

## Functionalized liposome purification *via* Liposome Extruder Purification (LEP)<sup>†</sup>

Cite this: *Analyst*, 2013, **138**, 4746

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Received 5th April 2013

Accepted 2nd July 2013

DOI: 10.1039/c3an00680h

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Liposome Extruder Purification (LEP) allows for the rapid purification of diverse liposome formulations using the same extrusion apparatus employed during liposome formation. The LEP process provides a means for purifying functionalized liposomes from non-conjugated drug or protein contaminants with >93% liposome recovery and >93% contaminant removal in a single step.

### Communication

Translational research using liposomes is expanding rapidly as liposomes advance through clinical trials as targeted and non-targeted drug delivery vehicles for cancer therapies.<sup>1–6</sup> When used as drug carriers, targeted liposomes are capable of selectively delivering chemotherapeutics to tumors while greatly reducing systemic toxicity allowing for improved patient outcomes.<sup>2,4</sup> Liposomes are also utilized for vaccine delivery<sup>7,8</sup> and for diagnostic imaging applications.<sup>9,10</sup> There are many different methods of forming and functionalizing liposomes, making the development of a universal method for purifying functionalized liposomes nearly impossible. The Liposome Extruder Purification (LEP) process, described here, allows for the inexpensive, rapid, and efficient purification of various liposome formulations with >93% contaminant removal and >93% liposomal recovery. The LEP process can also be implemented as an analytical quality control technique to validate product purity throughout the manufacturing process in the large scale production of pharmaceutical liposomes.

Depending upon the desired liposomal properties, different preparation techniques can be used that include: extrusion, sonication, reversed phase evaporation, and freeze-dried rehydration.<sup>11–13</sup> The preferred method for liposome preparation is extrusion due to its simplicity and its ability to reproducibly form unilamellar liposomes with low polydispersity in the most physiologically relevant size range of 30–200 nm. Liposomes within this diameter range display reduced clearance rates and increased tumor targeting *in vivo* as a result of the enhanced permeability and retention (EPR) effect.<sup>14–17</sup> After liposome formation is complete, the resulting liposomes are often chemically coupled with targeting moieties or are loaded with cytotoxic drugs, as in the case of nanoparticle drug delivery formulations. After conjugation, it is then necessary to purify the liposomes from the unreacted/excess components prior to evaluating the conjugated liposome product. While there are many optimized methods to form and functionalize liposomes, there is currently a lack of efficient methods to purify liposomes. Purification is a critical step to effectively validate liposome formulations for their clinical and diagnostic uses to ensure that the observed therapeutic effects are due to the liposome conjugate and not from non-conjugated contaminants.<sup>1,18</sup>

Due to the extensive variation in liposome formulations, various conjugation and loading strategies, vast size distributions, and complicated surface functionalization methods, a single liposome purification technique suitable in all cases has not been identified. There are some available techniques for the purification of liposomes, however, they negatively impact liposomal integrity and often need to be optimized for each liposome formulation as they rely upon the intrinsic properties of each unique formulation. For example, gel filtration, the most common liposomal purification technique due to commercial availability, can be a lengthy process that requires column packing and equilibration (~2 h), 20–30 min purification runs and pre-saturation of the column with a large excess of blank liposomes.<sup>19</sup> Column pretreatment is necessary to minimize the non-specific interactions between the column packing material and the functionalized liposomes. While gel filtration

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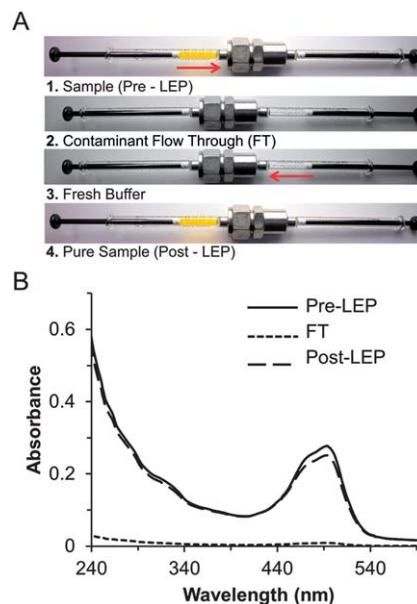
<sup>†</sup> Electronic supplementary information (ESI) available. See DOI: 10.1039/c3an00680h

provides for highly purified liposomal conjugates (>90% purity), functionalized liposome recovery depends highly upon liposome composition and can be as low as 50%, even after column pre-saturation, with the resulting liposome solution being significantly diluted.<sup>13,19</sup> Additionally, there is the potential for cross contamination between samples when using the same column to purify different liposome formulations, resulting in reduced sample purity. Dialysis is another common filtration method implemented to remove low molecular weight contaminants from liposomes but also suffers from relatively poor liposomal recovery (<80%), overnight incubations, and inefficient removal of large protein contaminants.<sup>20</sup> An alternate liposome purification technique utilizes an ultracentrifugation process by which very high centrifugal forces ( $100\,000$ – $160\,000 \times g$  for  $\sim 30$  min) are used to pellet liposomes while lower molecular weight contaminants remain in the supernatant.<sup>2,11</sup> Multiple washes of the liposome pellet provide for high functionalized liposome purities (>90%) however; the extreme centrifugal forces applied to the liposomes results in liposome fragmentation as well as leakage of loaded solutes from the intra-liposome aqueous phase.<sup>2,19</sup> Centrifugation can also be employed to purify liposomes in a different manner *via* density gradient fractionation utilizing high concentrations of metrizamide, Ficoll, or dextran.<sup>11</sup> While this technique is not detrimental to liposome integrity it does require additional purification *via* gel filtration or dialysis post centrifugation to remove the compound initially used to create the density gradient. All of these common purification processes suffer from poor recovery of the purified liposome product, long purification cycles, dilute liposome solutions, the requirement of specialized equipment, and can potentially damage the liposomal product.<sup>19</sup> Therefore, there is a clear need for a universal liposomal purification technique that efficiently removes a range of non-conjugated contaminants from functionalized liposomes without negatively impacting liposomal integrity or unnecessarily diluting the liposome solution.

Here, we describe an efficient purification technique for functionalized liposomes to allow for the inexpensive and rapid purification of diverse liposome formulations with high purity and recovery. The Liposome Extruder Purification (LEP) process utilizes the same components necessary for liposome formation *via* the extrusion method. This is the most commonly used method of liposome formation and only requires: an extruder, syringes, and track etched polycarbonate extrusion membranes.<sup>2</sup> To form liposomes using this method, lipids are mixed in their respective proportions in chloroform and then dried to form a lipid film. The film is then hydrated by adding an aqueous buffer, heated to a temperature above the lipid phase transition temperature ( $T_m$ ), and agitated. This temperature is determined by the lipid composition and can vary greatly between lipid formulations. The resulting large multilamellar vesicles (LMV) are then passed through a track etched polycarbonate membrane of a defined pore size while maintaining a temperature above the  $T_m$ . The process of forcing LMVs repeatedly through the membrane is known as extrusion and results in the formation of relatively monodisperse, small unilamellar liposome (SUV). The pore size and the number of

cycles the film is passed through the membrane dictate the ultimate liposome size and distribution.

The LEP process utilizes the same liposome extruder fitted with a membrane of a smaller pore diameter than the membrane used to form the liposomes and is carried out at temperatures below the lipid  $T_m$ . Performing the procedure below the  $T_m$  is essential as the fluidity of the lipid membrane is greatly reduced and the liposomes are unable to deform and pass through the membrane. Therefore, at temperatures below the  $T_m$  the membrane acts as a filter to concentrate the functionalized liposomes on the starting side of the membrane while smaller contaminants flow freely through the membrane. Unless otherwise noted, the LEP process was performed by loading the liposome sample into the starting syringe (250  $\mu$ L, Pre-LEP sample) which was then fitted to the extruder (Fig. 1A). Next, 245  $\mu$ L of the sample was passed through the membrane to a final sample volume of 5  $\mu$ L. The concentrated liposome solution was then brought back up to its initial volume of 250  $\mu$ L by flowing fresh buffer in the reverse direction through the membrane (Post-LEP sample) to complete one LEP cycle. Since the LEP technique removes contaminants by selective dilution of the contaminant the LEP process can be repeated as many times as necessary to attain the desired level of contaminant removal. This technique is simple to implement, reduces purification cost, and provides for the ability to rapidly purify liposomes from a wide array of contaminants ( $\sim 6$  min per LEP cycle).

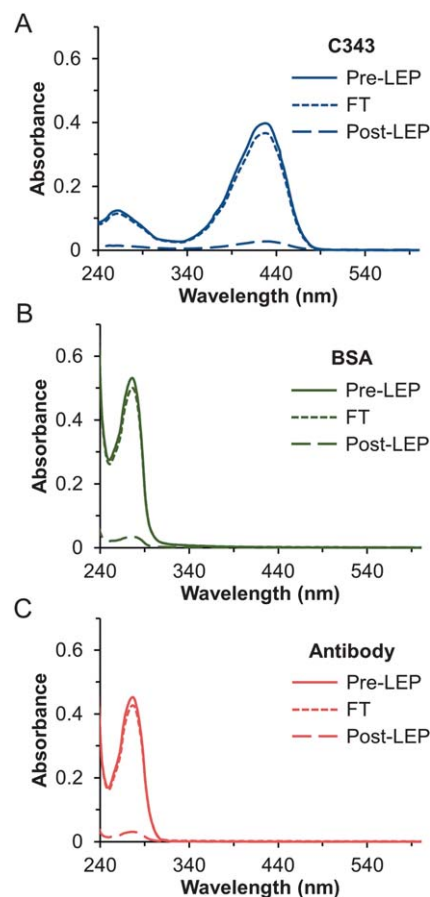


**Fig. 1** (A) Visual demonstration of the LEP procedure retaining fluorescein-labeled liposomes. (B) PEGylated liposomes were formed incorporating 1% carboxy-fluorescein-PE to aid in liposome visualization ( $\text{Abs}_{\text{max}} = 494$  nm). LEP was performed on a 250  $\mu$ L liposome sample and absorbance scans were performed Pre- and Post-LEP after the concentrate was diluted back to its original 250  $\mu$ L volume. Nearly no liposomes were observed in the FT fraction, as indicated by minimal absorbance at 494 nm, and a >94% liposome recovery was achieved Post-LEP. Experiments were conducted in triplicate, with representative absorbance scans shown.

The first step in validating the LEP technique was to determine the ability of a 50 nm membrane to retain 100 nm liposomes. For this experiment, LEP was performed on a 250  $\mu\text{L}$  solution of 100 nm diameter liposomes formulated as 94% HSPC, 5% PEG2000, 1% carboxy-fluorescein-PE and a 1 : 10 ratio of cholesterol : lipid. The carboxy-fluorescein lipid was included in the liposomal formulation to allow for visualization of the liposomes by absorbance at 494 nm to quantify liposomal recovery. After a single LEP cycle the liposome recovery (Post-LEP) was >94% (Fig. 1B). A LEP membrane of 50 nm was therefore selected as it was the largest pore diameter that was capable of retaining 100 nm liposomes and allowed for the greatest range of contaminants to pass through the membrane. This process was repeated using a 30 nm membrane and similar liposomal recovery was observed. However, large contaminants, such as intact antibodies (150 kDa), were not removed as efficiently due to the smaller pore diameter (data not shown).

We next evaluated the efficiency of the LEP method to remove contaminants. Three characteristic contaminants, ranging from small molecules to large proteins, were selected as examples to demonstrate the utility of the LEP process. Coumarin 343, a 285.29 Da fluorescent molecule, was selected based on its structure and relative size to mimic the removal of small molecules and chemotherapeutics from drug loaded liposomes (Fig. S1 in ESI†).<sup>2</sup> Bovine serum albumin (BSA), a 66 kDa protein, was selected as a medium sized protein to model removal of unconjugated antibody Fab and scFv, which are commonly conjugated to liposomes for targeting applications.<sup>1,12,21</sup> Finally, Trastuzumab, a 146 kDa pharmaceutical antibody, was selected as an example of removal of unbound, full length antibodies from liposome preparations.<sup>1,12,22,23</sup> For these experiments, LEP was performed on the contaminants to assess the maximum contaminant clearance through a 50 nm membrane (Fig. 2). LEP was performed by flowing 245  $\mu\text{L}$  of the 250  $\mu\text{L}$  sample through the membrane and then the sample was brought back up its original 250  $\mu\text{L}$  volume. Taking into account the dead volume present in the extrusion apparatus (10  $\mu\text{L}$ ) and the 5  $\mu\text{L}$  of sample remaining in the initial syringe, the theoretical maximum contaminant removal with each LEP cycle is 94%. As can be seen by absorbance scans of the Pre-LEP, Post-LEP, and flow through (FT) of the Coumarin 343 (32  $\mu\text{M}$ ), BSA (3  $\text{mg mL}^{-1}$ ) and Trastuzumab (1  $\text{mg mL}^{-1}$ ), all of the contaminants flow through the 50 nm membrane relatively unimpeded with  $93.3 \pm 0.24$ ,  $93.8 \pm 0.39$ , and  $93.7 \pm 0.49\%$  removal, respectively. These results demonstrated contaminant removal near the theoretical maximum of 94%.

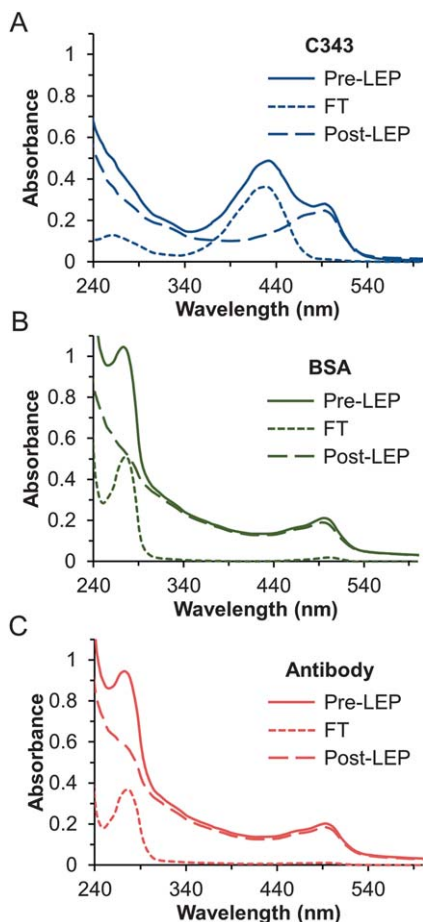
It was then necessary to demonstrate the ability of the LEP process to purify liposomes from mixed samples of contaminants and liposomes. For these experiments, standard extrusion techniques were used to form 100 nm liposomes (94% HSPC, 5% PEG2000, 1% carboxy-fluorescein-PE, 1 : 10 ratio of cholesterol : lipid) at 1 mM lipid concentration, which were then intentionally contaminated with 32  $\mu\text{M}$  Coumarin 343, 3  $\text{mg mL}^{-1}$  BSA or 1  $\text{mg mL}^{-1}$  Trastuzumab. The LEP process was carried out for each sample using a 50 nm membrane. Liposomes were concentrated to a volume of 5  $\mu\text{L}$  and then rehydrated to their original 250  $\mu\text{L}$  starting volumes.



**Fig. 2** (A) Removal of Coumarin 343 (32  $\mu\text{M}$ ,  $\text{Abs}_{\text{max}} = 427 \text{ nm}$ ), (B) BSA (3  $\text{mg mL}^{-1}$ ,  $\text{Abs}_{\text{max}} = 280 \text{ nm}$ ), and (C) Trastuzumab (1  $\text{mg mL}^{-1}$ ,  $\text{Abs}_{\text{max}} = 280 \text{ nm}$ ) contaminant through a 50 nm LEP membrane. Samples were prepared at an initial sample volume of 250  $\mu\text{L}$  and LEP was carried out to concentrate the samples to 5  $\mu\text{L}$ . Contaminant removal was calculated by comparison of the maximum absorbance for each contaminant Pre- and Post-LEP. Experiments were conducted in triplicate, with representative absorbance scans for each contaminant shown.

Absorbance scans of the Pre-LEP, FT, and Post-LEP samples were used to assess liposome recovery (494 nm) and contaminant clearance (Fig. 3). In all cases, >93% liposome recovery and >93% contaminant clearance was observed. Again, the contaminant removal was near the theoretical maximum of 94%. As expected, the yield of contaminant clearance in the presence of liposomes was slightly reduced when compared to contaminant clearance in the absence of liposome likely due to nonspecific interactions between the contaminants and the liposomes in solution. This is most evident in the case of Coumarin 343 as it is a hydrophobic small molecule capable of imbedding into the lipid bilayer. Adding the absorbance spectrums of the Post-LEP and FT nearly perfectly overlay with the Pre-LEP sample demonstrating very little loss of liposome or contaminant to the LEP apparatus (Fig. S2 in ESI†). Contaminant clearance results and total liposome recoveries are summarized in Table 1.

There are many characteristics of liposomes that can be adjusted depending upon their intended use. A few adjustable characteristics include: liposome size, percent PEG composition,



**Fig. 3** Absorbance scans of Pre-LEP sample (solid line), flow through (FT, short dashed line) and Post-LEP sample (long dashed line) for mixed 100 nm carboxy-fluorescein-PE labeled liposomes and the following contaminants: (A) Coumarin 343 at 32  $\mu\text{M}$ , (B) BSA at 3  $\text{mg mL}^{-1}$ , and (C) Trastuzumab at 1  $\text{mg mL}^{-1}$ . Contaminant removal and liposomal recovery was calculated by comparison of the maximum absorbance for each component Pre- and Post-LEP. Experiments were conducted in triplicate, with representative absorbance scans for each purification shown.

PEG length, incorporation of lipids with terminal reactive groups, incorporation of peptide conjugated targeting lipids, incorporation of hydrophobic molecules within the bilayer, loading of water soluble components within the liposome core, and the type of lipids used to form the liposomes.<sup>24</sup> To demonstrate the utility and breadth of the LEP technique,

**Table 2** Recovery of various liposome formulations after one LEP cycle

Sample	Liposome size (nm)	PEG (%)	PEG MW (Da)	Membrane (nm)	Liposome recovery (%)
1	100	0	0	50	97.58 $\pm$ 1.28
2	100	5	350	50	97.43 $\pm$ 0.37
3	100	5	1000	50	97.93 $\pm$ 0.40
4	100	5	2000	50	94.37 $\pm$ 2.90
5 <sup>a</sup>	100	5	2000	50	96.01 $\pm$ 4.81
6	100	1	2000	50	97.59 $\pm$ 1.02
7	100	5	2000	30	96.84 $\pm$ 1.11
8	50	5	2000	30	87.12 $\pm$ 1.55

<sup>a</sup> Indicates three LEP cycles.

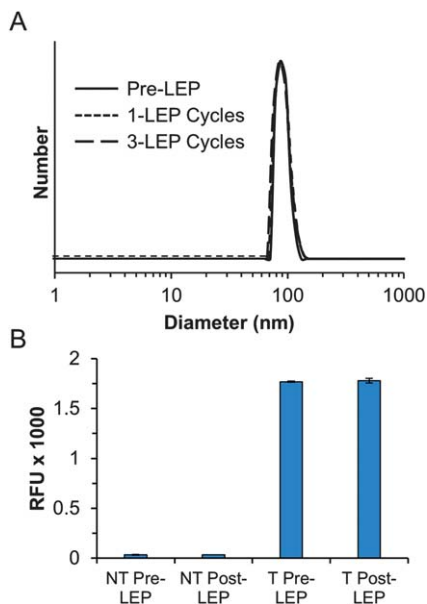
different liposome formulations of variable diameter, PEG length, and PEG coating percentage were tested to assess liposome recovery. As can be seen in Table 2, there is >94% recovery of the diverse liposome formulations after a single LEP cycle. A >96% liposome recovery was also attained after performing 3 LEP cycles, demonstrating a contaminant reduction of 2750–4100 fold (Fig. S3 in ESI†). It is important to note that each liposome formulation and contaminant must be independently tested to ensure the LEP technique is effective for each unique liposome application. In the case of 50 nm liposomes, a 30 nm membrane was used to perform LEP and the recovery was reduced to 87% due to the proximity in size of the liposomes to the LEP membrane pore diameter.

It is critical that the purification technique employed is not detrimental to the liposomes and that the process does not affect their ability to carry out their designated function. A significant indicator of liposome integrity is its hydrodynamic diameter which can be determined by dynamic light scattering (DLS). Common problems associated with liposome purification are aggregation or a reduction in liposome size due to excessive stress on the bilayer, both of which can be assessed *via* DLS. DLS, which was performed to verify that liposomal integrity was maintained after multiple LEP cycles were performed, indicated no change to the liposomal hydrodynamic diameter (Fig. 4A). DLS was also carried out on all liposome formulations before and after LEP and in all cases the process had no effect on the purified liposome size distributions (Fig. S4 in ESI†).

To further verify that the LEP process was not detrimental to functionalized liposomes, an *in vitro* cellular uptake assay was performed with ligand-targeted and non-targeted liposomes. A

**Table 1** Liposome recovery and contaminant removal of mixed liposome and contaminant samples

	Contaminant	Liposome recovery (%)	Contaminant removal (%)	Contaminant (fold reduction)
No liposome	C343	—	93.36 $\pm$ 0.24	15.1
	BSA	—	93.83 $\pm$ 0.39	16.2
	Trastuzumab	—	93.70 $\pm$ 0.49	15.9
Liposome	C343	93.79 $\pm$ 0.50	92.90 $\pm$ 1.24	14.1
	BSA	91.45 $\pm$ 1.12	93.66 $\pm$ 1.39	15.8
	Trastuzumab	92.19 $\pm$ 3.24	93.52 $\pm$ 0.85	15.4



**Fig. 4** (A) Liposome size distribution Pre- and Post-LEP as determined by dynamic light scattering (DLS). Liposomes were formed *via* extrusion through a 100 nm pore size membrane and LEP was carried out using a 50 nm pore size membrane. (B) *In vitro* cellular uptake assay using targeted liposomes (T) Pre- and Post-LEP compared to non-targeted control liposomes (NT) as determined *via* flow cytometry (data represents mean  $\pm$  SD of triplicate experiments).

cyclic peptide sequence targeting Very Late Antigen-4 (VLA-4) was conjugated to a lipid molecule and directly incorporated into the liposomes during nanoparticle formation for the preparation of targeted-liposomes.<sup>25,26</sup> Liposomes were extruded and subjected to the LEP process using a 50 nm membrane. Because the targeting moiety was directly incorporated into the liposome construct, LEP was performed only to determine its effect on liposomal targeting and not for purification purposes. Liposomes were then incubated with VLA-4 overexpressing NCI-H929 myeloma cells, and cellular uptake was evaluated by flow cytometry. As can be seen in Fig. 4B, no change was observed in the cellular uptake of the Pre- or Post-LEP, targeted or non-targeted liposomes, demonstrating that LEP did not negatively affect liposomal targeting. Absorbance spectrums for the targeted and non-targeted Pre- and Post-LEP samples can be found in Fig. S5 in ESI†. Combined, these results support the conclusion that LEP purification does not adversely affect liposomal integrity or targeting.

The LEP process can also be utilized to concentrate dilute liposomal samples. This is a useful application of the LEP process since many conjugation methods cause dilution of the liposome sample. While concentrating liposome samples can also be achieved through centrifugation the technique requires expensive equipment and as previously discussed the process can be detrimental to liposomal integrity. When performing LEP to concentrate liposome samples the degree of sample concentrating is directly dictated by the initial and final sample volumes selected. As a demonstration, a 0.2 mM lipid sample in 1 mL of buffer was concentrated 10 fold to a final volume of 100  $\mu$ L. This was accomplished by performing LEP on the

sample down to 5  $\mu$ L and then flowing 95  $\mu$ L of buffer back through the membrane. The LEP concentrating resulted in an average  $8.3 \pm 0.09$  fold increase in concentration with an overall liposome recovery of  $82.9 \pm 0.92\%$  (Fig. S6 in ESI†). The liposome sample can be concentrated further by flowing less buffer back through the membrane during the sample recovery step, however, reducing this volume will result in reduced liposomal recovery. In some instances sample purification and liposome concentrating can be combined in the same assay quickly and easily without the need of multiple techniques or additional sample handling.

As described here, the LEP method allows for the inexpensive and rapid purification of functionalized liposomes. Implementation of the LEP process in the development of complex liposomal formulations can increase throughput to more rapidly test various formulations allowing for faster selection of liposome designs that exhibit the desired characteristics. Utilizing the LEP technique, contaminants as large as intact antibodies can be efficiently separated from liposomes with a >93% liposome recovery and contaminant clearance that approaches the maximum theoretical clearance of 94% in a single process. In cases where larger quantities of functionalized liposomes are needed, the LEP technique can be scaled up by increasing the syringe volumes, increasing the lipid concentration in the sample or by employing a larger extruder system. For industrial and pharmaceutical applications, where functionalized liposome preparation and purification are performed on a very large scale, LEP can be implemented as a quality control validation method to determine liposomal product purity throughout the manufacturing process. While all experiments demonstrated here were performed manually, the LEP process can also be carried out incorporating syringe pumps to allow for constant flow rates to provide for a more consistent LEP process. The described technique can be nearly universally applied across many liposome formulations, for the efficient and economical purification of liposomes.

## Experimental details

Please see the ESI† section for a list of reagents and detailed experimental procedures.

## Acknowledgements

The authors thank the Center for Environmental Science and Technology at the University of Notre Dame for usage of the DLS. This work was supported by the NSF (grant award number CBET-1263713).

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