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Supplementary Materials for

Targeting neurons in the tumor microenvironment with bupivacaine nanoparticles reduces breast cancer progression and metastases

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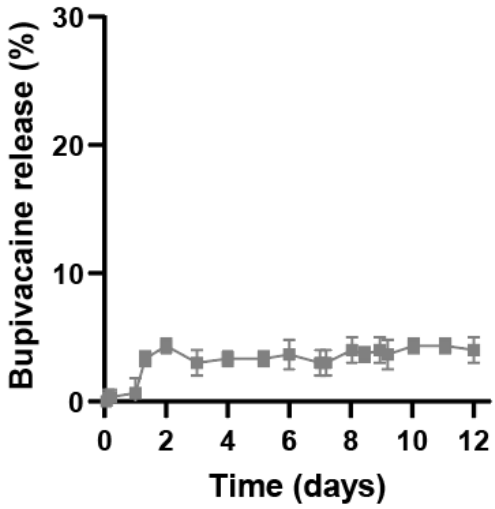
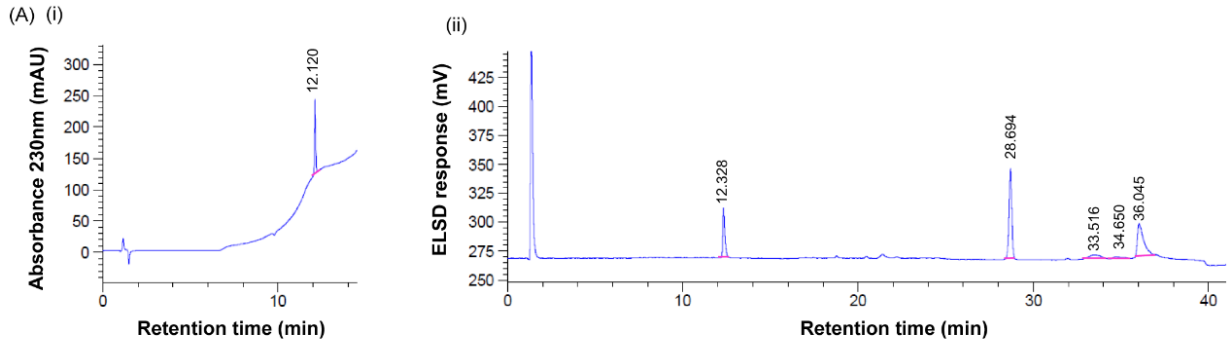


Figure S1. Bupivacaine release profile in 4°C. Liposomal bupivacaine release profile was conducted at 4°C for 12 days. Liposomes were composed of HSPC, cholesterol and DSPE-PEG2000 (molar ratio of 55:40:5). Results (three independent repetitions preformed in 3 replicates) are presented as mean±SD.



(B)

	Bupivacaine	Cholesterol	DSPE-PEG2000	HSPC	
				16:0	18:0
Retention time (min)	12.120	28.694	33.516	34.65	36.045
Area (mAUxs or mVxs)	755.851	874.181	205.467	922.275	
Concentration (mg/ml)	3.077	5.015	4.784	12.078	

Figure S2. HPLC analysis of liposomal bupivacaine. Chromatogram of liposomal bupivacaine using UV detector (**A**, i) and ELSD detector (**A**, ii). Analysis of the data regarding bupivacaine and the lipids obtained from UV and ELSD detectors respectively (**B**).

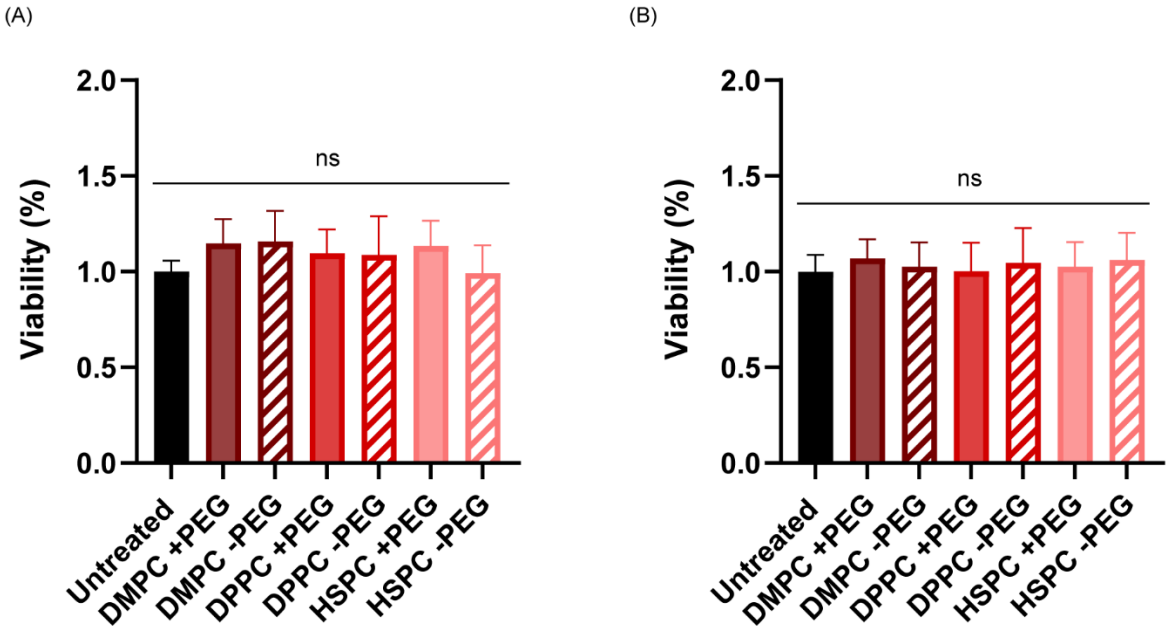


Figure S3. PEG effect on neuronal viability. The viability of PC12 after being treated with various lipid formulations: HSPC, DPPC, DMPC, cholesterol, with/without DSPE-PEG2000 was tested over 4 (**A**) and 24 hours (**B**). MTT viability values are normalized to the untreated group. One-way ANOVA was used for statistical analysis. Results represent 3 independent experimental repetitions preformed in 5 replicates in each experiment, presented as mean±SD.

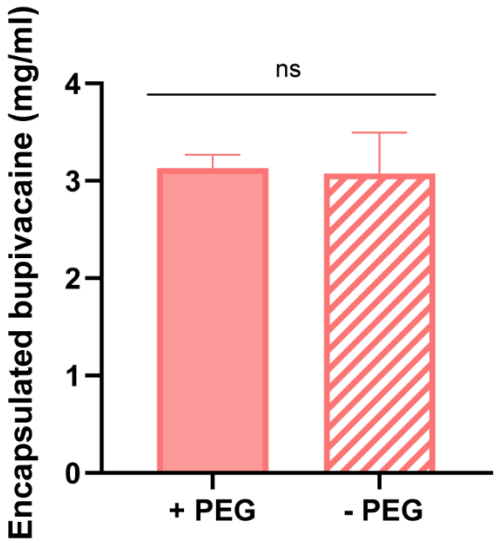


Figure S4. The effect of PEG on bupivacaine encapsulation efficiency. Liposomes, 100±20nm, composed of HSPC, cholesterol with/without DSPE-PEG2000 (55:40:5, or 60:40:0, molar ratio) were loaded with bupivacaine, and the drug loading efficiency was tested. Two-tailed unpaired Student's t-test was used for statistical analysis. Results represent three independent repetitions and presented as mean±SD..

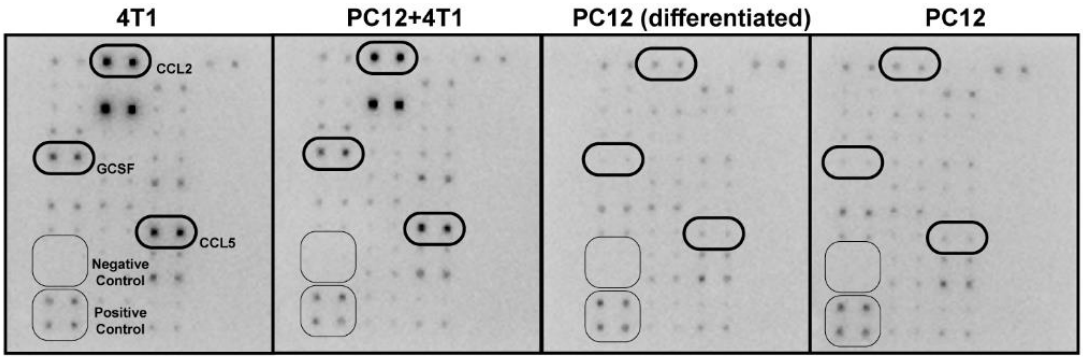


Figure S5. Cancer cells stimulate neurite outgrowth through cytokines secretion. Cancer cells secrete cytokines that promote neurites outgrowth, as was identified by cytokine antibody array. The analysis was conducted using conditioned media of different treatments after 72 hours from seeding.

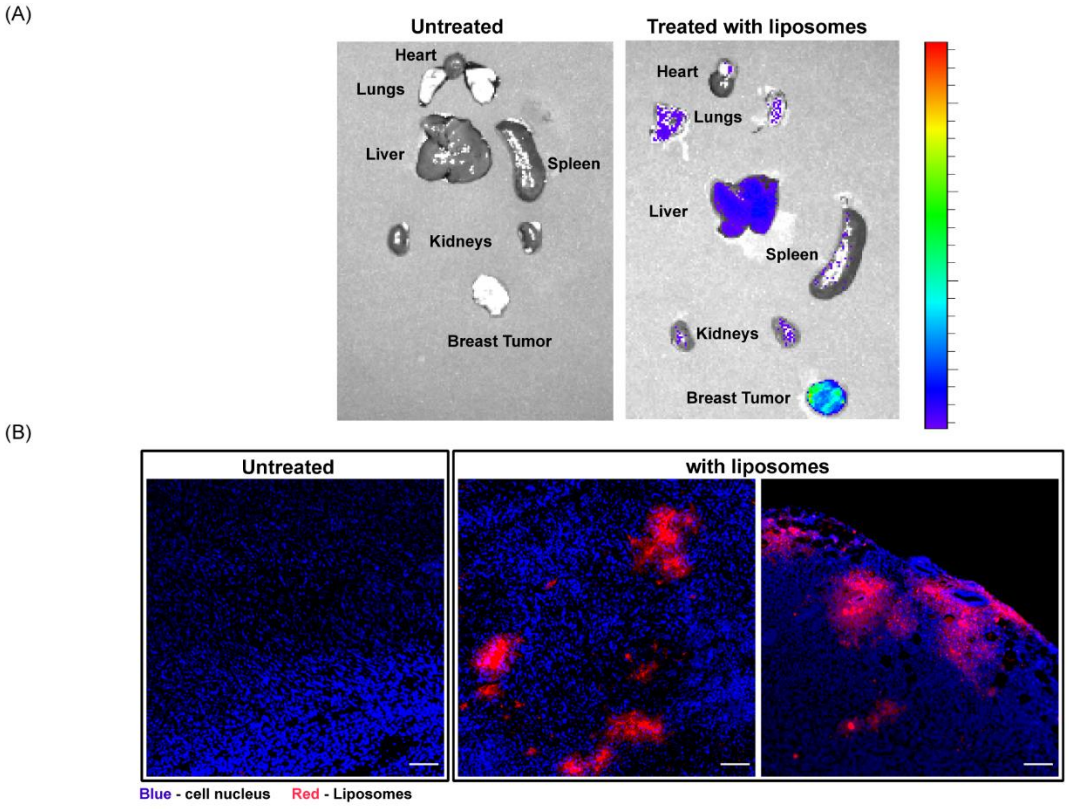


Figure S6. Liposomal delivery to the tumor. Liposomes labeled with Rhodamine were intravenously injected to mice bearing orthotopic 4T1 tumors, and after 24 hours their accumulation in different tissues was quantified using IVIS ex-vivo imaging (**A**). Liposome accumulation in the tumor tissue is also demonstrated by fluorescent histology images of the tumor (**B**, scale bar is 5cm).

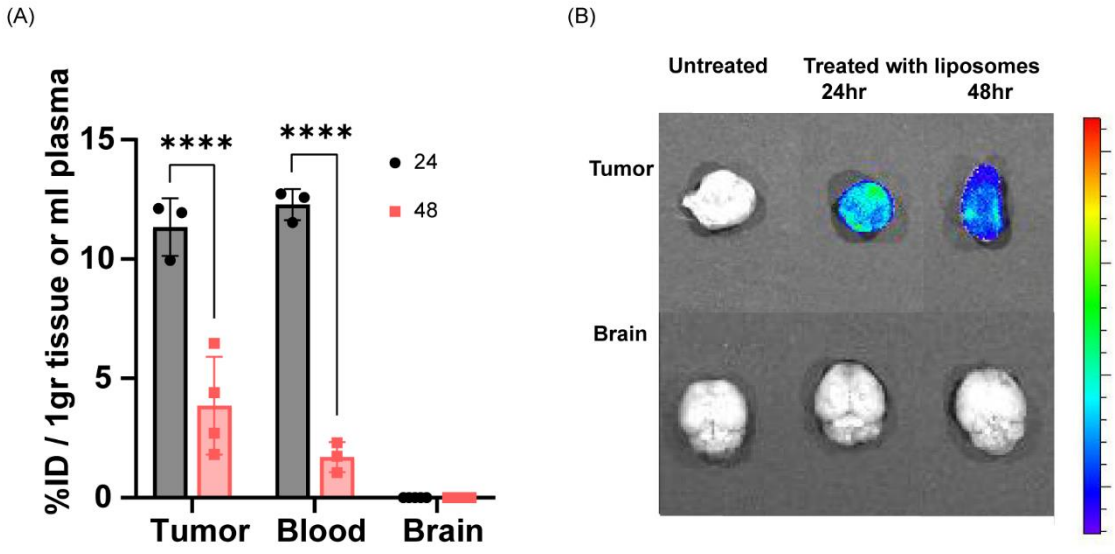


Figure S7. Nanoparticle' biodistribution to the tumor, brain and blood 24 and 48 hours after intravenous administration. Gadolinium-loaded liposomes were injected intravenously to mice bearing orthotopic 4T1 tumors. Twenty-four and 48 hours later the liposomal accumulation in the tumor, brain and blood plasma was quantified using Gadolinium elemental analysis (**A**), and whole animal imaging IVIS (**B**). Results ($3 \leq n \leq 4$) are presented as mean±SD. Two-way ANOVA was used for statistical analysis with multiple comparisons test adjusted *p* value; ****$p<0.0001$.

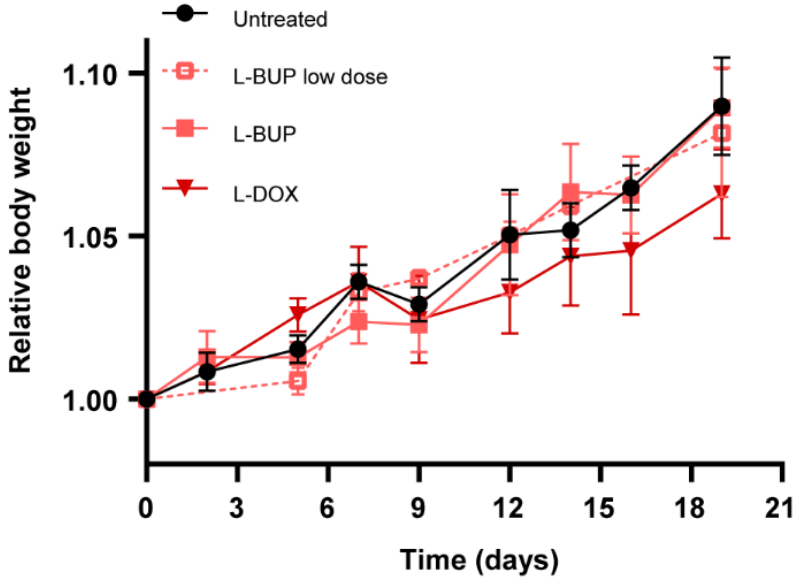


Figure S8. Liposomal bupivacaine treatment does not affect mice's body weight. No toxic effects were observed due to the treatments, and all mice's weight gradually increased. Body weight of mice was normalized to the initial weight at day 0. Results ($4 \leq n \leq 6$) are presented as mean±SEM.

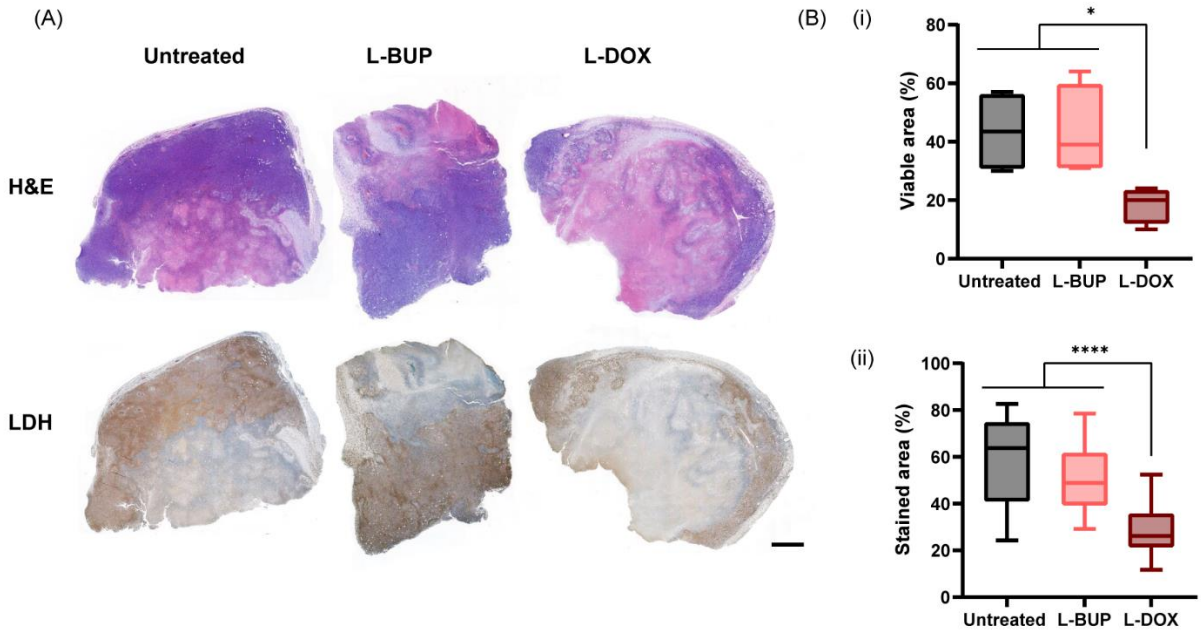


Figure S9. Histopathological H&E and molecular staining of glycolysis in tumors treated with liposomal bupivacaine and doxorubicin. Histological H&E (purple scale) and immunohistochemistry staining for anti-lactic dehydrogenase (LDH, brown scales) in mice bearing orthotopic 4T1 tumors, treated with either liposomal bupivacaine (L-BUP) or liposomal doxorubicin (L-DOX). Higher glycolysis was detected in the L-BUP treatment group compared to the L-DOX (**A**, scale bar is 1mm). The viable areas of the tumor tissue were quantified based on the H&E staining (**B**-i). The glycolytic metabolism was quantified based on positive anti-LDH staining (**B**-ii). One-way ANOVA (n=4) was used for statistical analysis of (B) with adjusted *p* value in multiple comparisons tests; *$p<0.05$ and ****$p<0.0001$