Electronic Supplementary Material (ESI) for Analyst


Supporting Information for:

# Functionalized Liposome Purification via Liposome Extruder Purification (LEP)

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**Table of Contents:**

**Experimental Section:**

## Experimental Section:

### Liposome Formation via Extrusion

Unless stated otherwise the general liposome composition was as follows: 94% HSPC, 5% mPEG2000, 1% carboxy-fluorescein-PE (CF-PE), and a 1:10 ratio of cholesterol to lipid at a final lipid concentration of 5 mM in 250 µL volume. The lipid components were mixed in chloroform and dried under nitrogen. Film hydration was carried out in PBS pH 7.4 at 65 °C (HSPC has a $T_m$ = 55 °C) for 30 min while rotating. Extrusion of the liposomes was carried out at 65 °C with 15 passes through a polycarbonate track etched membrane (100 nm) in a mini-extruder, with 250 µL syringes. The membranes (30, 50, and 100 nm), mini-extruder and all lipid components were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and were used for both extrusion and LEP purification processes. Cholesterol was purchased from Sigma Aldrich (St. Louis, MO).

### Liposome Extruder Purification (LEP)

Post extrusion, the liposome samples were allowed to cool to room temperature. Unless stated otherwise, LEP was performed on 100 nm liposome samples, with or without contaminant, at 1 mM lipid concentration (250 µL) by passing 245 µL of the sample through a 50 nm membrane. A reduction in liposomal recovery was observed at lipid concentration >4 mM. The flow through syringe was removed and the sample was saved for analysis. The syringe was then washed and PBS pH 7.4 was flowed the reverse direction through the membrane to return the sample syringe back to its starting volume of 250 µL. The sample syringe was removed from the mini-extruder only after fresh PBS was flowed through the membrane to enhance liposome recovery. When multiple LEP cycles were carried out, the sample syringe remained attached to

the mini-extruder until the final LEP cycle was completed. The dead volume associated with the syringe needles and mini-extruder membrane space was determined to be 10 μL. All experiments were performed in triplicate (± SD) with representative absorbance curves displayed.

**Quantification of Contaminant Removal and Liposome Recovery**

To determine the liposome recovery and the contaminant removal all samples (Pre-LEP, FT and Post-LEP) underwent either an absorbance scan from 220 – 650 nm or an endpoint absorbance reading specific to the liposome (494 nm) and contaminant used: Coumarin 343 (427 nm) purchased from Sigma Aldrich (St. Louis, MO), bovine serum albumin (BSA, 280 nm) purchased from EMD Millipore (Billerica, MA), and Trastuzumab (280 nm) was a gift from Dr. Rudolph Navari at the Indiana University School of Medicine (South Bend, IN). The liposome recovery was determined by absorbance readings at 494 nm to quantify CF-PE within the liposome. To quantify the amount of contaminant present in the sample the absorbance contribution of the CF-PE containing liposomes was subtracted from the mixed sample absorbance readings at 280 and 427 nm based on the liposomal absorbance at 494 nm. All absorbance readings were carried out in a 0.3 cm path length quartz cuvette using a Spectramax M5 plate reader. All experiments were performed in triplicate (± SD) with representative absorbance curves displayed.

**Liposome DLS**

To verify that the LEP process was not detrimental to the liposome integrity, dynamic light scattering (DLS) was carried out before and after the samples underwent the LEP process. Data

was collected on a Brookhaven ZetaPlus instrument and was averaged from five 1 minute measurements carried out at room temperature in PBS pH 7.4.

**Liposome Targeting Pre- and Post- LEP**

Liposome Preparation: The non-targeted liposome was formulated as 94.5:10:5:0.5 HSPC:CHOL:mPEG2000-DSPE:CF-PE and the targeted liposome was formulated as 93.5:10:5:1:0.5 HSPC:CHOL:mPEG2000-DSPE:VLA4-pep:CF-PE. VLA4-pep has been shown to bind with specificity to Very Late Antigen-4 (VLA-4; also known as $\alpha_4\beta_1$). Post-LEP, the absorbance intensities of the Pre-LEP and Post-LEP samples were matched to account for small losses during the LEP procedure. The NCI-H929 cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 media (Cellgro, Manassas, VA) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 55 uM 2-mercaptoethanol purchased from Gibco (Carlsbad, CA). To assess the liposome targeting, an *in vitro* uptake assay was carried out and flow cytometry was used to determine the amount of liposomes taken up by the cells. NCI-H929 cells (known to express VLA-4) were plated at a density of 1 x $10^5$ cells/well 24 h prior to the experiment in a 24 well dish. Liposomes were added at 100 μM phospholipid concentration and incubated for 3 h at 37 °C in 5% $CO_2$. After incubation, cells were washed 3 times with DPBS, diluted into growth media, and analyzed on a Guava easyCyte 8HT flow cytometer.

**Supporting Information Figures**

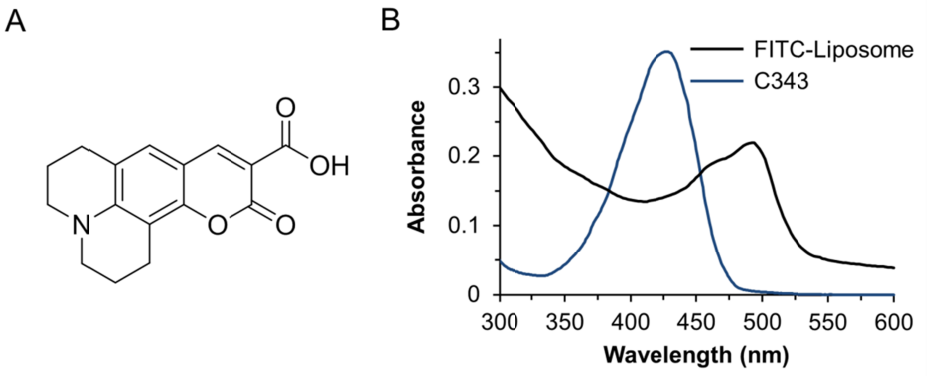


**Figure S1.** A) Coumarin 343 (C343) molecular structure, 285.29 Da. B) Absorbance scans of C343 (blue line) and 94% HSPC, 5% mPEG2000, 1% CF-PE, with a 1:10 ratio of cholesterol to lipid at 1 mM lipid concentration (black line) demonstrate that despite significant overlap in the spectrums, there is essentially no contribution of the C343 to the fluorescein present in the liposome formulation at its maximum absorbance wavelength of 494 nm. This allows for very accurate quantification of the liposome recovery and C343 contaminant separately.

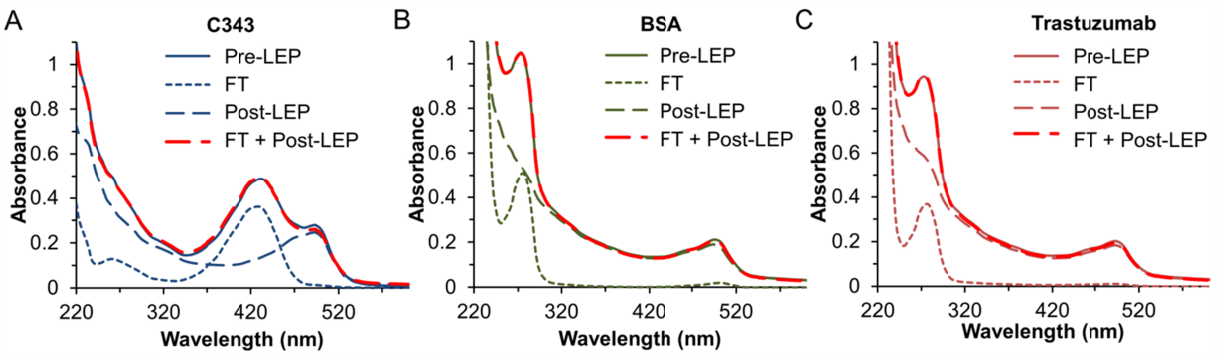


**Figure S2.** Absorbance scans for the purification of liposomes from C343 (A), BSA (B) and Trastuzumab (C). To demonstrate that there is a minimal loss of liposomes or contaminant to the LEP system the Post-LEP purified liposome absorbance curve and the flow through (FT) curve were summed. In all cases this new curve nearly perfectly overlays onto the Pre-LEP sample absorbance curve. If there were loses of any kind associated with the process we would expect the sum of the Post-LEP and FT curves to be less than the initial absorbance scan of the Pre-LEP sample.

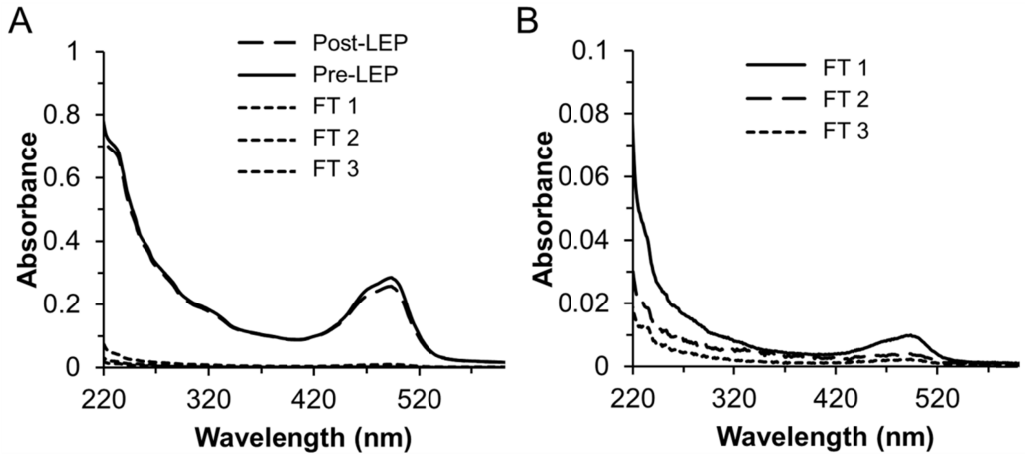


**Figure S3.** A) Absorbance scans of 3 cycles of LEP being performed on a single liposome sample (100 nm liposomes, 50 nm LEP membrane) demonstrating a liposome recovery of 96.01 ± 4.81%. B) Absorbance scans of the flow through (FT) from each of the 3 LEP cycles showing a reduced flow through of liposomes through the membrane with each consecutive LEP cycle. The initial flow through shows a liposome absorbance of 0.0096 at 494 nm which is <3.5% of the initial liposome sample with significantly less liposome passing through the membrane in the second and third FT fractions. This process demonstrates a ~14-16 fold reduction in contaminate after each LEP cycle with a final reduction in contaminate of ~ 2,750-4,100 fold after 3 cycles.

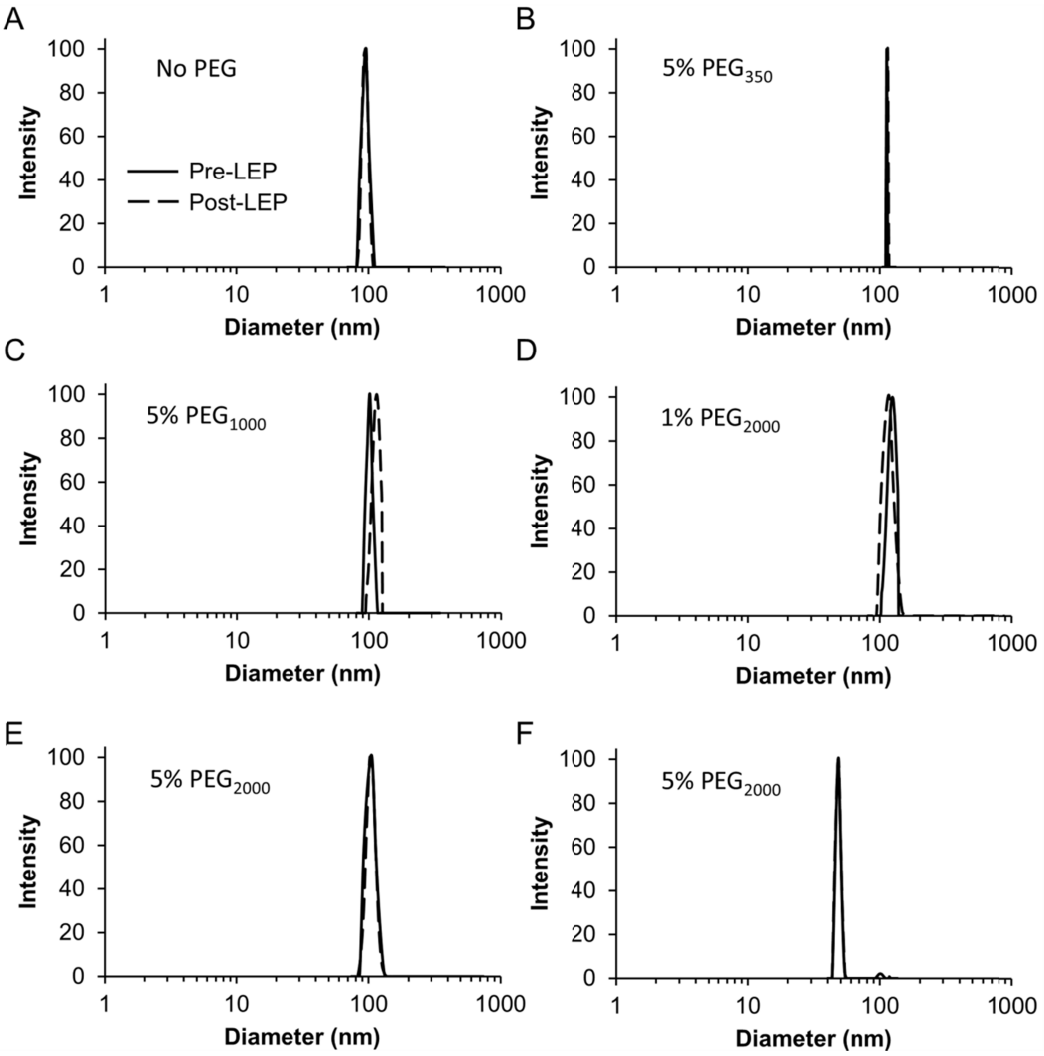


**Figure S4.** DLS data for the diverse liposome formulations tested to verify no aggregation or liposome fragmentation occurring as a result of the LEP process. A) No PEG, 100 nm liposome, 50 nm LEP membrane. B) 5% PEG350, 100 nm liposome, 50 nm LEP membrane. C) 5% PEG1000, 100 nm liposome, 50 nm LEP membrane. D) 1% PEG2000, 100 nm liposome, 50 nm LEP membrane. E) 5% PEG2000, 100 nm liposome, 30 nm LEP membrane. F) 5% PEG2000, 50 nm liposome, 30 nm LEP membrane.

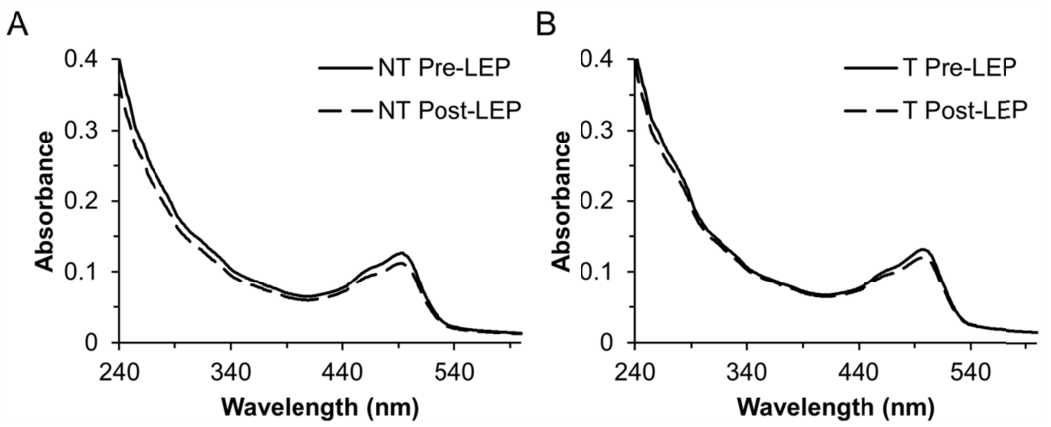


**Figure S5.** A) Absorbance scans of the non-targeted (NT) liposomes Pre- and Post-LEP used in the *in vitro* uptake assay demonstrating a liposome recovery of 89.20% (94.5:10:5:0.5 HSPC:CHOL:mPEG2000-DSPE:CF-PE). B) Absorbance scans of the targeted (T) liposomes Pre and Post-LEP used in the *in vitro* uptake assay demonstrating a liposome recovery of 91.30% (93.5:10:5:1:0.5 HSPC:CHOL:mPEG2000-DSPE:VLA4-pep:CF-PE).

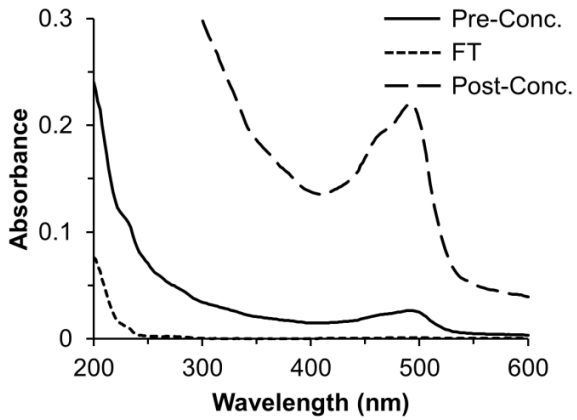


**Figure S6.** Absorbance scans of concentrating liposomes via the LEP process. The concentrating LEP process was carried out with a 1000 µL starting volume of 100 nm liposomes at 0.2 mM lipid concentration to a LEP volume of 5 µL using a 50 nm membrane. The sample was then brought up to a final volume of 100 µL to facilitate the maximum recovery of liposomes in the Post-Concentrated sample by flowing 95 µL of PBS pH 7.4 back through the membrane. This would result in a 10 fold concentration increase of the initial liposome sample but due to liposome loses the final liposome recovery was 82.9 ± 0.92% with a fold increase in concentration of 8.3 ± 0.09. The liposome recovery can be increased by flowing more PBS back through the membrane which would increase the final sample volume and decrease the overall enhancement in concentrating the sample.