way ANOVA for all statistics; n = 4-7 per treatment group; *p<0.05 compared to PEG, **p<0.01 compared to PEG*

Animal Analysis

Blood Chemistry

Test *(Normal Value)*	**Indication**	**Vehicle** *(n=4-5)*	**Uncoated** *(n=7)*	**PEG** *(n=6)*	**Palm** *(n=5)*	**DiPalm** *(n=7)*
ALT (60-125 U/L)	Liver Function	297 ± 196.7	200.3 ± 260.4	89.83 ± 98.6	215.8 ± 330.8	111 ± 149.9
ALP (15-45 U/L)	Liver Function	30 ± 10.6	36.1 ± 14.6**	58.5 ± 7.7	45.6 ± 12.5	42.57 ± 11.3*
BUN (15-33 mg/dL)	Renal Function	22.4 ± 3.6	31.3 ± 6.63	36.17 ± 7.1	28 ± 2.6*	25.71 ± 3.3**

Histopathology

Organ	**Findings**
Tumor	Increased necrosis in HEP-DiPalm compared to PEG coating (p=0.0017)
Heart	No evidence of toxicity or other pathologic process
Lung	Evidence of micrometastasis in all groups; reduced by PEG and DiPalm ($p<0.0001$ and $p<0.05$, respectively, compared to uncoated dox-lipoosomes)
Kidney	No definitive evidence of toxicity
Liver	Occasional mitosis; no definitive evidence of toxicity or apoptosis
Spleen	Strain-related extramedullary hematopoiesis; no evidence of toxicity or lymphoid apoptosis