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Supporting Information

Bortezomib Immunoliposomes for CD44-Positive Macrophage Targeting: A New Paradigm in Inflammatory Therapeutics

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	Zeta potential (mV)	Hydrodynamic Size (DLS)	Size (TEM)	PDI
Empty Liposome	-8.55	119.6 ± 57.8 nm	107.9 ± 34 nm	0.158
BTZ-Liposome	-9.35	123.21 ± 61.2 nm	99.7 ± 29 nm	0.190
Empty Immunoliposome	-8.33	176.43 ± 86.2 nm	220.1 ± 135 nm	0.206
BTZ-Immunoliposome	-6.13	144.26± 74.4 nm	158.46 ± 45 nm	0.205

Table S1. DLS data for all the synthesized liposomes.

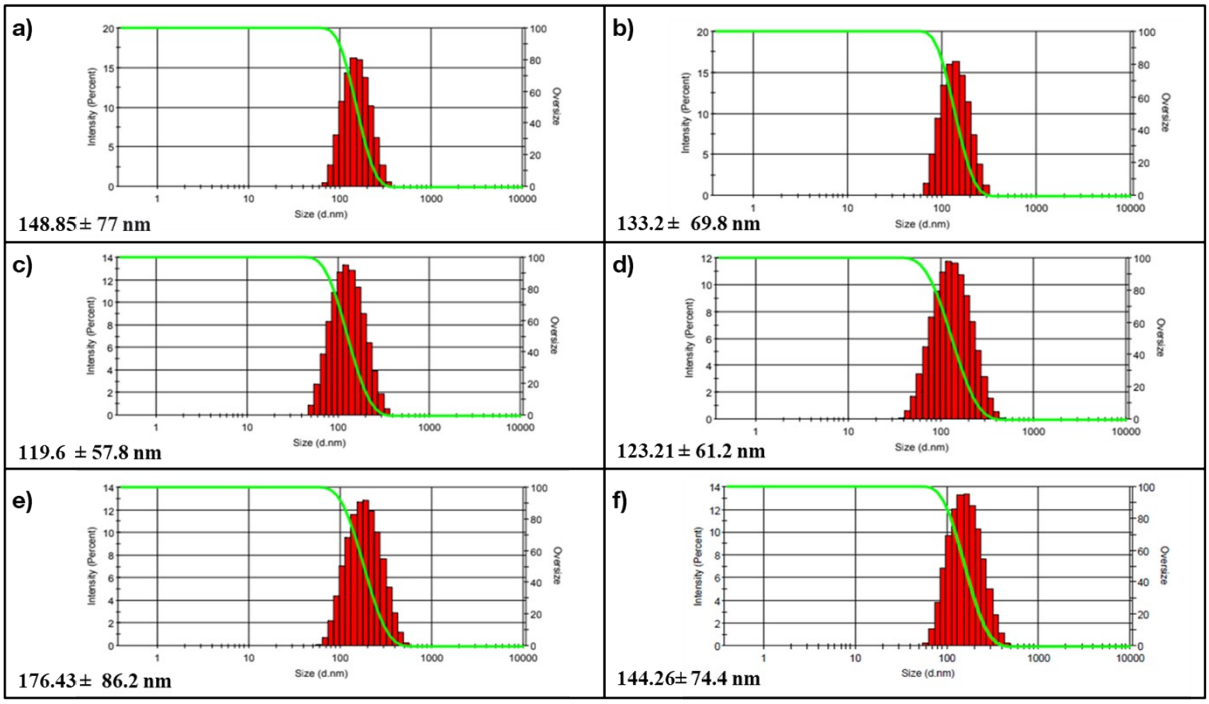

Figure S1. Size distribution by intensity of a) Empty liposome without DSPE-PEG b) Bortezomib liposome without DSPE-PEG c) Empty liposome with DSPE-PEG d) Bortezomib liposome with DSPE-PEG e) Empty immunoliposome f) Bortezomib immunoliposome.

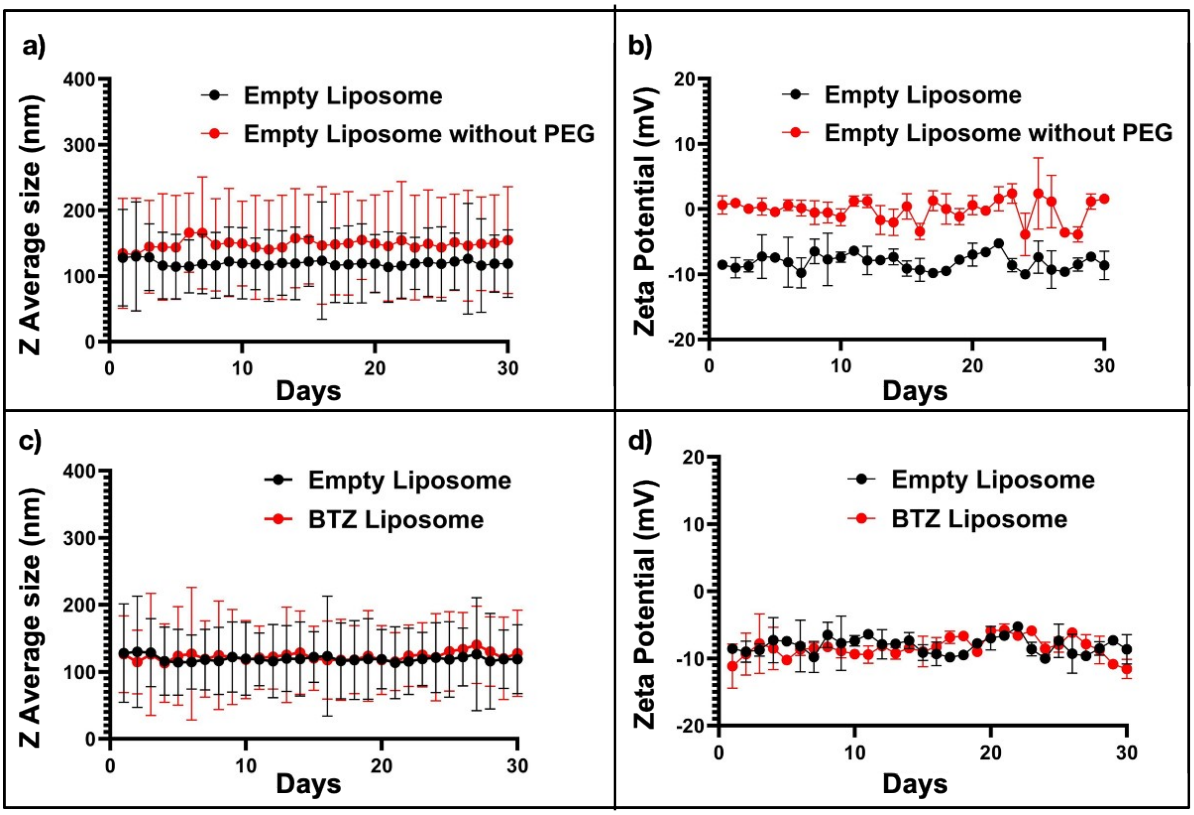

Figure S2. Relative analysis of the hydrodynamic size and zeta potential of a) Empty liposome without DSPE-PEG and empty liposome with DSPE-PEG b) Empty liposome with Bortezomib liposome

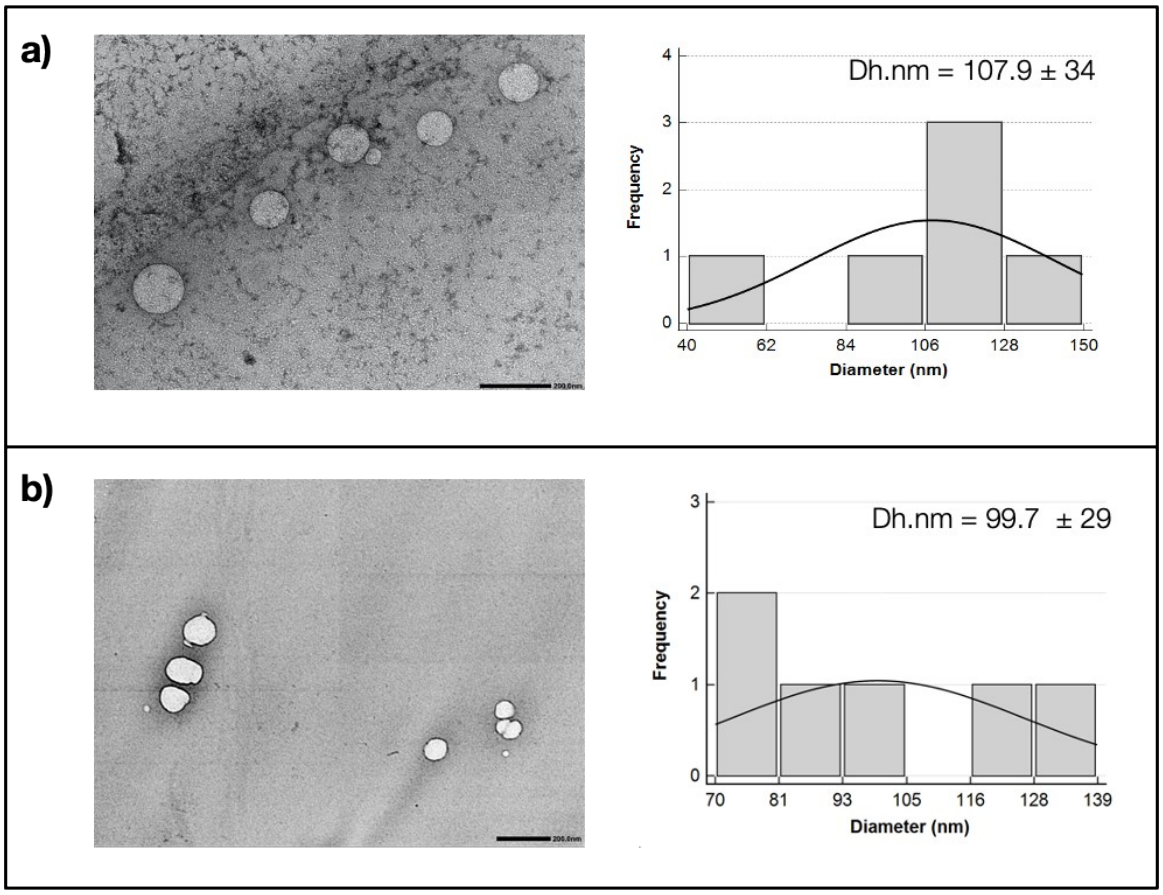

Figure S3. TEM images and size-distribution histogram of liposome samples before antibody loading a) Empty liposome, average size calculated is 107.9 ± 34 b) Bortezomib Liposome, average size calculated is 99.7 ± 29 nm

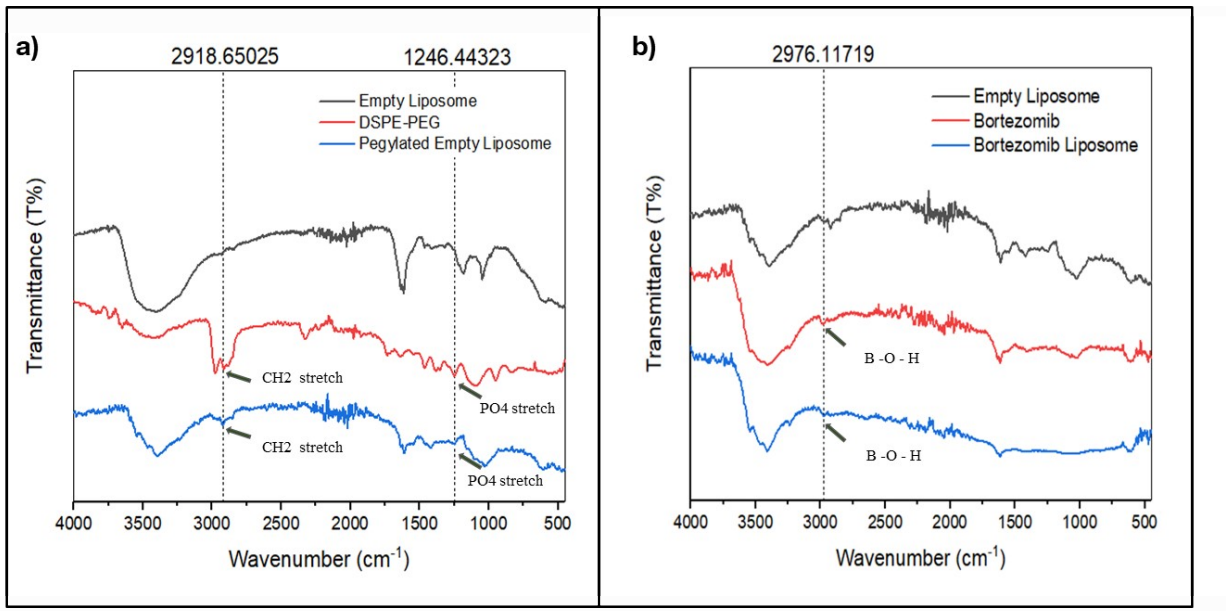

Figure S4. a) Pegylated empty liposome spectra showing CH2 stretch (2918.6 cm-1) and PO4 stretch (1246.4 cm-1) incorporated similar to DSPE-PEG spectra depicting successful pegylation

of liposome. b) Bortezomib liposome spectra showing B-O-H bond (2976.1 cm-1) incorporated similar to Bortezomib drug spectra depicting successful loading of drug in liposome.

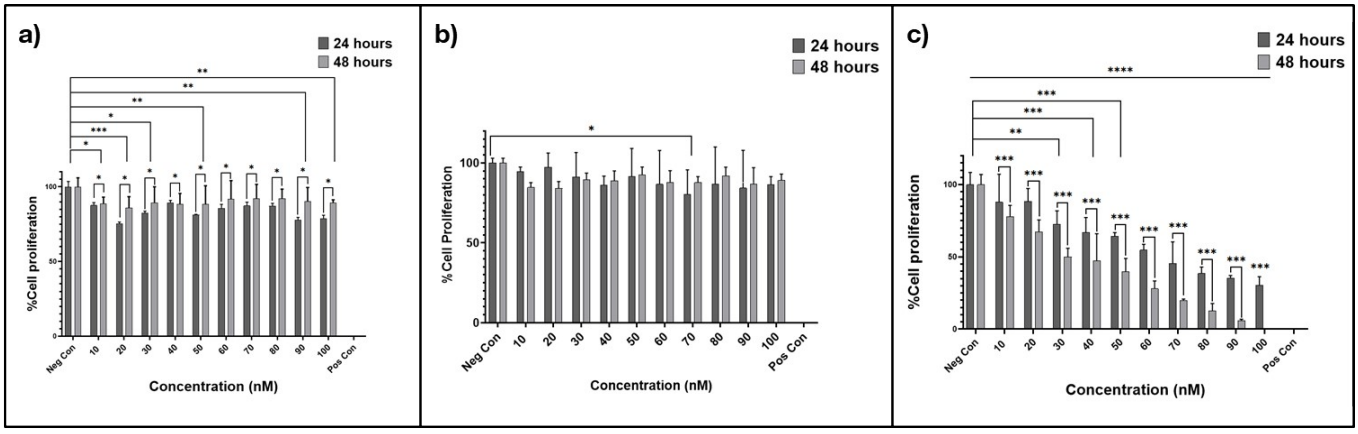

Figure S5. Relative analysis of 24 and 48 hrs MTT of a) Empty Liposome b) Empty immunoliposome c) Bare BTZ drug, IC50 = 62.87 nM (24 hrs)

The p-values for this graphs are represented as * for p<0.05, ** for p<0.01, * for p<0.001**

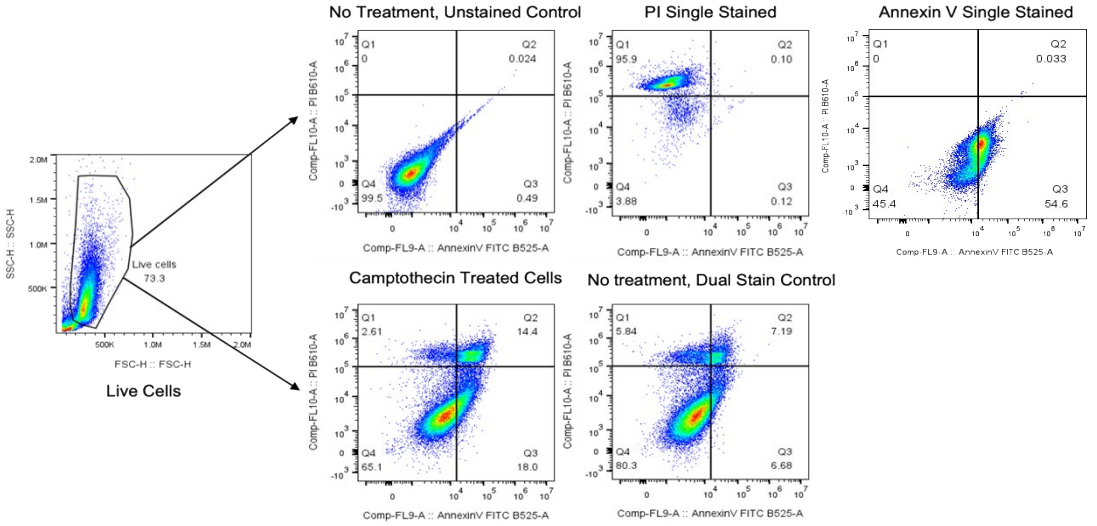

Figure S6. Figure depicting the gating strategy for the Annexin-V/PI apoptosis assay. -

RAW 246.7 cells were gated on forward (FSC) versus side scatter (SSC) to select the live cell population. Next, the cells were gated on Annexin V-FITC vs side scatter (SSC) to represent the Early Apoptotic cells with Annexin V positive, obtained through serum deprivation. (Heat-treated cells were gated on Annexin V-FITC vs PI to achieve late apoptotic gating. The necrotic population was gated by forward (FSC) scatter vs PI gating.

Four distinct populations were defined after achieving the gating strategy - Live cells (Annexin V and PI negative) - Q4, Early Apoptotic cells (Annexin V positive) - Q3, Late Apoptotic cells (Annexin V and PI positive) - Q2, and dead cells (PI positive) - Q1

No.	Genes	Forward primer	Reverse primer
1	GAPDH	GTGTTTCCTCGTCCCGTAGA	ATGAAGGGGTCGTTGATGGC
2	CD206	GTGCAGTGATGGAACCCCAG	CTGTCCGCCCAGTATCCATC
3	Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
4	IL-6	CACGGCCTTCCCTACTTCACA	TGCAAGTGCATCATCGTTGTTC
5	IL-1	TGCCACCTTTTGACAGTGATG	TGATACTGCCTGCCTGAAGC
6	iNOS	TCCTGGACATTACGACCCCT	CTCTGAGGGCTGACACAAGG
7	TNF-alpha	AGCCCACGTCGTAGCAAACCAC	AGGTACAACCCATCGGCTGGCA
8	PD-L1	AAGTCAATGCCCCATACCGC	CTCTTCCCACTCACGGGTTG
9	BAX	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
10	Bcl2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
11	Caspase-3	TGTTTGTGTGCTTCTGATAAGT	CATGGCTCAGAAGCACACAAAC
12	Caspase-8	GGTCACTTGAACCTTGGGAA	AGGCCAGATCTTCACTGTCC
13	Caspase-9	GTGGACATTGGTTCTGGAGGAT	CGCAACTTCTCACAGTCGATG
14	JNK	TGGACTTGGAGGAGAGAACC	ACGATGATGATGGATGCTGA
15	ERK	TGCAGATCCAGACCATGATC	GAATGCAGCCTACAGACCAA

Table S2. Primers used in RT-PCR studies.

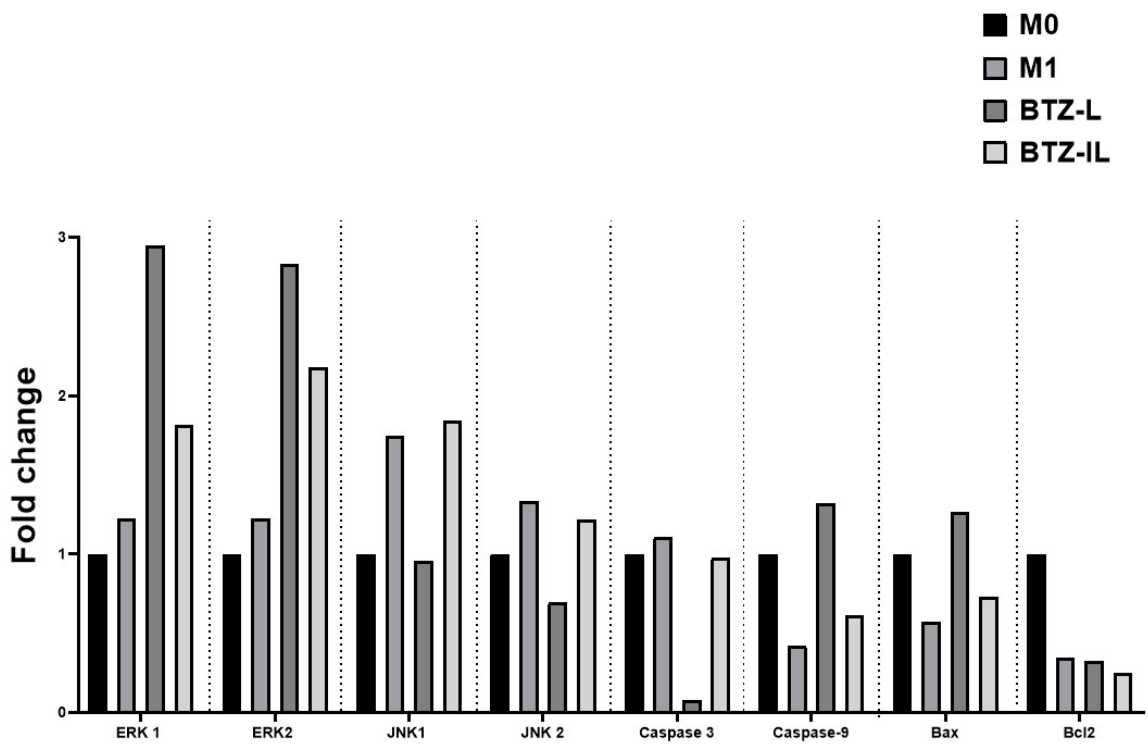

Figure S7. The figure depicts the relative fold change in the protein expression of ERK 1, ERK 2, JNK1, JNK 2, Caspase-3, Caspase-9, Bax and Bcl2 in RAW 264.7 after liposome treatment on LPS activated (M1) macrophages.

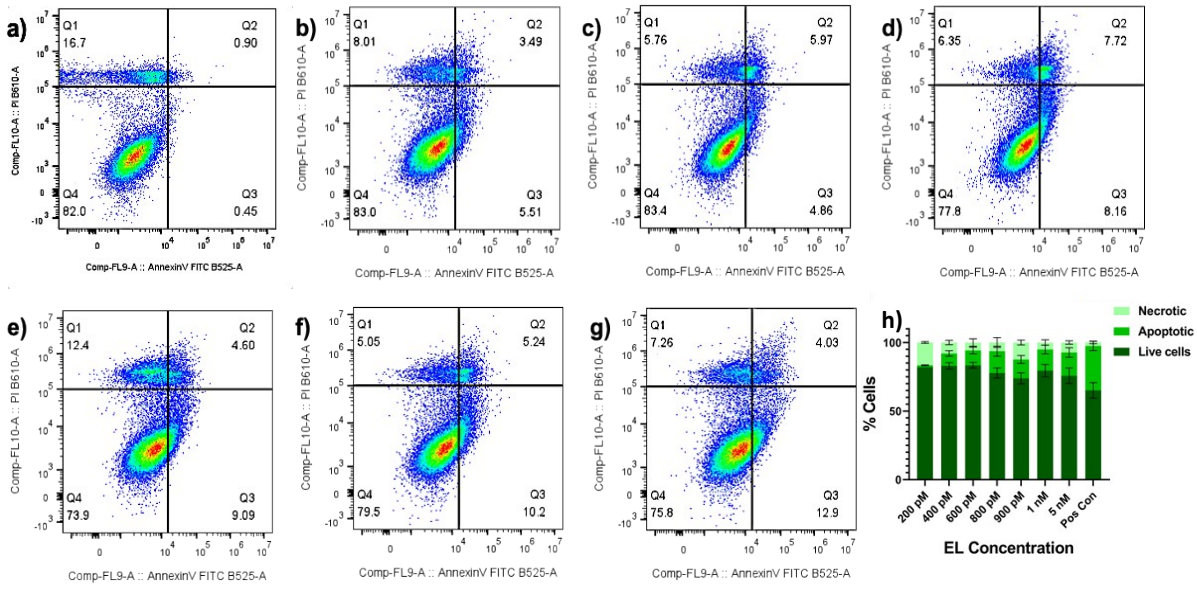

Figure S8: The Annexin V PI apoptosis assay was performed across a range of Empty Liposome concentrations (200, 400, 600, 800, 900 pM, 1nM and 5 nM). Most cells remain in a live state, even at the highest concentration, suggesting the vehicle control of the experiment doesn't induce apoptosis.

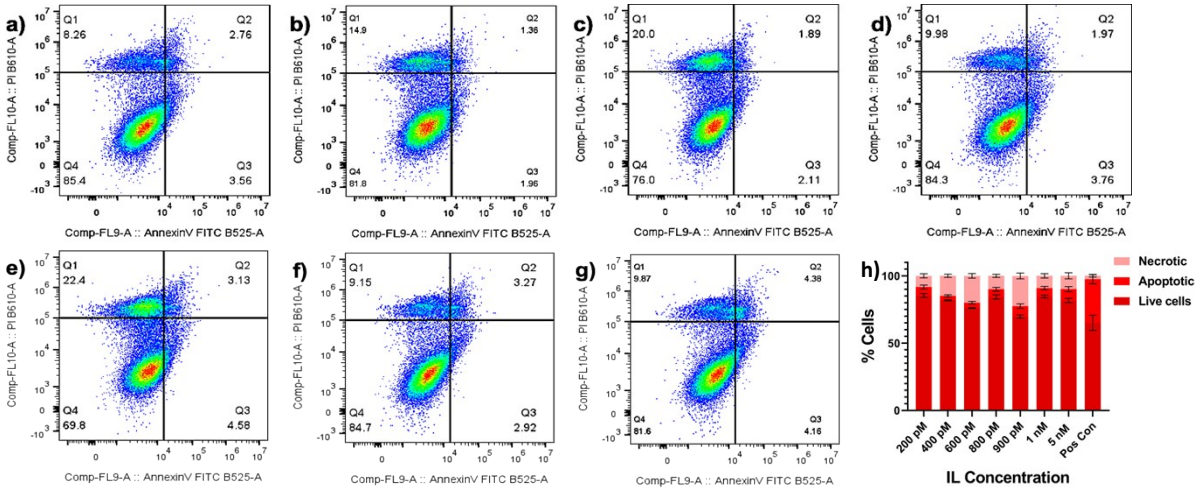

Figure S9: The Annexin V PI apoptosis assay was performed across a range of Empty CD44 Immunolipoosme concentrations (200, 400, 600, 800, 900 pM, 1nM and 5 nM).

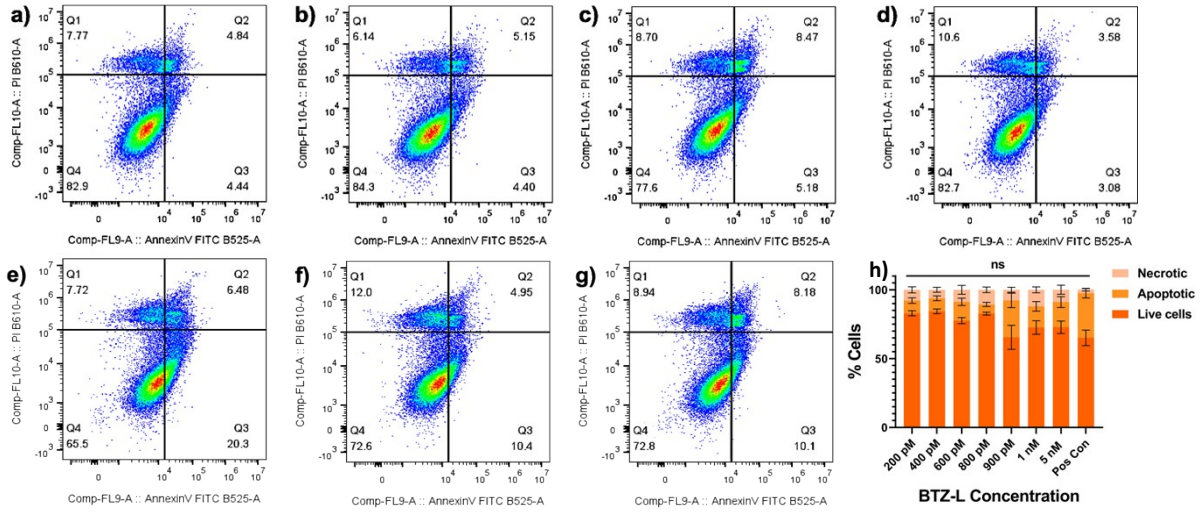

Figure S10: The Annexin V PI apoptosis assay was performed across a range of BTZ-loaded Liposome concentrations (200, 400, 600, 800, 900 pM, 1nM and 5 nM).

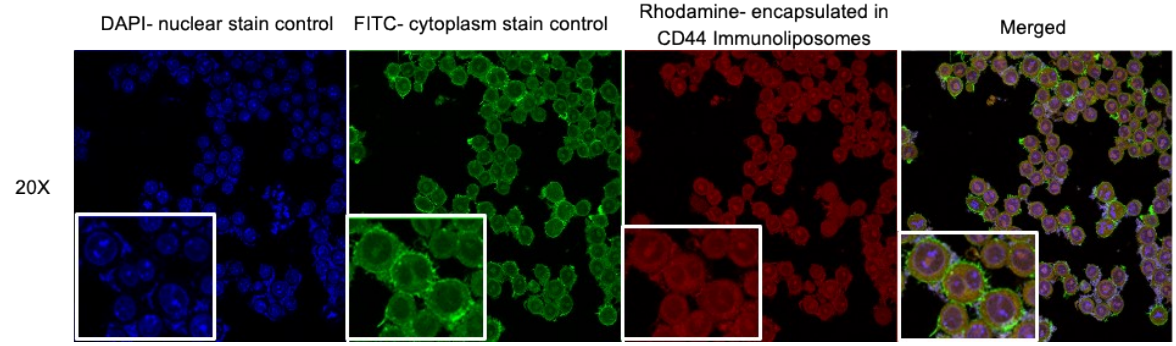

Figure S11: Single channel images for DAPI, FITC, and Rhodamine liposome treated cells to study internalization of liposomes in RAW264.7.