Supporting Information: Evaluating Methods to Create Protein Functionalized Catanionic Vesicles

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I. Protein-Functionalization Studies

BCA Assay Data

A. Representative BCA Assay Data of Vesicle formulations.

The following is representative BCA assay data depicting results of vesicle samples prepared and analyzed throughout the study. Standard solutions of acid phosphatase in PBS (1X, pH =7.4) were prepared from solid acid phosphatase. Vesicle samples were purified via SEC with PBS (1X, pH =7.4) as the eluent before vesicle samples were analyzed. Absorbance values were measured at 562 nm and were corrected by subtracting the PBS blank. The enhanced test tube BCA assay protocol was used.

Table 1: Absorbance values at 562 nm from a BCA Assay

Sample	Raw Absorbance at 562 nm	Corrected Absorbance at 562 nm (PBS blank subtracted)
PBS blank	0.150	0.000
12.5 µg/mL standard	0.218	0.068
25 µg/mL standard	0.249	0.099
50 µg/mL standard	0.321	0.171
100 µg/mL standard	0.484	0.334
200 µg/mL standard	0.734	0.584
TX100 sample	0.181	0.031
TXAP sample	0.188	0.038
ETXAP sample-Trial 1	0.271	0.121
ETXAP sample-Trial 2	0.242	0.092

Sample Key

TX100 = Triton X-100 enriched vesicles

TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase—Trials 1 and 2

Figure 1: Standard curve generated from the BCA assay data in Table 1. Absorbance was measured at 562 nm.

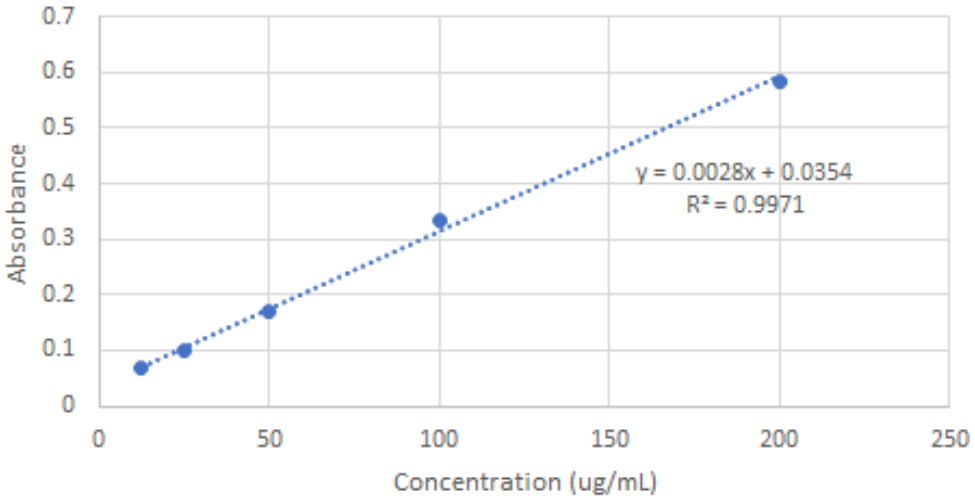


Figure 2: Absorbance spectra of acid phosphatase standard solutions.

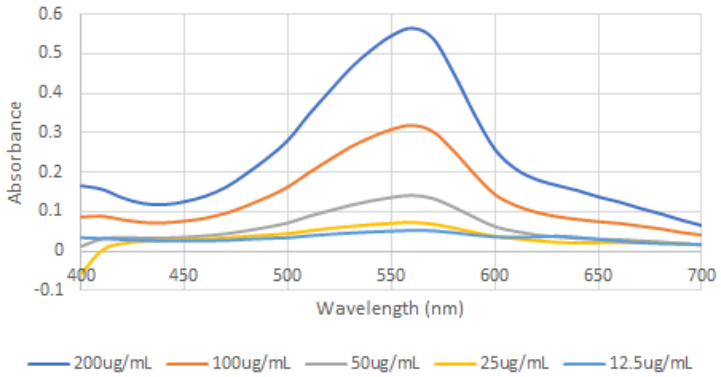


Figure 3: Absorbance spectra of acid phosphatase standard solutions and sample solutions.

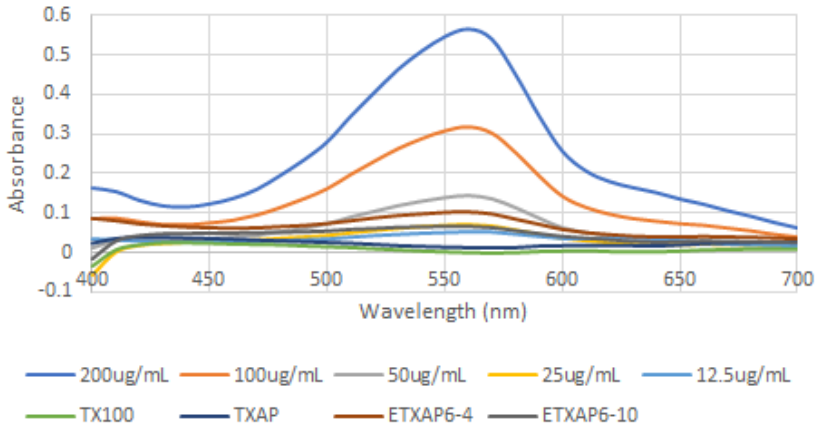
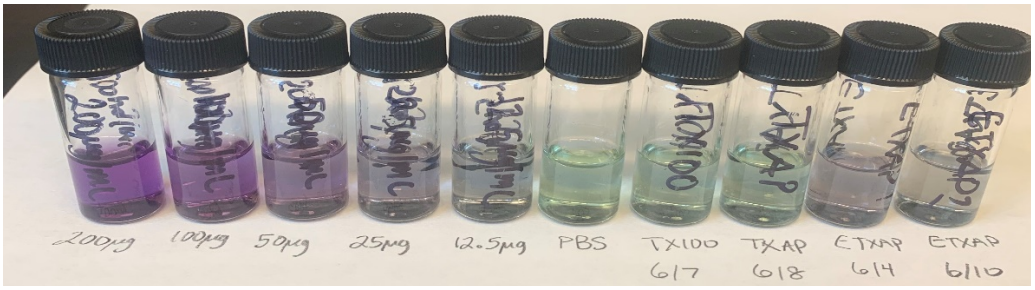


Figure 4: Picture of acid phosphatase standard solutions and sample solutions after BCA assay development.



During the BCA assay, samples that contain protein undergo a color change from green to purple. The purple color intensifies with increasing protein concentrations. The 12.5, 25, 50, 100, and 200 µg markings refer to standard solutions of 12.5, 25, 50, 100, and 200 µg/mL. The sample marked "PBS" is the PBS blank sample.

Sample Key and Concentration Calculations

TX100 = Triton X-100 enriched vesicles

0.031 = 0.0028x + 0.0354 = -1.57ug/mL (Below LOD)

TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase

0.038 = 0.0028x + 0.0354 = 0.92ug/mL (Below LOD)

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase—Trial 1

0.121 = 0.0028x + 0.0354 = 30.6 ug/mL

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase—Trial 2

0.092 = 0.0028x + 0.0354 = 20.2 ug/mL

Control Experiment: BCA Assay of Esterified Triton X-100 enriched Vesicles Without Acid Phosphatase

To ensure esterified Triton X -100 functionalized vesicles do not interfere with the BCA assay, a control experiment was conducted: esterified Triton X -100 functionalized vesicles that were not mixed with acid phosphatase were analyzed by the BCA assay. The results of that study are below.

Figure 5: Standard curve generated from the BCA assay

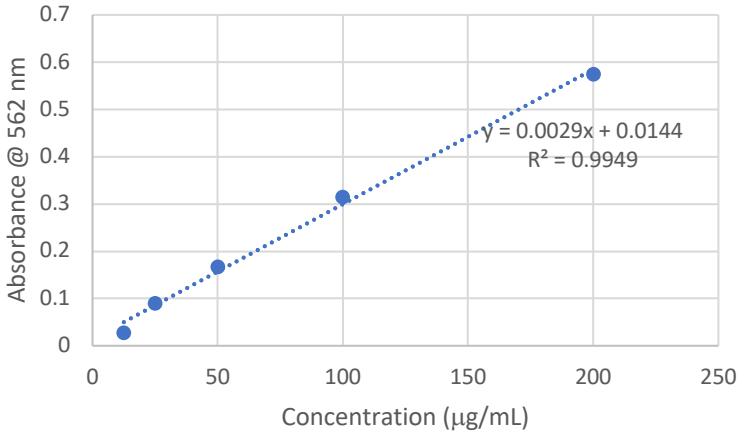


Figure 6: Absorbance spectra of acid phosphatase standard solutions and sample solutions.

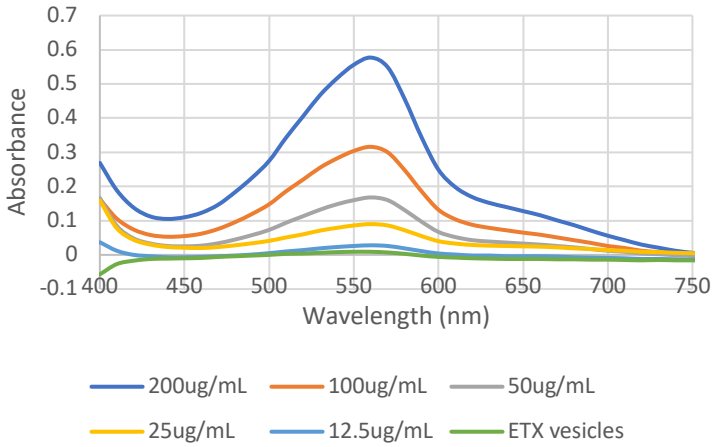
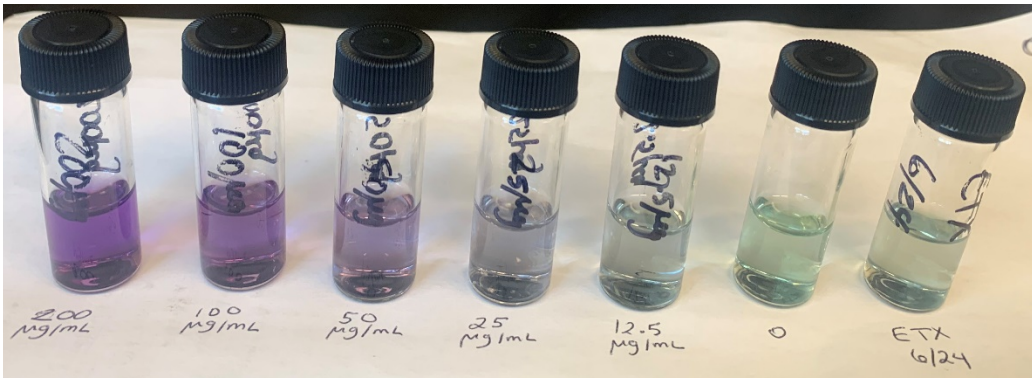


Figure 7: Picture of acid phosphatase standard solutions and sample solution after BCA assay development.



During the BCA assay, samples that contain protein undergo a color change from green to purple. The purple color intensifies with increasing protein concentrations. The sample marked with "0" is the PBS blank.

Sample Key and Concentration Calculation

ETX = Esterified Triton X-100 vesicles with NO acid phosphatase added.

0.009 = 0.0029x + 0.0144 = -1.86ug/mL (Below LOD)

B. Triton X-100 enriched vesicles mixed with acid phosphatase

To demonstrate that acid phosphatase does not nonspecifically bind to Triton X-100 enriched vesicles, Triton X-100 enriched vesicles were mixed with acid phosphatase and the vesicles were then purified by SEC. The purified vesicles were then analyzed for protein content via the BCA assay. The results of 6 trials are below.

Table 2: **BCA assay data from Trial 1 of Triton X-100 enriched vesicles mixed with acid phosphatase**

Sample	Corrected Absorbance at 562 nm (PBS blank subtracted)
PBS blank	0.000
12.5 µg/mL standard	0.068
25 µg/mL standard	0.099
50 µg/mL standard	0.171
100 µg/mL standard	0.334
200 µg/mL standard	0.584
TX100 sample	0.031
TXAP sample	0.038

Sample Key

TX100 = Triton X-100 enriched vesicles
TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase

Note: this is the sample data from Table 1. See Figures 1-4 for graphical data.

Table 3: BCA assay data from Trial 2 of Triton X-100 enriched vesicles mixed with acid phosphatase

Sample	Raw Absorbance at 562 nm	Corrected Absorbance at 562 nm (PBS blank subtracted)
Phosphatase (200 µg/mL)	0.703	0.520
Phosphatase (100 µg/mL)	0.537	0.354
Phosphatase (50 µg/mL)	0.352	0.169
Phosphatase (25 µg/mL)	0.240	0.057
PBS	0.183	0.000
TX100 sample	0.162	-0.021
TXAP sample	0.195	0.012

Sample Key

TX100 = Triton X-100 enriched vesicles
TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase

Figure 8: Standard curve from Trial 2 of Triton X-100 enriched vesicles mixed with acid phosphatase

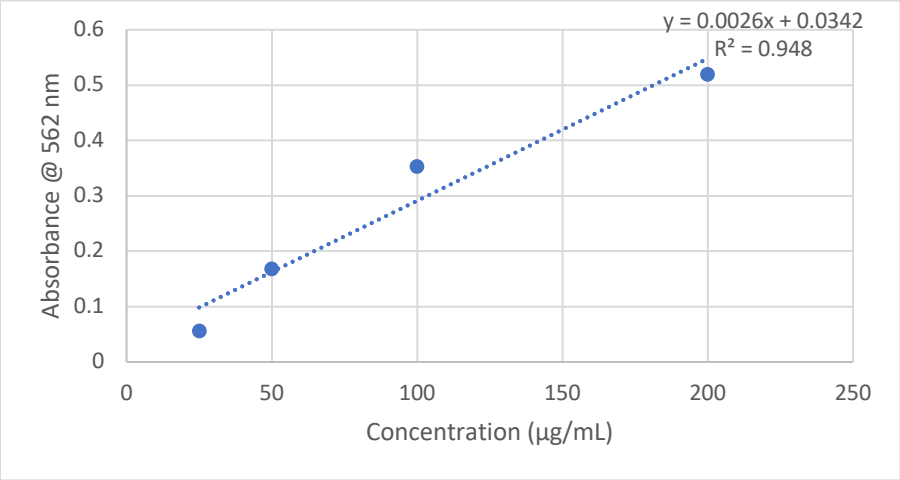


Table 3: BCA assay data from Trial 3 of Triton X-100 enriched vesicles mixed with acid phosphatase

Sample	Raw Absorbance at 562 nm	Corrected Absorbance at 562 nm (PBS blank subtracted)
Phosphatase (200 µg/mL)	0.796	0.615
Phosphatase (100 µg/mL)	0.530	0.349
Phosphatase (50 µg/mL)	0.356	0.175
Phosphatase (25 µg/mL)	0.270	0.089
PBS	0.181	0.000
TX100 sample	0.207	0.026
TXAP sample	0.219	0.038

Sample Key

TX100 = Triton X-100 enriched vesicles
TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase

Figure 9: Standard curve from Trial 3 of Triton X-100 enriched vesicles mixed with acid phosphatase

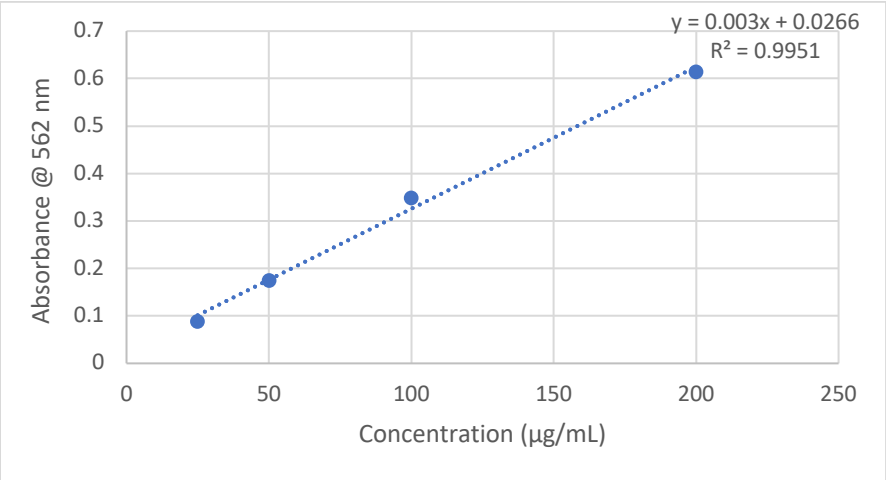


Table 4: BCA assay data from Trials 4-6 of Triton X-100 enriched vesicles mixed with acid phosphatase

	Sample	Trial 4	Trial 5	Trial 6
Raw Absorbance Values at 562 nm	PBS	0.178	0.187	0.206
	TX100 sample	0.206	0.202	0.204
	TXAP sample	0.242	0.239	0.234
	200 µg/mL	0.836	0.824	0.797
	100 µg/mL	0.529	0.571	0.545
	50 µg/mL	0.393	0.385	0.381
	25 µg/mL	0.288	0.277	0.233

Sample Key

TX100 = Triton X-100 enriched vesicles
TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase

Figure 10: Standard curve from Trial 4 of Triton X-100 enriched vesicles mixed with acid phosphatase

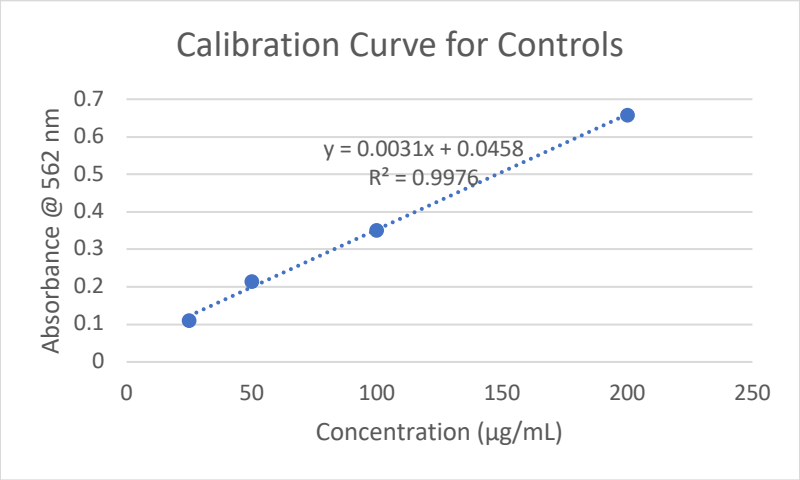


Figure 11: Standard curve from Trial 5 of Triton X-100 enriched vesicles mixed with acid phosphatase

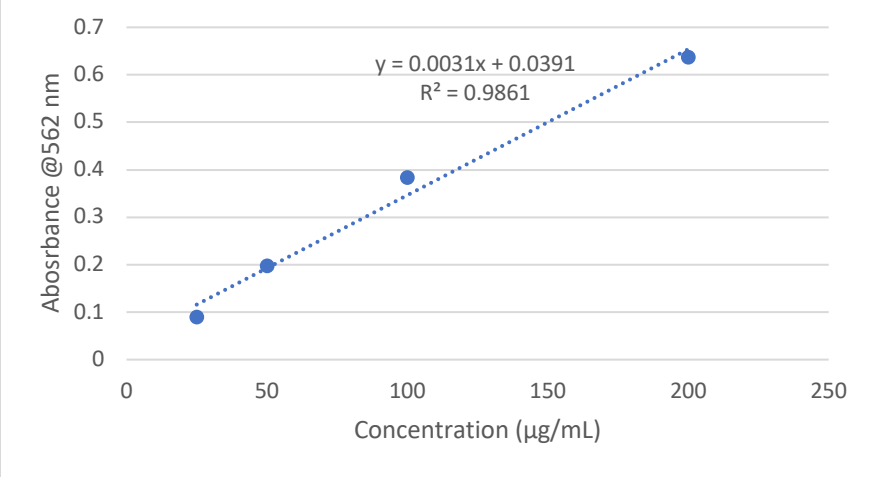
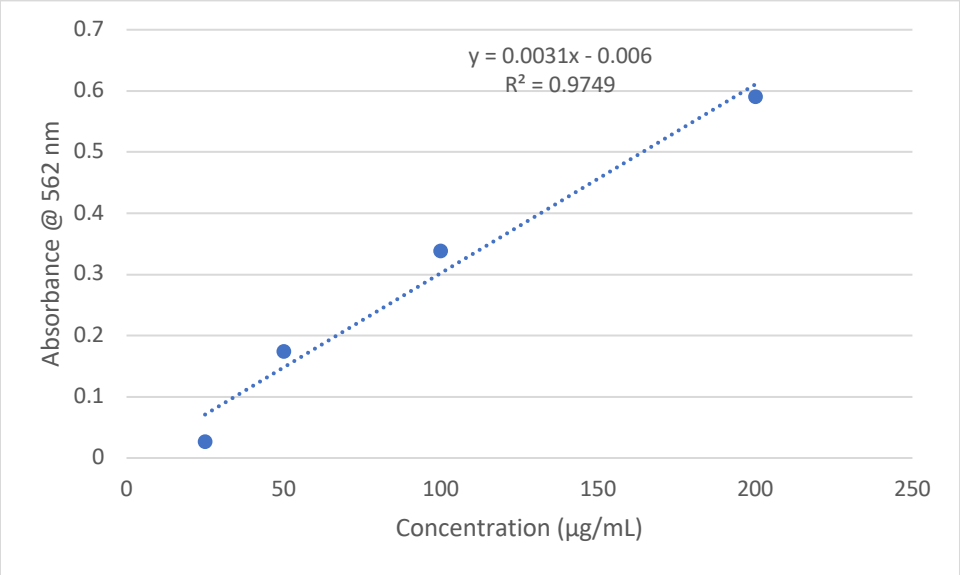


Figure 12: Standard curve from Trial 6 of Triton X-100 enriched vesicles mixed with acid phosphatase



Calculated concentration of acid phosphatase in Triton X-100 enriched vesicles mixed with acid phosphatase

Trial 1: 0.92 µg/mL
Trial 2: -8.5 µg/mL
Trial 3: 3.8 µg/mL
Trial 4: 5.9 µg/mL
Trial 5: 4.1 µg/mL
Trial 6: 11 µg/mL

Average: 2.9 +/- 6.5 µg/mL

Average with trial 2 = 0: 4.2 +/- 3.9 µg/mL

C. Maleimide-Functionalized Vesicles Mixed with Acid Phosphatase

DSPE-PEG-Maleimide was incorporated into vesicles. Maleimide-functionalized vesicles were mixed with acid phosphatase and purified by SEC. The purified vesicles were then analyzed for protein content via the BCA assay. The results of 5 trials are below.

Table 5: BCA assay data from Trials 1-4 of maleimide functionalized vesicles mixed with acid phosphatase

	Sample	Trial 1	Trial 2	Trial 3	Trial 4
Corrected Absorbance Values at 562 nm	25 µg/mL	0.038	0.078	0.096	0.077
	50 µg/mL	0.079	0.145	0.234	0.2
	100 µg/mL	0.407	0.249	0.366	0.329
	200 µg/mL	0.545	0.436	0.638	0.625
	Maleimide-AP sample	-0.028	0.013	0.044	0.054

Figure 13: Standard curve from Trial 1 of maleimide functionalized vesicles mixed with acid phosphatase

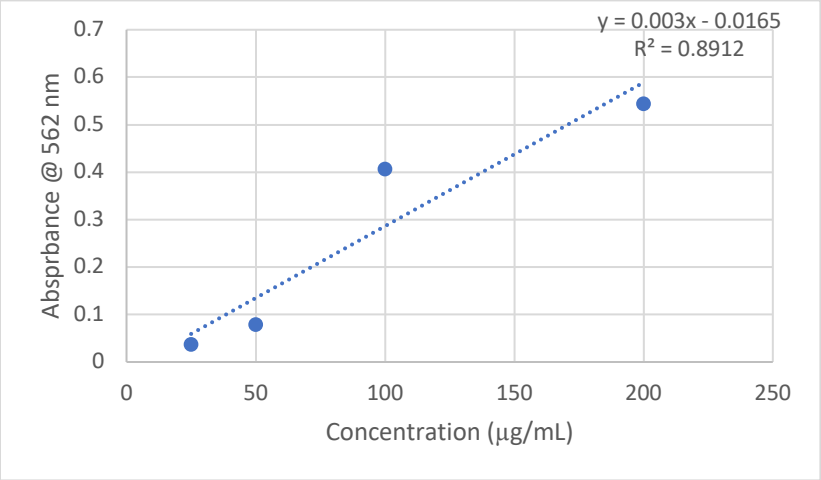


Figure 14: Standard curve from Trial 2 of maleimide functionalized vesicles mixed with acid phosphatase

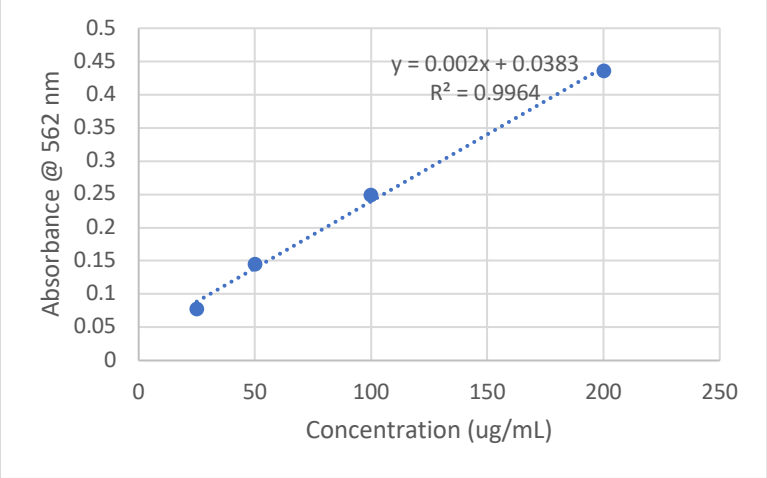


Figure 15: Standard curve from Trial 3 of maleimide functionalized vesicles mixed with acid phosphatase

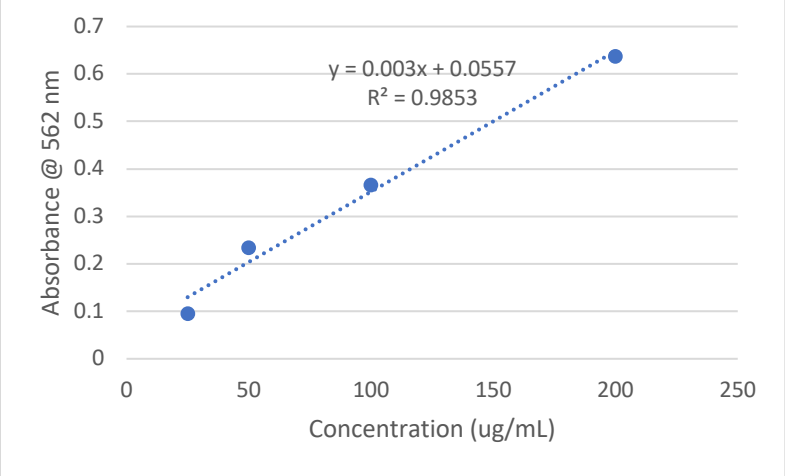


Figure 16: Standard curve from Trial 4 of maleimide functionalized vesicles mixed with acid phosphatase

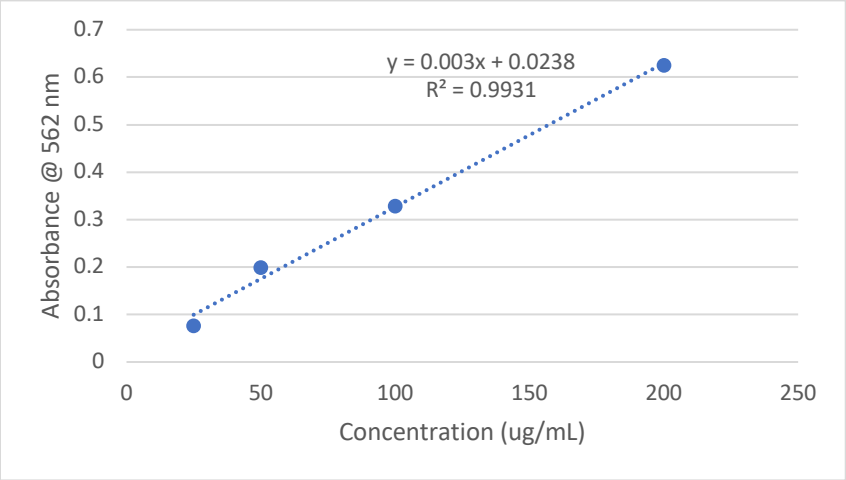
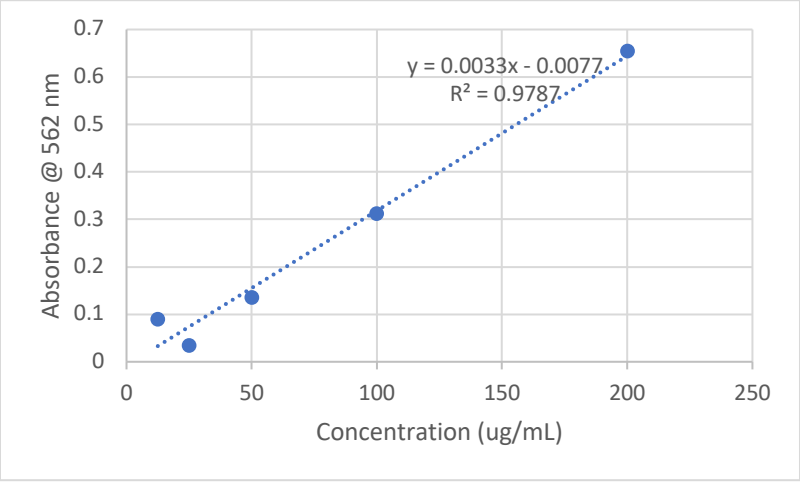


Table 6: BCA assay data from Trial 5 of maleimide functionalized vesicles mixed with acid phosphatase

Sample	Corrected Absorbance at 562 nm (PBS blank subtracted)
12.5 µg/mL	0.090
25 µg/mL	0.034
50 µg/mL	0.135
100 µg/mL	0.313
200 µg/mL	0.654
Maleimide-AP sample	0.005

Figure 17: Standard curve from Trial 5 of maleimide functionalized vesicles mixed with acid phosphatase



Calculated concentration of acid phosphatase in maleimide-functionalized vesicles mixed with acid phosphatase

Trial 1: -3.8 µg/mL
Trial 2: -12.7 µg/mL

Trial 3: -3.9 µg/mL
Trial 4: 10.1 µg/mL
Trial 5: 3.8 µg/mL

Average concentration +/- standard deviation: -1.3 +/- 8.6 µg/mL

Average and standard deviation of five trials with concentrations of trials 1, 2 and 3 = 0: 3 $\pm$ 4 µg/mL

D. Esterified Triton X-100 Vesicles Mixed with Acid Phosphatase

Esterified Triton X-100 was incorporated into vesicles. Functionalized vesicles were mixed with acid phosphatase and purified by SEC. The purified vesicles were then analyzed for protein content via the BCA assay. The results of 6 trials are below.

Table 7: BCA assay data from Trials 1-2 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase

Sample	Raw Absorbance at 562 nm	Corrected Absorbance at 562 nm (PBS blank subtracted)
PBS blank	0.150	0.000
12.5 µg/mL standard	0.218	0.068
25 µg/mL standard	0.249	0.099
50 µg/mL standard	0.321	0.171
100 µg/mL standard	0.484	0.334
200 µg/mL standard	0.734	0.584
ETXAP sample-Trial 1	0.271	0.121
ETXAP sample-Trial 2	0.242	0.092

Sample Key

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase—Trials 1 and 2
Note: this is the same data from Table 1. See Figures 1-4 for graphical data.

Table 8: BCA assay data from Trial 3 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase

Sample	Absorbance @562nm	Corrected Absorbance
200 µg/mL standard	0.595	0.434
100 µg/mL standard	0.413	0.252
50 µg/mL standard	0.285	0.124
25 µg/mL standard	0.215	0.054
12.5 µg/mL standard	0.182	0.021
PBS	0.161	0
TX100	0.179	0.018
ETXAP—Trial 3	0.227	0.066

Sample Key

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase
TX100 = Triton X-100 enriched vesicles

Figure 18: Standard curve from Trial 3 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase

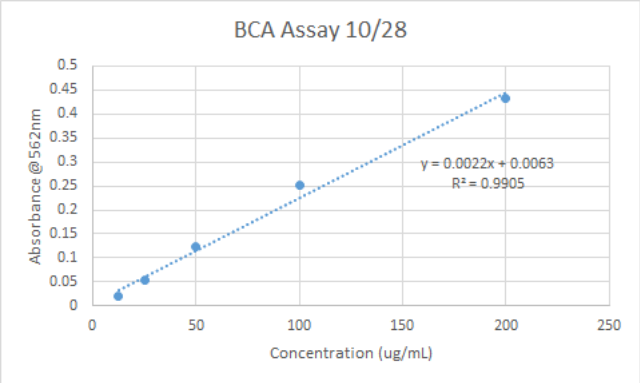


Table 9: BCA assay data from Trials 4-5 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase

Sample	Corrected Absorbance at 562 nm (PBS blank subtracted)
25 µg/mL standard	0.317
50 µg/mL standard	0.401
100 µg/mL standard	0.654
200 µg/mL standard	1.091
ETXAP sample-Trial 4	0.340
ETXAP sample-Trial 5	0.407

Sample Key

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase

Figure 19: Standard curve from Trials 4-5 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase

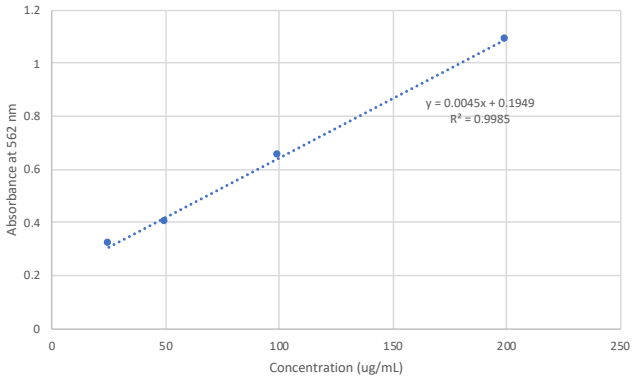


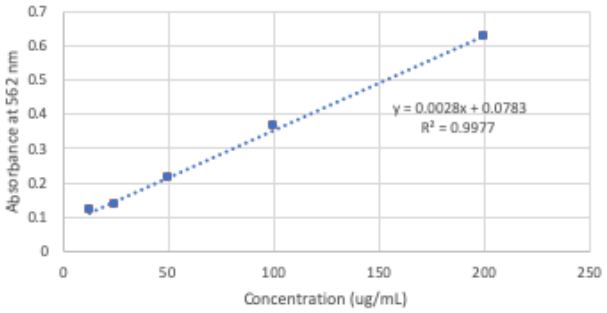
Table 10: BCA assay data from Trial 6 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase

Sample	Corrected Absorbance at 562 nm (PBS blank subtracted)
12.5 µg/mL standard	0.122
25 µg/mL standard	0.135
50 µg/mL standard	0.212
100 µg/mL standard	0.365
200 µg/mL standard	0.625
ETXAP sample-Trial 6	0.157

Sample Key

ETXAP = Esterified Triton X-100 enriched vesicles mixed with acid phosphatase

Figure 20: Standard curve from Trial 6 of esterified Triton X-100 enriched vesicles mixed with acid phosphatase



Calculated concentration of acid phosphatase in esterified Triton X-100-functionalized vesicles mixed with acid phosphatase. These samples are also called AP vesicles.

Trial 1: 30.6 µg/mL
Trial 2: 20.2 µg/mL
Trial 3: 27.1 µg/mL
Trial 4: 32.2 µg/mL
Trial 5: 47.1 µg/mL
Trial 6: 28.1 µg/mL

Average of 6 trials: 30.8 µg/mL

Standard deviation of 6 trials: 8.9 µg/mL

E. Bare Vesicles Mixed with Acid Phosphatase

Bare vesicles were prepared, purified and mixed with acid phosphatase. After a second round of purification via SEC, vesicle samples were analyzed via the BCA assay. The results of two trials are below.

Table 11: BCA assay data from trial 1 of bare vesicles mixed with acid phosphatase.

Sample	Absorbance @562nm	Corrected
200	1.517	1.359
100	1.022	0.864
50	0.669	0.511
25	0.417	0.259
12.5	0.313	0.155
0	0.158	0
Bare w/ AP	0.21	0.052

Table 12: BCA assay data from trial 2 of bare vesicles mixed with acid phosphatase.

Sample	Abosrbance @562nm	Corrected
200	0.772	0.638
100	0.492	0.358
50	0.344	0.21
25	0.223	0.089
12.5	0.182	0.048
0	0.134	0
Bare w/ AP	0.157	0.023

Sample Key

Bare w/ AP = bare vesicles mixed with acid phosphatase

Calculated concentration of acid phosphatase in bare vesicles mixed with acid phosphatase

Trial 1:

$0.052=0.0064x + 0.1356$

$X = -13$ µg/mL => 0 µg/mL

Trial 2:

$0.023=0.0031x+0.0265$

$X = -1.1$ µg/mL => 0 µg/mL

F. Electrostatic anchors

Acid phosphatase was labelled with cationic moieties that could be used to anchor the proteins to the surface of anionic vesicles. Acid phosphatase was labelled with a quaternary ammonium group and Rhodamine B. See Table 1, entries 3 and 4, in the article for the bifunctional linkers used to label acid phosphatase with cationic groups. Labelled acid phosphatase was mixed with pre-formed, purified anionic vesicles. Vesicles were then purified by SEC. The purified vesicles were then analyzed for protein content via the BCA assay. The results of the BCA assays are below.

Table 13: BCA assay data and calculated protein concentration from cation-labelled acid phosphatase mixed with preformed anionic vesicles.

Sample	Corrected Absorbance at 562 nm (PBS blank subtracted)	Calculated Protein Concentration (μg/mL)
$N(CH_3)_4^+$-AP in BV—Trial 1	0.0245	12.5
$N(CH_3)_4^+$-AP in BV—Trial 2	-0.0168	0
Rhodamine B-AP in BV	-0.0362	0

Sample Key

$N(CH_3)_4^+$-AP in BV = quaternary ammonium labelled acid phosphatase mixed with purified bare anionic vesicles.
Rhodamine B-AP in BV = Rhodamine B labelled acid phosphatase mixed with purified bare anionic vesicles.

II. Acid Phosphatase Assay Results

A. Percent of attached protein that retained enzymatic activity

Once conjugated to vesicles, the enzymatic activity of acid phosphatase was measured using a standard acid phosphatase assay with 4-nitrobenzene phosphate as the substrate. Activity was monitored by measuring the absorbance of the product (4-nitrophenol) generated by the enzymatic hydrolysis of the phosphate substrate. Absorbances were normalized by subtracting the absorbance values of negative samples—samples that contain the substrate and buffer but no enzyme. The percentage of enzyme that remained functional after conjugation was calculated based on the ratio of normalized absorbance values of vesicle samples to positive control samples and the concentration of protein in the samples (based on BCA assay results).

Table 14: Acid phosphatase assay data from analysis of acid phosphatase functionalized vesicles.

Sample	Absorbance @ 410 nm	Normalized Absorbance	Vesicle Sample : Positive Sample Ratio
negative	0.07	0	
positive (30 μg/mL)	0.546	0.476	
AP vesicles —Trial 3	0.124	0.054	0.113445378

Sample Key

Negative = negative control sample

Positive = Positive control sample = solution of acid phosphatase in PBS.

AP vesicles = acid phosphatase functionalized vesicles.

AP vesicles are esterified Triton X-100 enriched vesicles mixed with acid phosphatase (ETXAP). AP vesicles—Trial 3 is the same as ETXAP—Trial 3.

See Table 7 above for BCA assay data. BCA analysis indicated that sample contained 27 μg/mL of acid phosphatase.

Sample calculations to determine the amount of functional protein attached to vesicles

$$30 \times 0.113445 = 3.40336$$

$$\frac{3.40336}{27} \times 100 = 12.6\% \text{ functional protein}$$

Table 15: Acid phosphatase assay data from analysis of acid phosphatase functionalized vesicles.

Sample	Absorbance @ 410 nm	Normalized Absorbance	Vesicle Sample:Positive Sample Ratio
Negative	0.016	0	
Positive (50 μg/mL)	0.6762	0.6602	
AP vesicles -Trial 4	0.0737	0.0577	0.087397758
AP vesicles -Trial 5	0.1121	0.0961	0.145561951

Sample Key

Negative = negative control sample

Positive = Positive control sample = solution of acid phosphatase in PBS.

AP vesicles = acid phosphatase functionalized vesicles.

AP vesicles are esterified Triton X-100 enriched vesicles mixed with acid phosphatase (ETXAP). AP vesicles—Trial 4 is the same as ETXAP—Trial 4. AP vesicles—Trial 5 is the same as ETXAP—Trial 5.

See Table 8 above for BCA assay data. BCA analysis indicated that Trial 4 contained 32 μg/mL of acid phosphatase and Trial 5 contained 47 μg/mL.

Table 16: Acid phosphatase assay data from analysis of acid phosphatase functionalized vesicles.

Sample	Absorbance @ 410 nm	Normalized Absorbance	Vesicle Sample : Positive Sample Ratio
negative	0.0192	0	
positive (50 μg/mL)	0.54487	0.52567	
AP vesicles -Trial 6	0.07371	0.05451	0.103696235

Sample Key

Negative = negative control sample

Positive = Positive control sample = solution of acid phosphatase in PBS.

AP vesicles = acid phosphatase functionalized vesicles.

AP vesicles are esterified Triton X-100 enriched vesicles mixed with acid phosphatase (ETXAP). AP vesicles—Trial 6 is the same as ETXAP—Trial 6.

See Table 9 above for BCA assay data. BCA analysis indicated that Trial 4 contained 28 μg/mL of acid phosphatase.

Calculated percent of functional protein attached to vesicles.

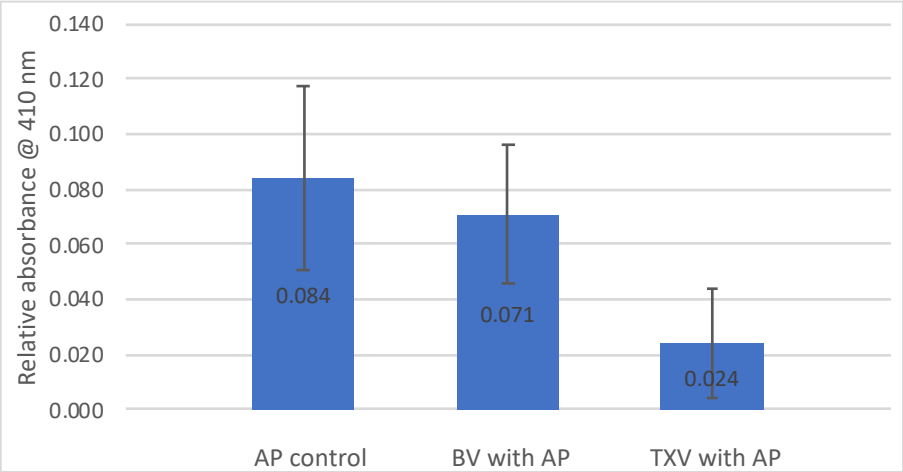
AP vesicles —Trial 3: 12.6%
AP vesicles —Trial 4: 13.7%
AP vesicles —Trial 5: 15.5%
AP vesicles —Trial 6: 18.5%

Average percent of functional protein attached to vesicles: 15.1%

B. Statistical analysis of acid phosphatase assay data: comparing acid phosphatase standard solutions to mixtures of acid phosphatase with vesicles.

Mixtures of vesicles and acid phosphatase were analyzed via the acid phosphatase to determine if the presence of vesicles interferes with the assay. The data indicated that the presence of Triton X-100 enriched vesicles may be interfering with the assay. Below is the statistical analysis of acid phosphatase standard solutions (positive control samples of acid phosphatase in PBS) and solutions of acid phosphatase mixed with Triton X-100 enriched vesicles. The concentration of acid phosphatase in all samples was 50 μg/mL.

Figure 21: Effect of vesicles on the acid phosphatase assay



Sample key: AP control = acid phosphatase control sample; BV with AP = bare vesicles mixed with acid phosphatase; TXV with AP: Triton X-100 enriched vesicles mixed with acid phosphatase. Average values and standard deviation are represented. For AP control samples, n = 4; Mixtures of bare vesicles with acid phosphatase (BV with AP) and mixtures of Triton X-100 enriched vesicles with acid phosphatase (TXV with AP) show diminished activity compared to samples that do not contain vesicles (AP control). The difference between control samples and Triton X-100 enriched vesicles mixed with acid phosphatase (TXV with AP) was significant at the 99% confidence level. All samples contained 50 μg/mL of acid phosphatase and were analyzed in triplicate. Negative control samples (samples containing the substrate but no acid phosphatase) were used to normalize absorbance values.

Table 17: Acid phosphatase assay data for comparing acid phosphatase solutions to mixtures of acid phosphatase and Triton X-100 enriched vesicles.

Entry	Sample	Normalized Absorbance at 410 nm
1	Positive 1	0.078
2	Positive 2	0.128
3	Positive 3	0.082
4	Positive 4	0.048
5	TXV with AP 1	0.057
6	TXV with AP 2	0.043
7	TXV with AP 3	0.005
8	TXV with AP 4	0.005
9	TXV with AP 5	0.018
10	TXV with AP 6	0.022
11	TXV with AP 7	0.017

Sample Key

Positive = Positive control sample = solution of acid phosphatase in PBS.

TXV with AP = mixtures of acid phosphatase and Triton X-100 enriched vesicles.

Absorbance values were normalized by subtracting absorbance values of negative controls from sample absorbance values.

Statistical Values

Average normalized absorbance values of positive control samples: 0.084
Standard deviation of normalized absorbance values of positive control samples: 0.03302524
Variance of normalized absorbance values of positive control samples: 0.00109067

Average normalized absorbance values of acid phosphatase and Triton X-100 mixture samples: 0.02385714
Standard deviation of normalized absorbance values of positive control samples: 0.01941158
Variance of normalized absorbance values of positive control samples: 0.00037681

To determine if there is a significant difference between the means of the positive controls and mixtures of acid phosphatase and Triton X-100 enriched vesicles a T-test was performed using the following equations (see reference 17, Chapter 4):

$$t_{\text{exp}} = \frac{|\overline{X}_A - \overline{X}_B|}{s_{\text{pool}} \times \sqrt{\frac{1}{n_A} + \frac{1}{n_B}}} = \frac{|\overline{X}_A - \overline{X}_B|}{s_{\text{pool}}} \times \sqrt{\frac{n_A n_B}{n_A + n_B}}$$

where s_{pool}, the pooled standard deviation, is

$$s_{\text{pool}} = \sqrt{\frac{(n_A - 1)s_A^2 + (n_B - 1)s_B^2}{n_A + n_B - 2}}$$

F-test to determine if variance can be pooled

F_{exp} = : 0.00109067/0.00037681 = 2.89447744
Critical value for two tailed F-test = F(0.05, 3, 6) = 6.599

F_{exp} < F(0.05, 3, 6); therefore, variances were pooled.

T-test analysis

$$S_{pool} = \sqrt{\frac{(4-1)(0.00109067) + (7-1)(0.00037681)}{4+7-2}} = 0.02479439$$

$$t_{exp} = \frac{(0.084000 - 0.023857)}{0.02479439} \times \sqrt{\frac{(4)(7)}{4+7}} = 3.87002$$

Critical value for two tailed T-test = t(0.01, 9) = 3.250

$t_{exp} > t(0.01, 9)$; therefore, there is a significant difference between the means at a 99% confidence level. We conclude that Triton X-100 enriched vesicles interfere with the acid phosphatase assay.

C. Comparing the enzymatic activity of acid phosphatase standard solutions with mixtures of acid phosphatase and Triton X-100 enriched vesicles.

Using the average normalized absorbance values of acid phosphatase standard solutions and mixtures of Triton X-100 enriched vesicles and acid phosphatase from Table 14, the percent of acid phosphatase in mixtures of Triton X-100 enriched vesicles and acid phosphatase was estimated to be 30%.

Average normalized absorbance values of positive control samples: 0.084
Average normalized absorbance values of acid phosphatase and Triton X-100 mixture samples: 0.024

III. Dynamic Light Scattering and Zeta Potential Analyses

Table 18: Dynamic Light Scattering Data

Sample	Cumulant Measurement Z-Ave, nm	PdI	Distribution Measurement Mean Diameter (stdev), nm
bare vesicles—Measurement 1	118.3	0.245	126.6 (55.33)
bare vesicles—Measurement 2	121	0.236	144.9 (74.16)
bare vesicles—Measurement 3	119.9	0.246	141.4 (73.76)
bare vesicle averages	**119.7**	**0.242**	**137.6 (67.75)**
Triton X-100 enriched vesicles—Measurement 1	116.4	0.183	138.8 (64.33)
Triton X-100 enriched vesicles—Measurement 2	116.2	0.181	141.8 (69.81)
Triton X-100 enriched vesicles—Measurement 3	114.3	0.172	140.5 (66.72)
Triton X-100 enriched vesicles average	**115.6**	**0.179**	**140.4 (66.95)**
TXAP—Trial 1—Measurement 1	101.6	0.104	113.9 (39.56)
TXAP—Trial 1—Measurement 2	102.2	0.105	114.8 (40.58)
TXAP—Trial 1—Measurement 3	102.5	0.097	115.0 (40.22)
TXAP—Trial 1 averages	**102.1**	**0.102**	**114.6 (40.12)**
AP vesicles—Trial 1—Measurement 1	141.2	0.316	169.5 (79.68)
AP vesicles —Trial 1—Measurement 2	142.5	0.251	171.4 (74.14)
AP vesicles —Trial 1—Measurement 3	141.6	0.270	166.4 (66.88)
AP vesicles —Trial 1 averages	**141.8**	**0.279**	**169.1 (73.57)**
AP vesicles —Trial 2—Measurement 1	141.5	0.200	177.1 (95.00)
AP vesicles —Trial 2—Measurement 2	141.1	0.172	173.5 (85.46)
AP vesicles —Trial 2—Measurement 3	140.9	0.167	190.1 (109.5)
AP vesicles—Trial 2 averages	**141.2**	**0.180**	**180.2 (96.65)**

Sample Key

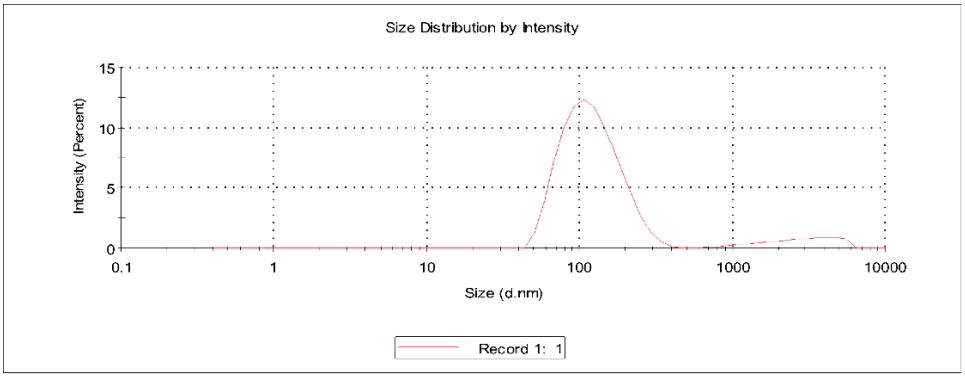
TXAP = Triton X-100 enriched vesicles mixed with acid phosphatase
AP vesicles = acid phosphatase functionalized vesicles.

AP vesicles are esterified Triton X-100 enriched vesicles mixed with acid phosphatase (ETXAP). AP vesicles—Trial 1 is the same as ETXAP—Trial 1.

BCA data for TXAP and ETXAP samples can be found in Table 1 and Figures 1-4.

A Malvern ZetaSizer ZS was used for DLS analyses. Each DLS measurement is an average of 12 runs. The buffer material setting on the instrument was used for each measurement. The instrument indicated that the results of each measurement were good quality. The polydispersity index for each sample is relatively high and indicates that the distribution algorithm should be used for measurement and thus, average diameters reported in the article are based on intensity distribution algorithms. Representative size distribution by intensity graphs for each sample are below.

Figure 22: Representative size distribution by intensity of bare vesicles



Note: The peak around 5 µm is attributed to dust or air bubbles in the sample. The average size and standard deviation of bare vesicles was based on peaks centered around 100 nm.

Figure 23: Representative size distribution by intensity of Triton X-100 enriched vesicles

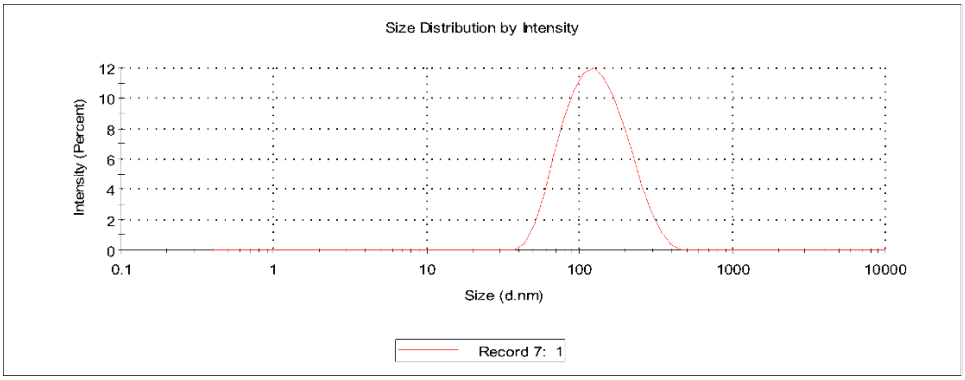


Figure 24: Representative size distribution by intensity of Triton X-100 enriched vesicles mixed with acid phosphatase

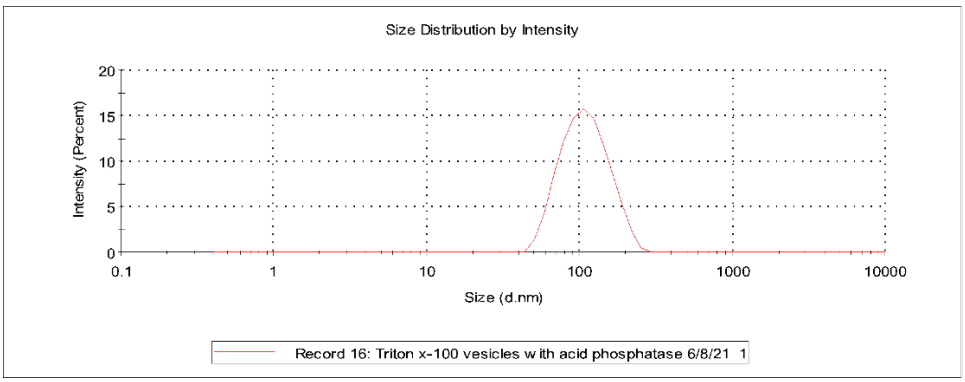
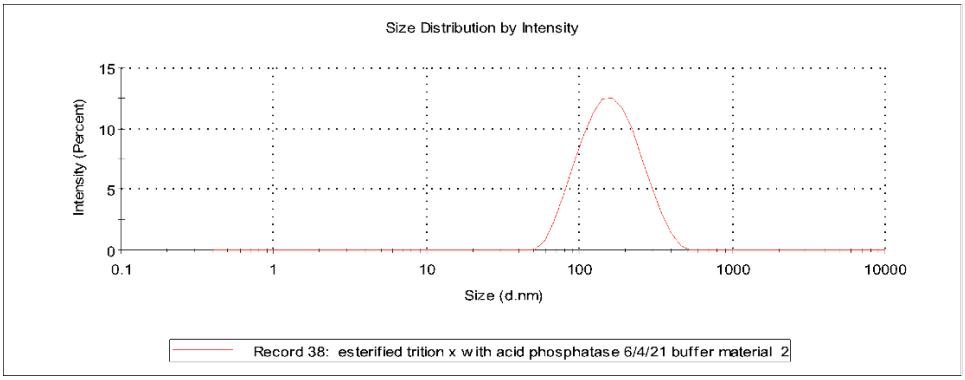


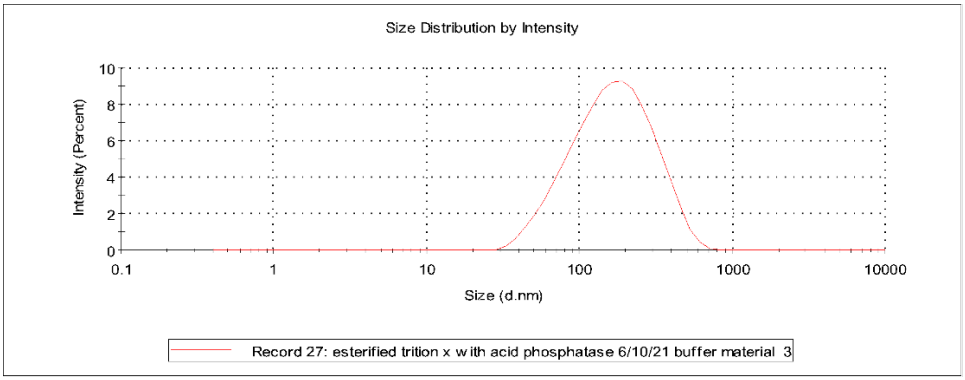
Figure 25: Representative size distribution by intensity of AP vesicles —Trial 2



AP vesicles = acid phosphatase functionalized vesicles.

AP vesicles are esterified Triton X-100 enriched vesicles mixed with acid phosphatase (ETXAP). AP vesicles—Trial 1 is the same as ETXAP—Trial 1.

Figure 26: Representative size distribution by intensity of AP vesicles —Trial 3



AP vesicles = acid phosphatase functionalized vesicles.

AP vesicles are esterified Triton X-100 enriched vesicles mixed with acid phosphatase (ETXAP). AP vesicles—Trial 2 is the same as ETXAP—Trial 2.

IV. BCA Assay Limit of Detection Calculations

A. LOD based on PBS blank samples and external calibration curves

The concentration of protein in PBS blank samples was determined by calculating the concentration of protein at an absorbance value of 0.000 using the equation from the linear regression line of external standard curves. All standard curves had R^2 values greater than 0.90.

Twenty-one external calibration curves were analyzed and the equations of the linear regression lines are below:

Table 19: BCA assay data used to determine the limit of detection based on external calibration curves.

Entry	Equation of linear regression line	Concentration of protein in PBS blank, μg/mL
1	y = 0.0021x + 0.0199	-9.476190476
2	y = 00031x + 0.0458	-14.77419355
3	y = 0.0028x + 0.0114	-4.071428571
4	y = 0.0030x + 0.0266	-8.866666667
5	y = 0.0030x + 0.0238	-7.933333333
6	y = 0.0028x + 0.0354	-12.64285714
7	y = 0.0029x + 0.0144	-4.965517241
8	y = 0.0023x + 0.0107	-4.652173913
9	y = 0.0022x + 0.0063	-2.863636364
10	y = 0.0039x + 0.0438	-11.23076923
11	y = 0.0031x + 0.0265	-8.548387097
12	y = 0.0039x + 0.0438	-11.23076923
13	y = 0.0018x + 0.0121	-6.722222222
14	y = 0.0019x + 0.0009	-0.473684211
15	y = 0.0035x + 0.0069	-1.971428571
16	y = 0.0036x + 0.0122	-3.388888889
17	y = 0.0020x + 0.0383	-19.15
18	y = 0.0028x – 0.0044	1.571428571
19	y = 0.0032x + 0.0007	-0.21875
20	y = 0.0074x + 0.0498	-6.72972973
21	y = 0.0091x + 0.0791	-8.692307692

Average calculated protein concentrations: -7.001 μg/mL
Standard deviation of calculated protein concentrations: 5.135

The average and standard deviation of the calculated protein concentration in the PBS blank samples were used to calculated the limit of detection (LOD) for the BCA assay using the following equation:

$$LOD = \bar{C} + 2.528s$$

were $\bar{C}$ is the average calculated protein concentration and s is the standard deviation of the calculated concentrations. The average calculated concentration was a negative value, therefore zero was used as the average value. 2.528 is the single-tailed Student's t-value for 21 samples at a 99% confidence level with n-1 degrees of freedom.

$$LOD = 0 + 2.528\ (5.135) = 13.0\ \mu g/mL$$

B. LOD based on bare vesicle and Triton X-100 enriched vesicles.

The limit of detection was also estimated from the average and standard deviation of absorbance values of purified bare vesicles and Triton X-100 enriched vesicles that did not contain protein (n = 12). The minimum distinguishable absorbance signal based on the 12 blank vesicle samples was calculated using the formula:

$$A_{min} = \bar{A} + 2.718s$$

where A_{min} is the minimum distinguishable absorbance value, $\bar{A}$ is the average absorbance signal of the 12 samples, and s is the standard deviation of the 12 absorbance signals. 2.718 is the single-tailed Student's t-value for 12 samples at a 99% confidence level with n-1 degrees of freedom

The corrected absorbance values (PBS blanks subtracted) of bare vesicles and Triton X-100 enriched vesicles used to determine the minimal distinguishable signal are below:

Table 20: BCA assay data used to determine the limit of detection based on blank vesicle samples.

Entry	Corrected Absorbance at 562 nm
1	0.041
2	-0.021
3	0.026
4	0.028
5	0.015
6	-0.002
7	0.000
8	0.016
9	0.018
10	0.031
11	0.010
12	0.009

Average corrected absorbance values: 0.01425
Standard deviation of corrected absorbance values: 0.01677728
Minimum distinguishable signal at 99% confidence level:

$$A_{min} = \bar{A} + 2.718s$$

$$A_{min} = 0.01425 + 2.718(0.01677728) = 0.05985066$$

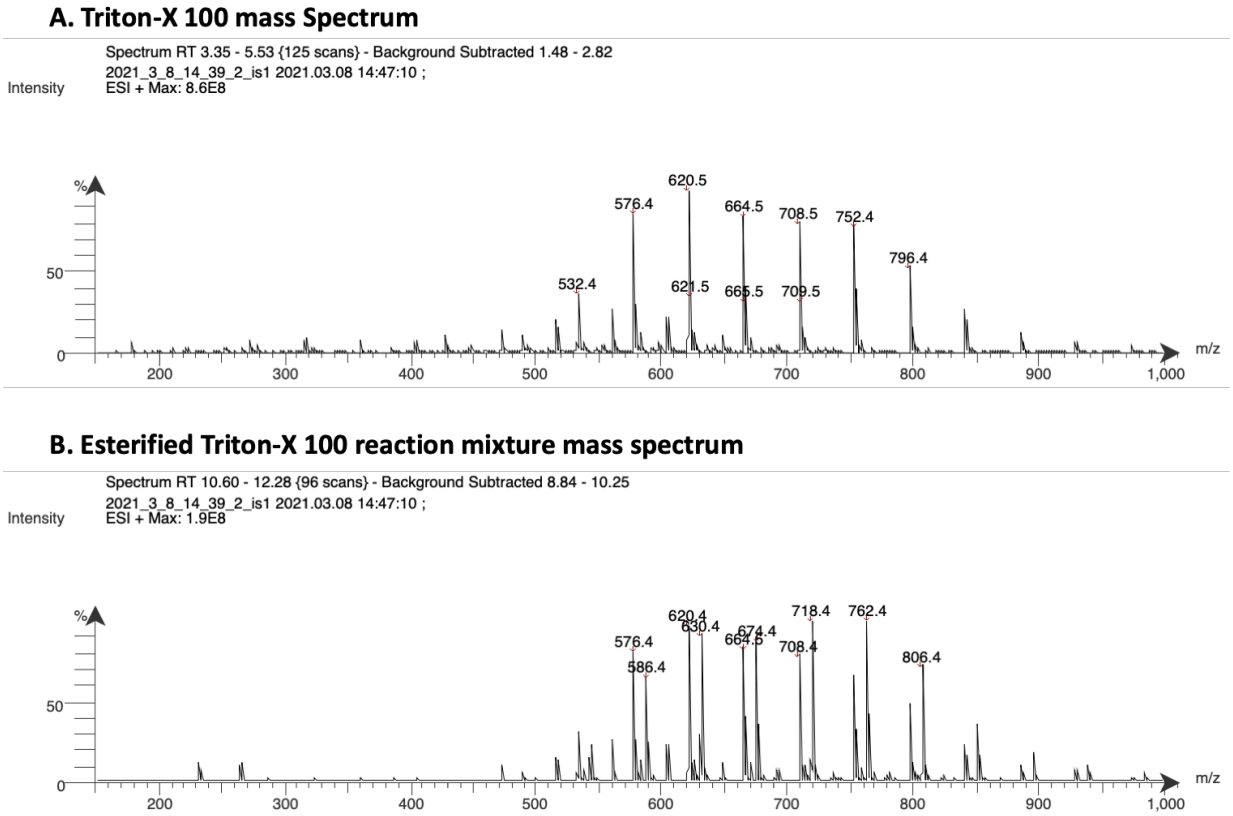
LOD based on A_{min} of blank vesicle samples : 12.3 μg/mL

The LOD based on A_{min} is the average calculated concentration from seven BCA assay external calibration curves with R^2 values greater than 0.99.

V. Thin-Layer Chromatography Mass Spectrometry of Esterified Triton X-100

To prepare the esterified Triton X-100 derivative, **1**, Triton X-100 was reacted with acryloyl chloride following a method reported by Oppolzer and coworkers. The reaction mixture was analyzed by thin layer chromatography-mass spectrometry. The mass spectra of Triton X-100 and the reaction mixture are depicted in Figure 27. Figure 27A is the mass spectrum of Triton X-100 and Figure 27B is the mass spectrum of the reaction mixture from the esterification of Triton X-100. In both spectra, peaks at 532.4, 576.4, 620.5, 664.5, 708.5, 752.5, and 796.4 m/z are attributed to Triton X-100 molecules with different numbers of ethylene glycol units. In the spectrum of the reaction mixture, peaks appearing at 586.4, 630.4, 674.4, 718.4, 762.4, and 806.4 m/z are attributed to esterified Triton X-100 molecules. The esterification adds 54 atomic mass units to Triton X-100 molecules. The difference between 532.4 and 586.4 m/z, 576.4 and 630.4 m/z, 620.5 and 674.4 m/z, 664.5 and 718.4 m/z, 708.5 and 762.4 m/z, and 752.5 and 806.4 m/z, is 54 m/z units. The shift of 54 m/z units indicates that the conversion to the ester was successful. The reaction did not go to completion, as unmodified Triton X-100 was still present in the reaction mixture, but the amount of esterified conjugate in the mixture was enough to carry the mixture forward into vesicle formulations.

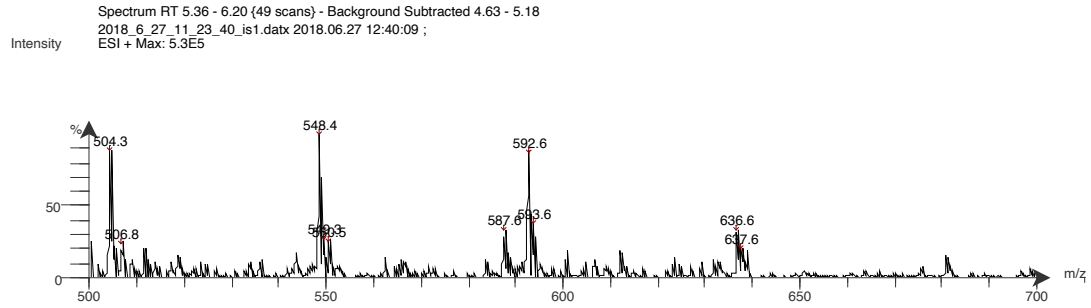
Figure 27: Electrospray ionization (ESI) mass spectra of Triton X-100 and esterified Triton X-100 reaction mixture.



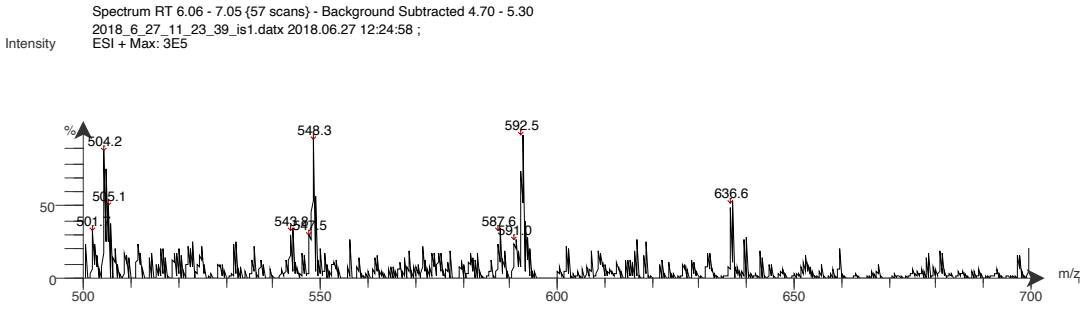
Positive mode ESI-MS of Triton X-100 (A) and esterified Triton X-100 reaction mixture (B). Peaks appearing at 586.4, 630.4, 674.4, 718.4, 762.4, and 806.4 m/z in the esterified Triton X-100 reaction mixture are attributed to esterified Triton X-100 molecules.

Figure 28: Electrospray ionization (ESI) mass spectra of esterified Triton X-100 enriched vesicles.

A. Unpurified vesicle mixture



B. SEC purified vesicle fraction



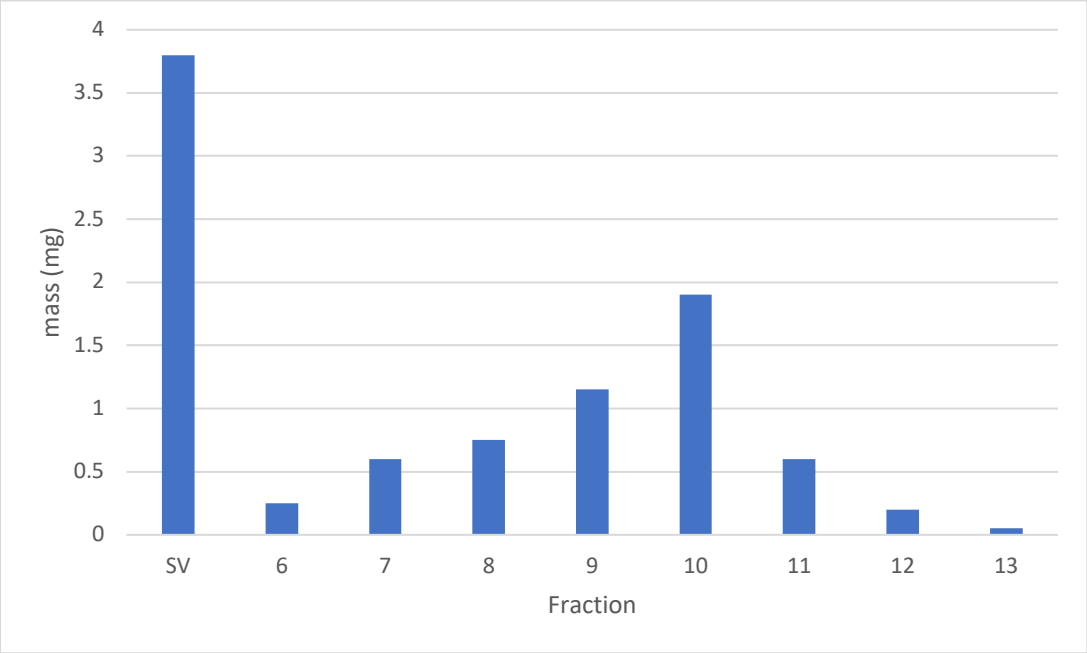
VI. Size Exclusion Chromatography Analyses

To assess the ability of Sepharose CL-2B size exclusion chromatography (SEC) to separate vesicles from excess surfactant and ligands, gravimetric, thin layer chromatography (TLC), ^{1}H-NMR, and TLC-MS analyses were performed.

Gravimetric, TLC, and ^{1}H-NMR Analyses of SDBS/CTAT Vesicles

A CD-10 column was packed with Sepharose CL-2B to a height of 5.5 cm. Anionic SDBS:CTAT vesicles were prepared in Millipore water at a total surfactant concentration of 1% by weight (70:30 SDBS:CTAT weight ratio). One milliliter of the vesicle solution was purified by SEC with Millipore water as the eluent and 1 mL fractions were collected up to fraction 13. Vesicles eluted in fractions 3.1-5.0 mL, so those fractions were combined. This process was repeated two more times and the fractions from the three purifications were pooled in pre-weighed vials. The samples were lyophilized, the mass of each sample was obtained, and the average mass of each fraction was calculated. The results are provided in Figure 28.

Figure 28: Gravimetric analysis of anionic SDBS/CTAT vesicles.



The SV fraction is the combination of fractions 3 to 5 (mL 3.1-5.0)

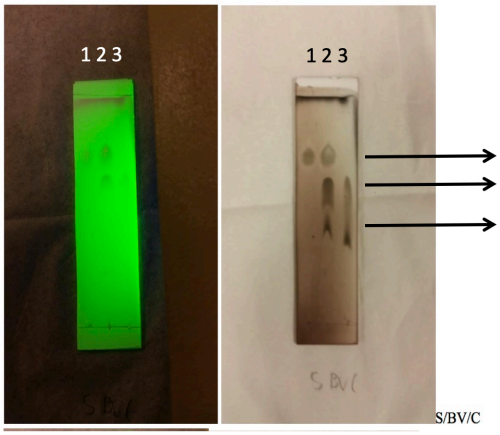
Fractions from the gravimetric analysis were further analyzed by TLC and ^{1}H-NMR. TLC analyses were performed on silica plates developed in $CHCl_3$:MeOH:H_20:acetic acid (10:5:1:1) and visualized under UV and phosphoric acid and copper char. TLC analyses showed that SDBS and CTA (cetyltrimethylammonium cation) were present in the SV fraction and SDBS and tosylate were present in later fractions.

Figure 29: TLC Analyses

A: TLC Analysis of Crude (Unpurified) Vesicles and SDBS and CTAT

Lane 1: SDBS
Lane 2: **Crude** BV
Lane 3: CTAT

SDBS and Tosylate are UV active



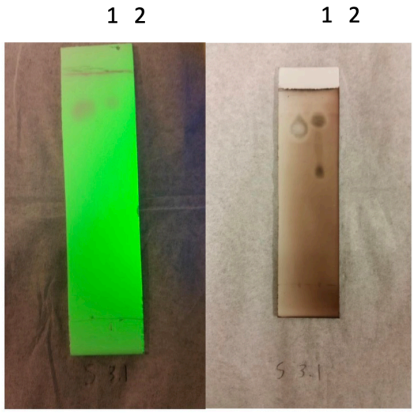
SDBS
Tosylate
CTA

UV

$H_3PO_4/CuSO_4$ char

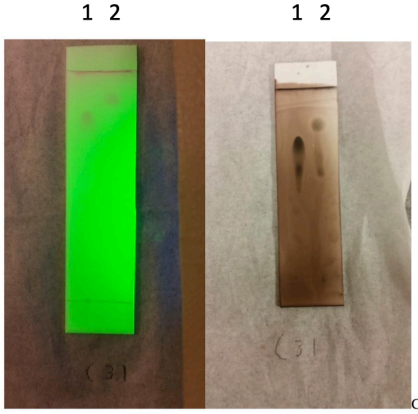
B: TLC Analysis of Purified Vesicles (SV fraction)

Lane 1: SDBS
Lane 2: **Purified** BV



Purified BV contain SDBS

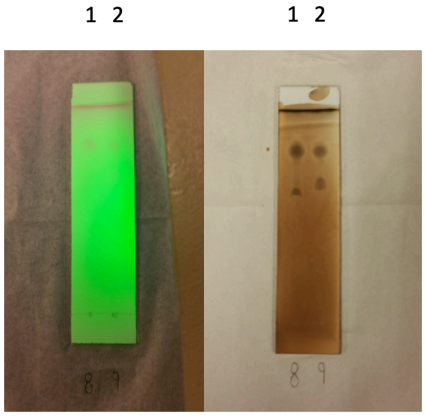
Lane 1: CTAT
Lane 2: **Purified** BV



Purified BV contain CTA
but do not contain tosylate

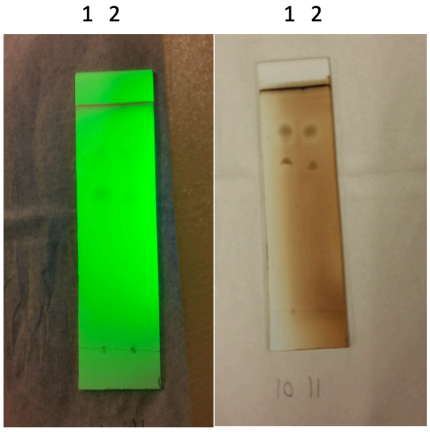
C: TLC Analysis of Fractions 8-11

Lane 1: Fraction 8
Lane 2: Fraction 9



Later fractions contain SDBS and tosylate

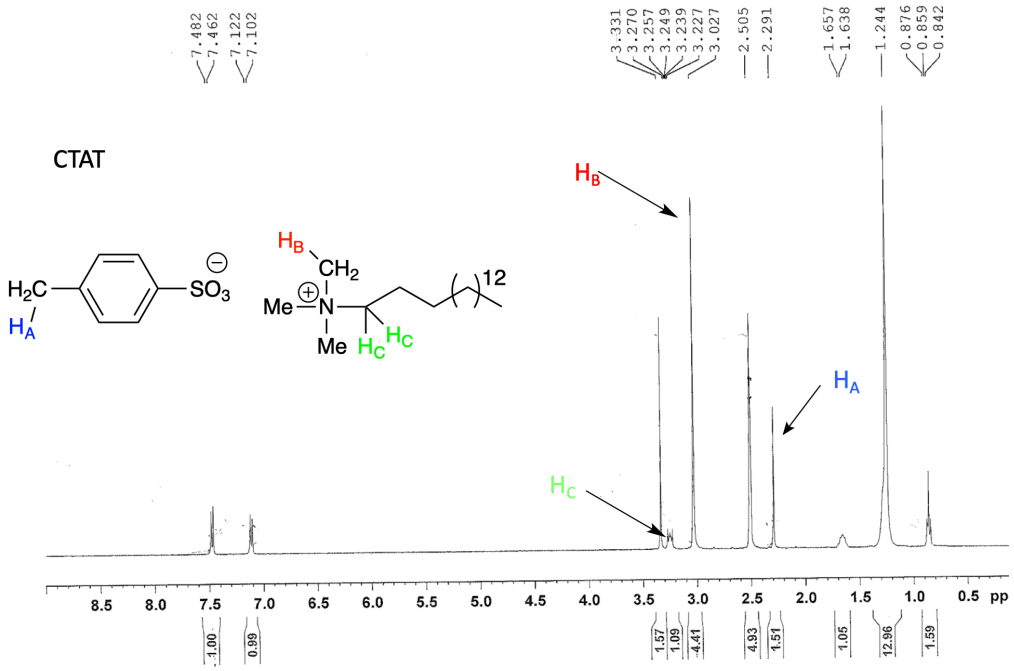
Lane 1: Fraction 10
Lane 2: **Purified** 11



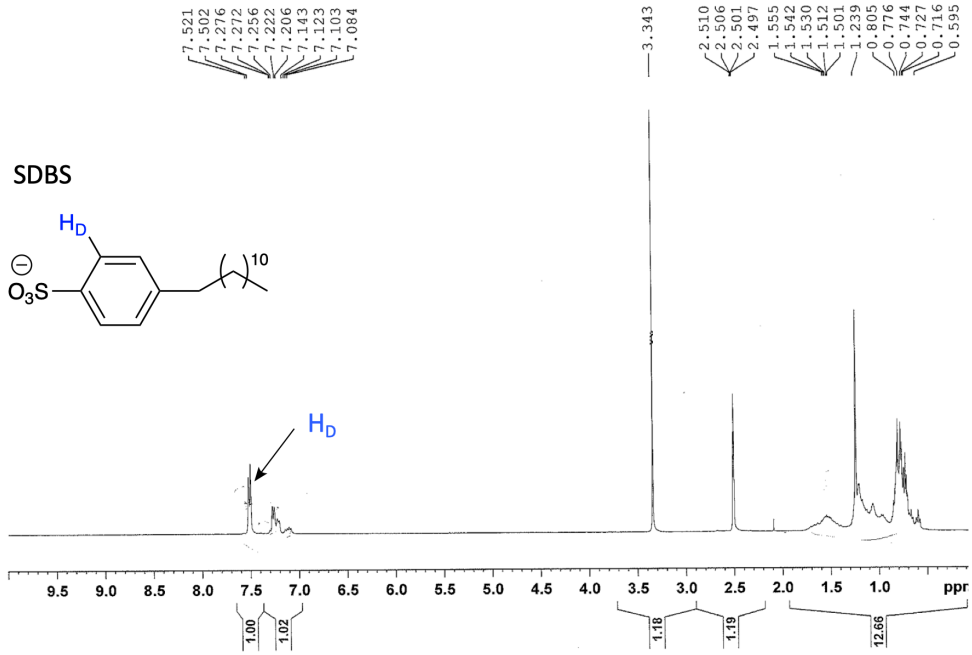
The SV fraction was analyzed by ^{1}H-NMR. The spectrum confirms that purified vesicles contain SDBS and CTA but not tosylate.

Figure 30: ^{1}H-NMR Spectra

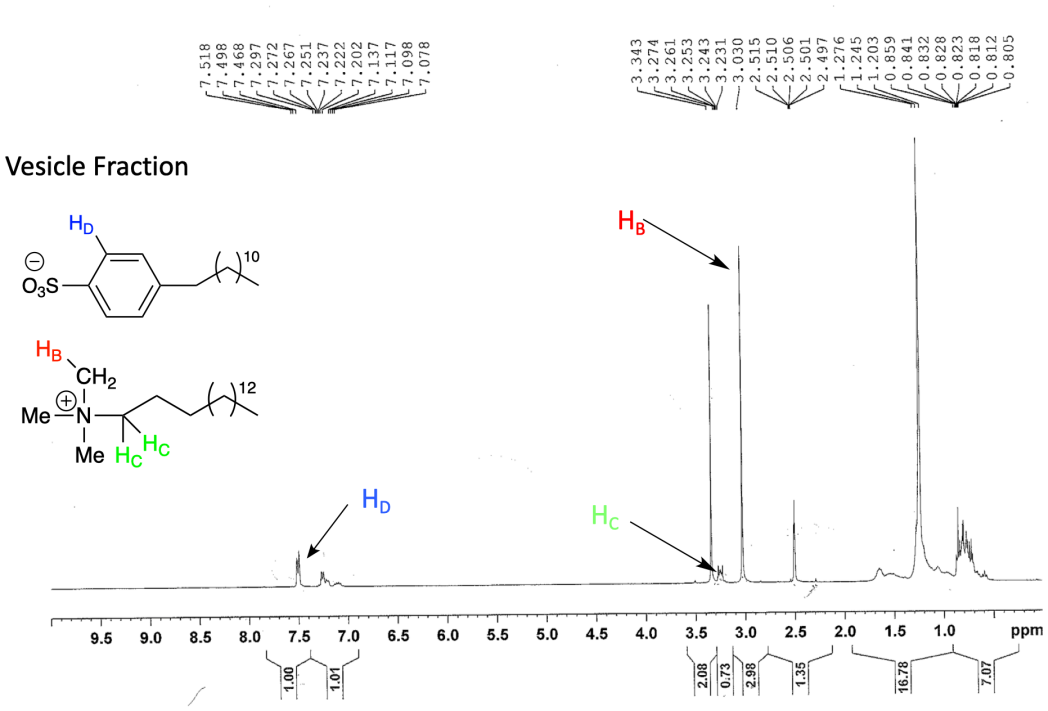
A: ^{1}H-NMR Spectrum of CTAT



B: ^{1}H-NMR Spectrum of SDBS



C: ^{1}H-NMR Spectrum of purified vesicles (SV fraction)

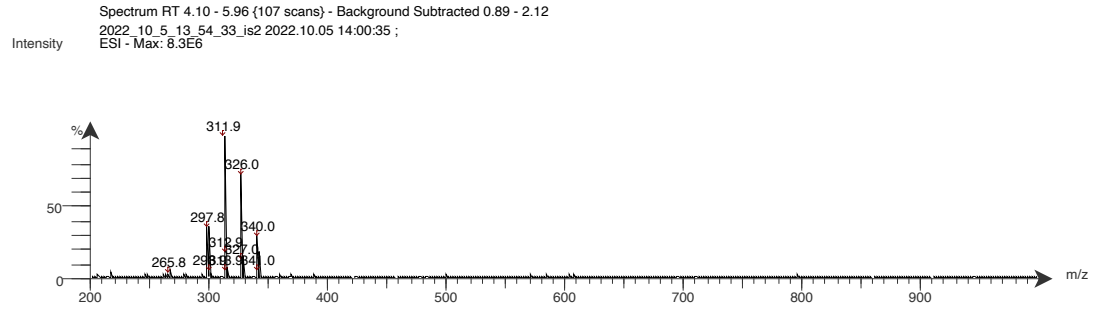


Based on the integrations of peaks H_B and H_D, the molar ratio of SDBS to CTAT in the vesicle fraction (purified vesicles) is 1.5:1. The SDBS:CTAT molar ratio in the crude vesicle mixture was 3:1; thus, the 1.5:1 SDBS:CTAT molar ratio in the SEC purified sample confirms that vesicles were separated from free surfactant during the SEC process. The absence of a peak at 2.291 ppm (the H_A peak in the spectrum of CTAT) shows that tosylate is not present in purified vesicles. A similar analysis was performed on vesicles prepared in PBS and the molar ratio of SDBS to CTAT is 2:1.

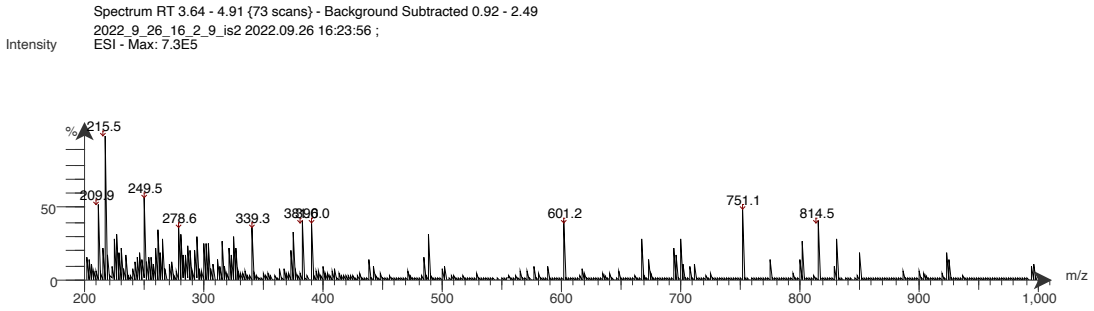
To further examine the ability of Sepharose CL-2B SEC to separated free surfactants and micelles from vesicles, a solution of Triton X-100 (0.25% by weight) and SDBS (0.5% by weight) was prepared to simulate free surfactant concentration in vesicle formulations. CTAT was not added to the mixture, so vesicles were not present in the solution. One milliliter of the solution was purified by Sepharose CL-2B SEC with PBS as the eluent. Fractions were collected in the same manner as in the gravimetric analysis and analyzed by TLC-MS. Negative mode ESI-MS was used to detect SDBS (Figure 31) and positive mode ESI-MS was used to detect Triton X-100 (Figure 32). The results of the TLC-MS analysis were consistent with the gravimetric analysis: SDBS was detected in fractions 6 -12, and Triton X-100 was detected in fractions 6-11. Most notably, neither SDBS or TX-100 was detected in SV fractions (3.1-5.0 mL) even when the SV fraction was concentrated by a factor of 10.

Figure 31: Negative mode ESI-mass spectra of SEC fractions of a solution of SDBS and Triton X-100 dissolved in PBS. SDBS is detected in negative mode ESI-MS.

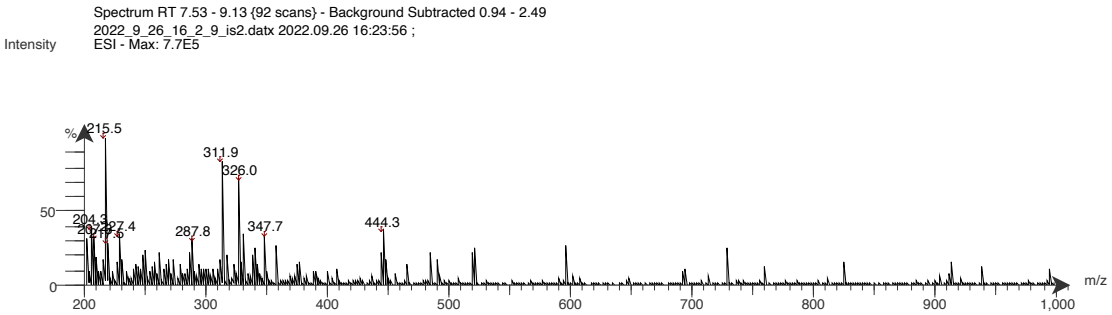
A: Mass spectrum of SDBS standard



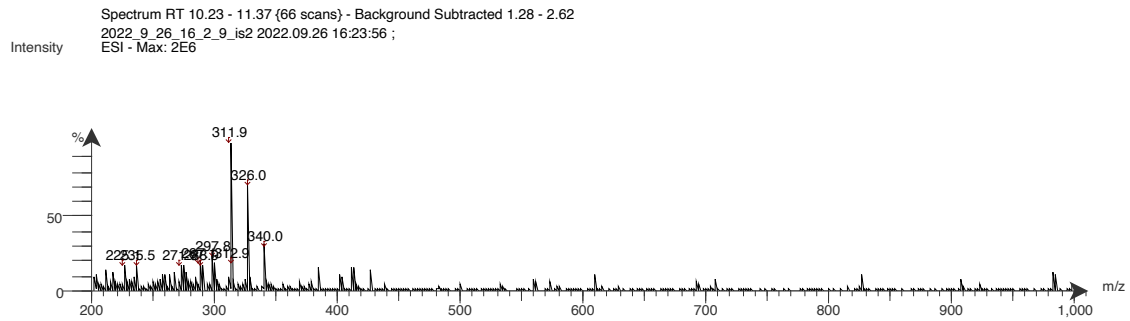
B: Mass spectrum of SV fraction



C: Mass spectrum of fraction 6

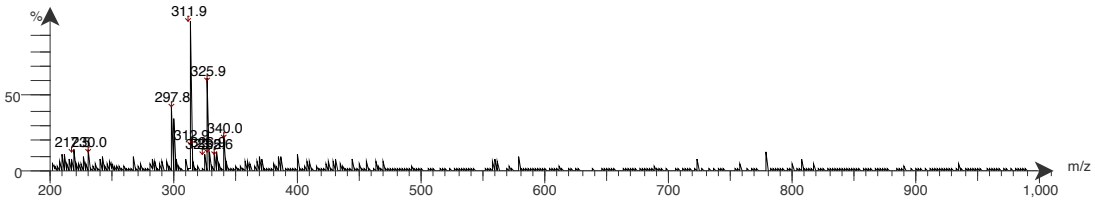


D: Mass spectrum of fraction 7



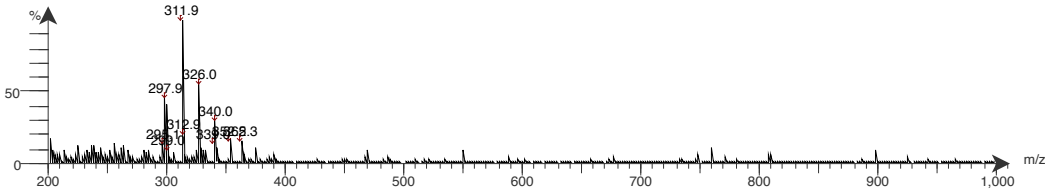
E: Mass spectrum of fraction 8

Spectrum RT 12.71 - 14.17 {84 scans} - Background Subtracted 1.33 - 2.53
2022_9_26_16_2_9_is2 2022.09.26 16:23:56 ;
Intensity ESI - Max: 2.6E6



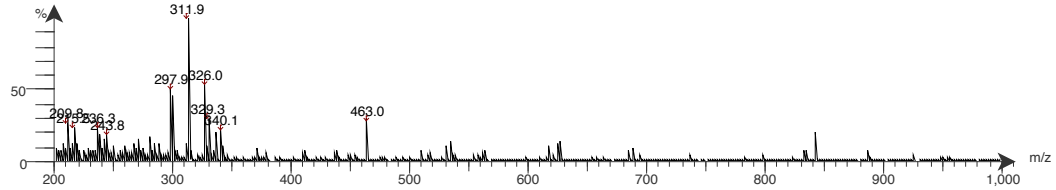
F: Mass spectrum of fraction 9

Spectrum RT 3.57 - 5.26 {97 scans} - Background Subtracted 0.84 - 2.65
2022_9_27_10_44_52_is2 2022.09.27 10:52:03 ;
Intensity ESI - Max: 1.6E6



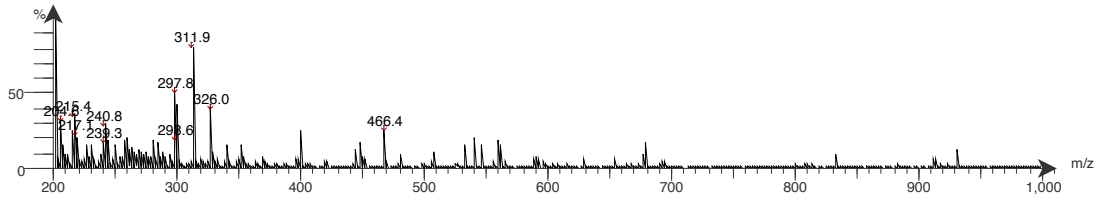
G: Mass spectrum of fraction 10

Spectrum RT 6.17 - 7.74 {90 scans} - Background Subtracted 0.92 - 2.72
2022_9_27_10_44_52_is2 2022.09.27 10:52:03 ;
Intensity ESI - Max: 1E6



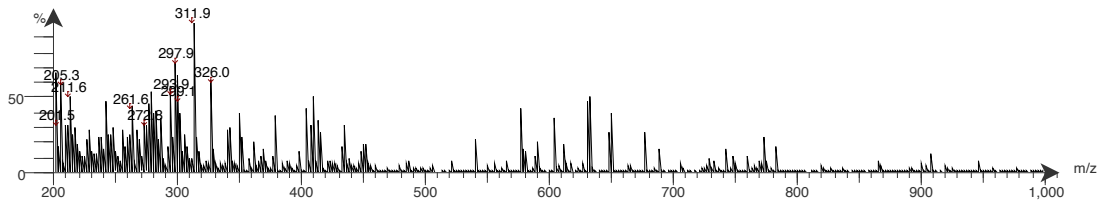
H: Mass spectrum of fraction 11

Spectrum RT 8.99 - 10.24 {72 scans} - Background Subtracted 1.08 - 2.88
2022_9_27_10_44_52_is2 2022.09.27 10:52:03 ;
Intensity ESI - Max: 8.1E5



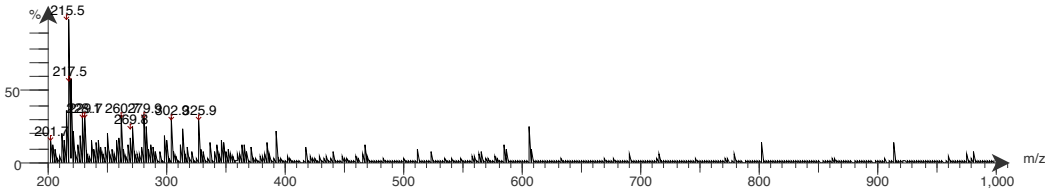
I: Mass spectrum of fraction 12

Spectrum RT 11.76 - 13.17 {81 scans} - Background Subtracted 1.17 - 3.21
2022_9_27_10_44_52_is2 2022.09.27 10:52:03 ;
Intensity ESI - Max: 4.3E5



J: Mass spectrum of fraction 13

Spectrum RT 15.14 - 16.39 {72 scans} - Background Subtracted 1.07 - 2.72
2022_9_27_10_44_52_is2 2022.09.27 10:52:03 ;
Intensity ESI - Max: 9.3E5



K: Mass spectrum of SV fraction concentrated by a factor of 10

Spectrum RT 3.94 - 5.42 {85 scans} - Background Subtracted 0.94 - 2.07
2022_9_27_15_59_57_is2.datx 2022.09.27 16:19:22 ;
Intensity ESI - Max: 9.4E5

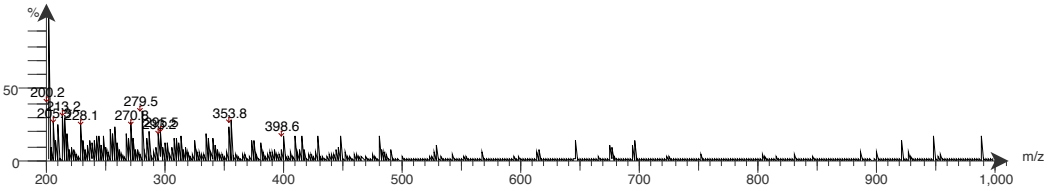
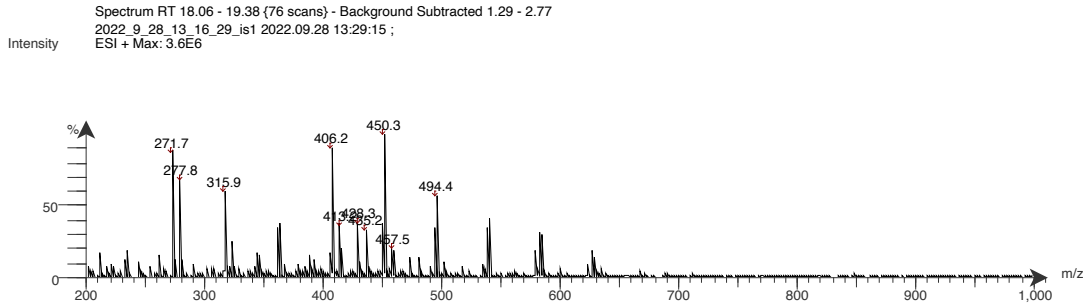
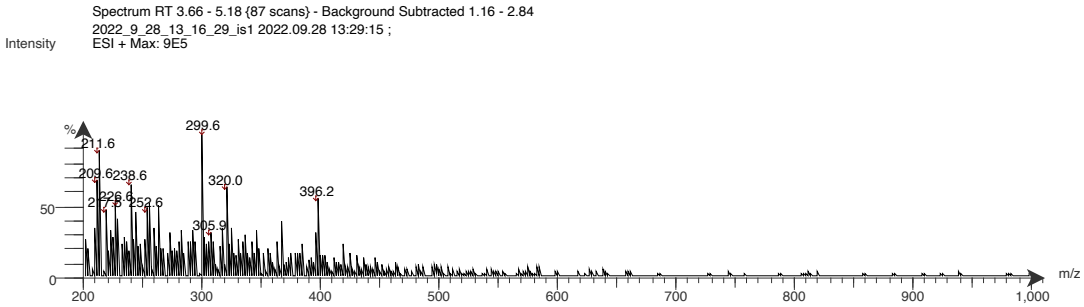


Figure 32: Positive mode ESI-mass spectra of SEC fractions of a solution of SDBS and Triton X-100 dissolved in PBS. Triton X-100 is detected in positive mode ESI-MS.

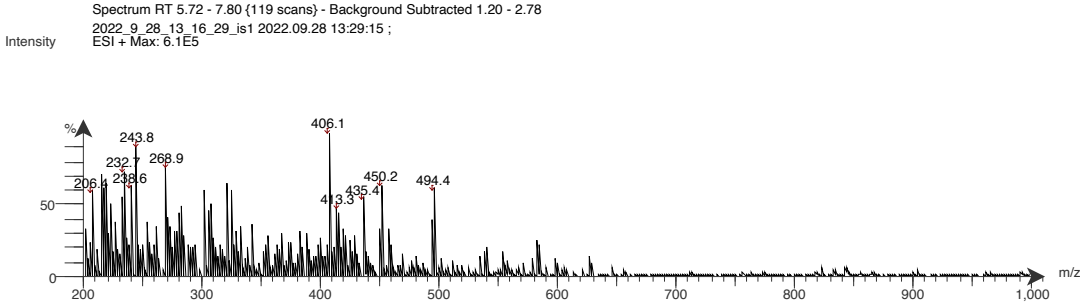
A: Mass spectrum of Triton X-100 standard



B: Mass spectrum of SV fraction

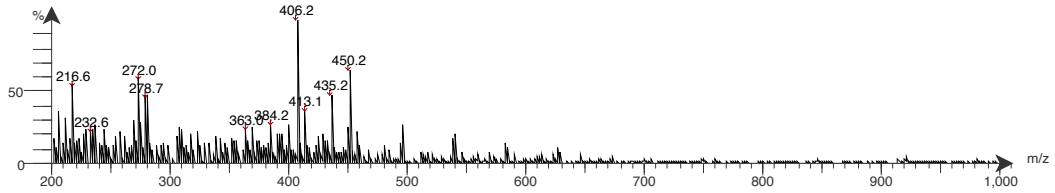


C: Mass spectrum of fraction 6



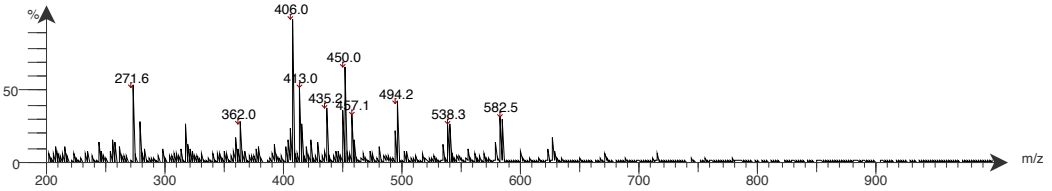
D: Mass spectrum of fraction 7

Spectrum RT 8.74 - 10.67 {111 scans} - Background Subtracted 1.27 - 2.92
2022_9_28_13_16_29_is1 2022.09.28 13:29:15 ;
Intensity ESI + Max: 9.1E5



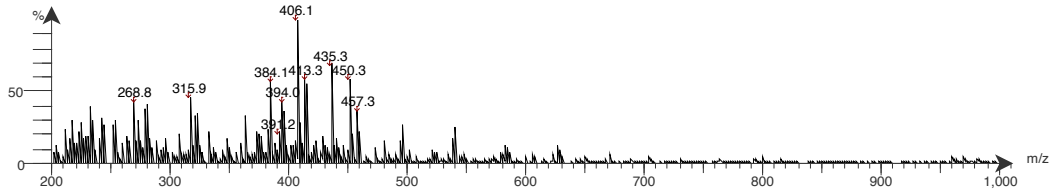
E: Mass spectrum of fraction 8

Spectrum RT 3.68 - 4.98 {75 scans} - Background Subtracted 1.41 - 2.82
2022_10_4_14_3_34_is1 2022.10.04 14:07:48 ;
Intensity ESI + Max: 4.3E6



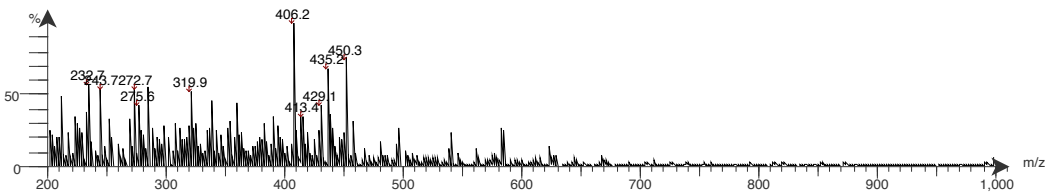
F: Mass spectrum of fraction 9

Spectrum RT 12.03 - 13.65 {93 scans} - Background Subtracted 1.41 - 2.92
2022_9_28_13_16_29_is1 2022.09.28 13:29:15 ;
Intensity ESI + Max: 9.7E5

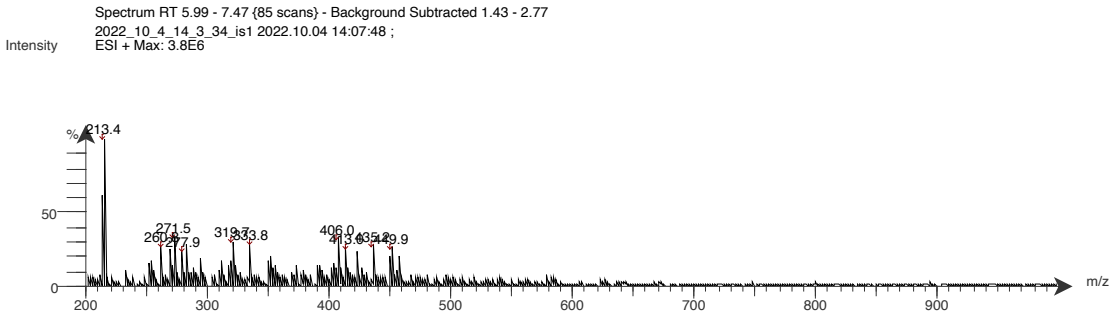


G: Mass spectrum of fraction 10

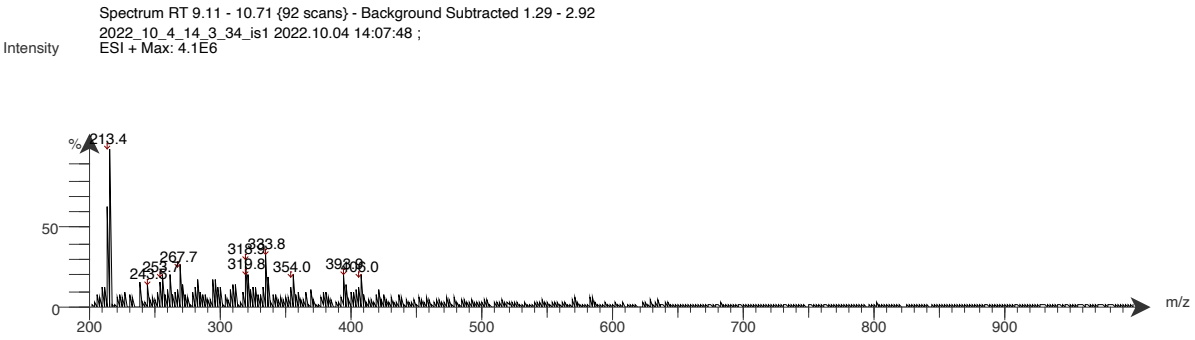
Spectrum RT 15.54 - 17.07 {88 scans} - Background Subtracted 1.27 - 2.78
2022_9_28_13_16_29_is1 2022.09.28 13:29:15 ;
Intensity ESI + Max: 9.1E5



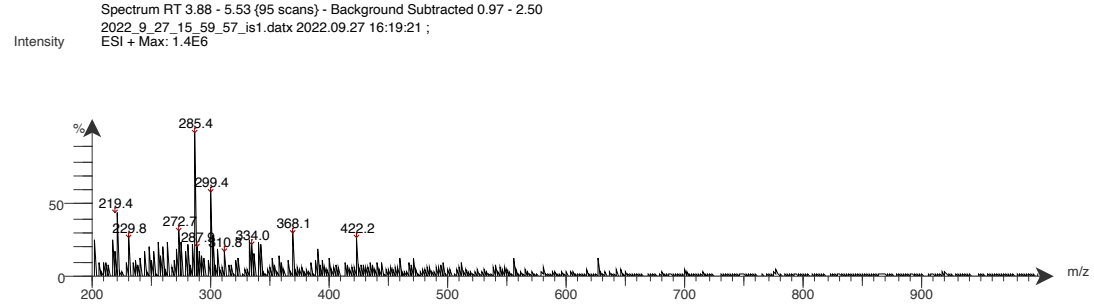
H: Mass spectrum of fraction 11



I: Mass spectrum of fraction 12



J: Mass spectrum of SV fraction concentrated by a factor of 10

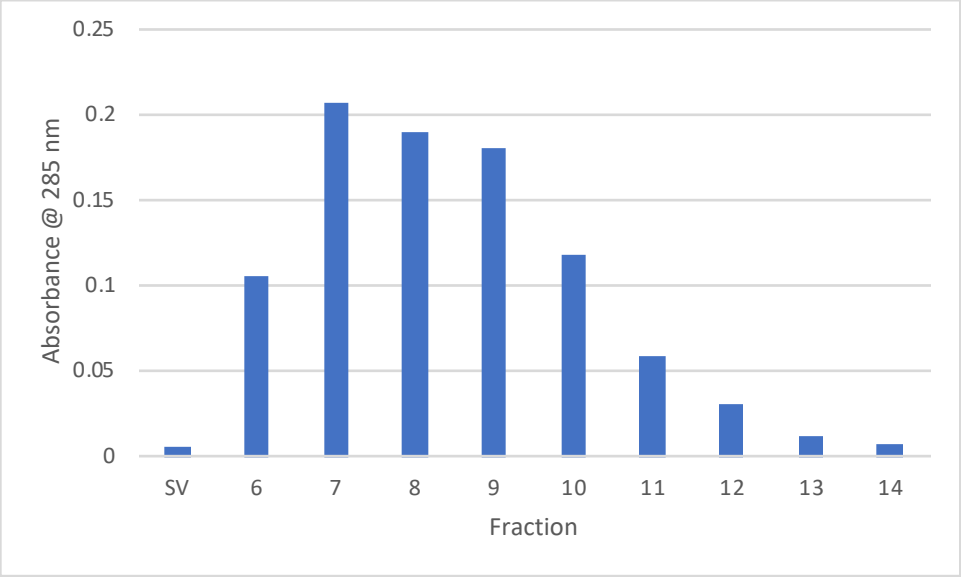


Triton X-100 can be detected and quantified via UV-Vis spectroscopy. SEC fractions of a solution of SDBS and Triton X-100 dissolved in PBS were analyzed at 285 nm (this wavelength has minimal interference from SDBS). The results were consistent with the ESI-MS analysis.

Table 21: UV-Vis spectroscopy data of size exclusion chromatography fractions of a solution of SDBS and Triton X-100 dissolved in PBS.

Sample	Corrected Absorbance at 285 nm
SV fraction	0.0062
6	0.1054
7	0.2059
8	0.1894
9	0.1794
10	0.1172
11	0.0593
12	0.0298
13	0.0117
14	0.0064

Figure 33: UV-Vis spectroscopy data of size exclusion chromatography fractions of a solution of SDBS and Triton X-100 dissolved in PBS.



SV = purified Triton X-100 vesicle fraction

Based on absorbance values, 99% of Triton X-100 molecules elute after the vesicle fraction.

VII. Percent Incorporation and Retention Calculations

To determine the percent of Triton X-100 incorporated into Triton X-100 enriched SVs, Triton X-100 enriched SV samples were purified by SEC and the collected fractions were analyzed by UV-Vis spectroscopy. Triton X100 can be quantified used the absorbance at 285 nm.

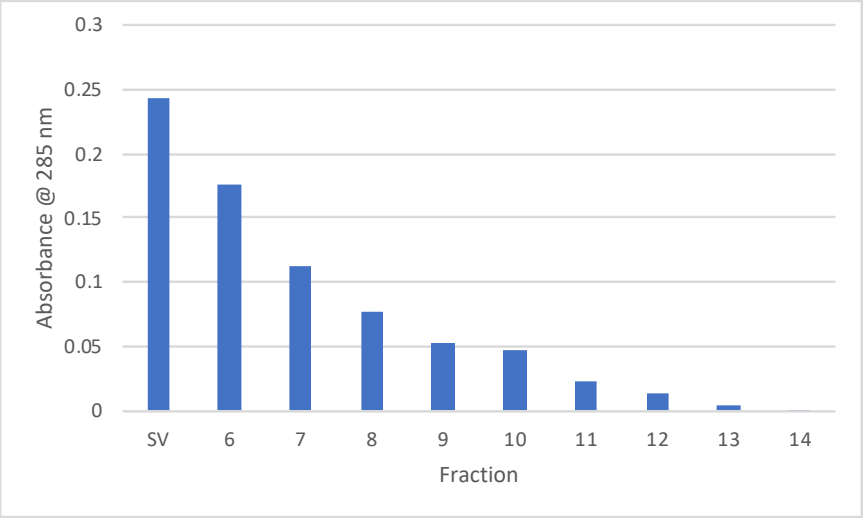
Table 22: UV-Vis spectroscopy data of exclusion chromatography fractions of Triton X-100 enriched SVs.

Sample	Absorbance at 285 nm
Crude SV	0.2867
SV fraction	0.2423
Fraction 6	0.1765
Fraction 7	0.1114
Fraction 8	0.0762
Fraction 9	0.0534
Fraction 10	0.0462
Fraction 11	0.0235
Fraction 12	0.0138
Fraction 13	0.0032
Fraction 14	-0.0008

Crude SV = unpurified Triton X-100 enriched vesicles
SV fraction = purified Triton X-100 vesicle fraction

For analysis, SEC fraction samples were prepared with 4-times more sample than the crude SV sample. During SEC, SVs are diluted by a factor of 2. The SV fraction is 2 mL, while the other SEC fractions are 1 mL.

Figure 34: UV-Vis spectroscopy data of size exclusion chromatography fractions of Triton X-100 enriched SVs.



SV = purified Triton X-100 vesicle fraction

Percent incorporation based on SV fraction versus crude SV sample: 44%
Percent incorporation based on total absorbance of SEC fractions: 49%

Average % incorporation: 47%

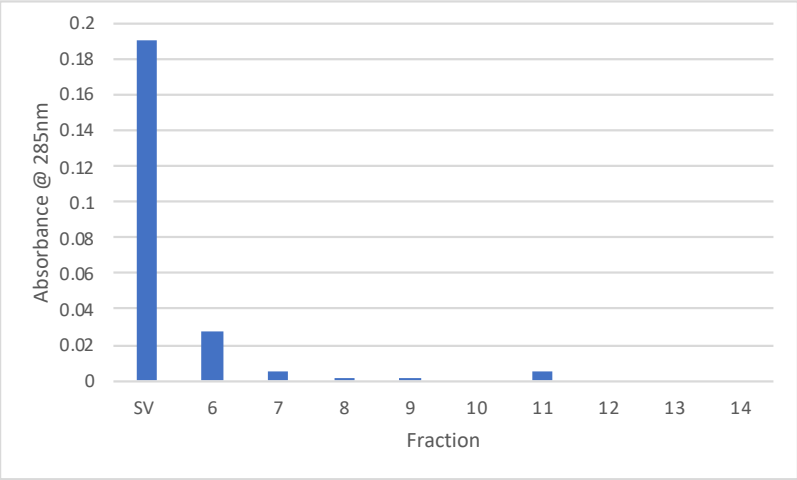
To determine if Triton X-100 molecules leach out of vesicles during the conjugation step, 1 mL of purified SEC fractions of SVs were purified by SEC a second time and the fractions were analyzed by UV-Vis spectroscopy.

Table 23: UV-Vis spectroscopy data of size exclusion chromatography fractions of twice purified Triton X-100 enriched vesicles

Sample	Corrected Absorbance at 285 nm
SV 1X pure	0.2123
SV 2X pure	0.1903
Fraction 6	0.0272
Fraction 7	0.0046
Fraction 8	0.0009
Fraction 9	0.0017
Fraction 10	-0.0012
Fraction 11	0.0045
Fraction 12	-0.0042
Fraction 13	-0.006
Fraction 14	-0.0072

SV 1X pure = Triton X-100 enriched SVs that were purified once.
SV 2X pure = Triton X-100 vesicle fraction from second purification

Figure 35: UV-Vis spectroscopy data of size exclusion chromatography fractions of twice purified Triton X-100 enriched vesicles.



SV = purified Triton X-100 vesicle fraction

Percent retention based on SV 1X pure sample versus crude SV 2X pure sample: 90%
Percent retention based on total absorbance of SEC fractions: 91%

Average % retention: 90%

VIII. Vesicle Concentration Calculations

Vesicle concentrations were calculated via encapsulation experiments. Vesicles were formed in solutions containing known concentrations of an analyte. Sodium ion, pemetrexed (disodium salt), carboplatin, and cisplatin were used as analytes in separate studies. Vesicles were prepared in a sodium chloride solution to evaluate the encapsulation of sodium ion, while the other vesicle formulations were prepared in solutions of PBS (1X, pH = 7.4) containing pemetrexed (disodium salt), carboplatin, and cisplatin, respectively. Millipore water was used as the eluent for vesicles containing sodium ion, while PBS was used as the eluent for the other vesicle samples. Analyte concentration in purified samples was determined using FAAS (sodium ion), ^{1}H-NMR (pemetrexed and carboplatin), and ICP-AES (cisplatin).

Table 24: Encapsulation Data

Analyte	[Analyte] in crude SVs, mM	[Analyte] in purified SVs, mM	% incorporation	[SVs], vesicles/mL
Sodium ion	200	1.2	1.2	9.5E+12
Pemetrexed	84	0.45	0.8	6.4E+12
Carboplatin	40	0.5	1.9	1.5E+13
Cisplatin	3.3	0.05	2.3	2.0E+13

Percent incorporation values were calculated using a dilution factor to account for dilution during SEC purification.

The analytes are water soluble and are expected to become encapsulated within the vesicles' lumen, and are not expected to incorporate into the vesicles' bilayers. Therefore, the percent incorporation values are an approximation of the volume percent of solution contained within crude SVs' lumen. This approximation does not account for possible ionic interactions between the analytes and the vesicles' surfaces. However, under the experimental conditions (pH = 7.4), pemetrexed is expected to be predominantly anionic and is not expected to associate with the surface of SDBS-rich vesicles. In addition, [analyte] in the various crude formulations were significantly different, but the % incorporation in the resulting purified samples were similar, indicating that the analytes are not simply binding electrostatically to the surface of vesicles. Based on TLC and ^{1}H-NMR analyses of purified vesicles, the estimated concentration of "available" dodecylbenzenesulfonate molecules (dodecylbenzenesulfonate molecules not paired with cetyltrimethylammonium molecules) in unpurified (crude) vesicles prepared in water is 2.8 mM and 4.0 mM for unpurified vesicles prepared in PBS.

Sample Calculation

Average concentration of sodium ion in purified vesicle samples = 1.2 mM.
Vesicles were diluted by a factor of two during SEC purification: 1.2mM x 2 = 2.4mM
(2.4 mM/200mM) x 100 = 1.2%
1.2% of 1 mL = 0.012 mL = total lumen volume in crude sample
Lumen radius of a vesicle with a total radius of 70 nm = Outer diameter – Leaflet = 70nm – 3nm = 67 nm
Lumen volume of vesicles with 70 nm radius = 1.26×10^{-15} mL
Concentration of vesicles (vesicles/mL) = 0.012 mL/1.26×10^{-15} mL = 9.5×10^{12}

Average vesicle concentration in crude vesicle samples based on encapsulation studies: 1.3×10^{13} vesicles/mL

IX. Acid Phosphatase per Vesicle and Percent Surface Area Coverage Calculations

Average acid phosphatase concentration in acid phosphatase functionalized vesicles: 31 μg/mL

The MW of acid phosphatase (from Sigma): 58 kDa

31μg/mL = 3.2×10^{14} acid phosphatase molecules/mL

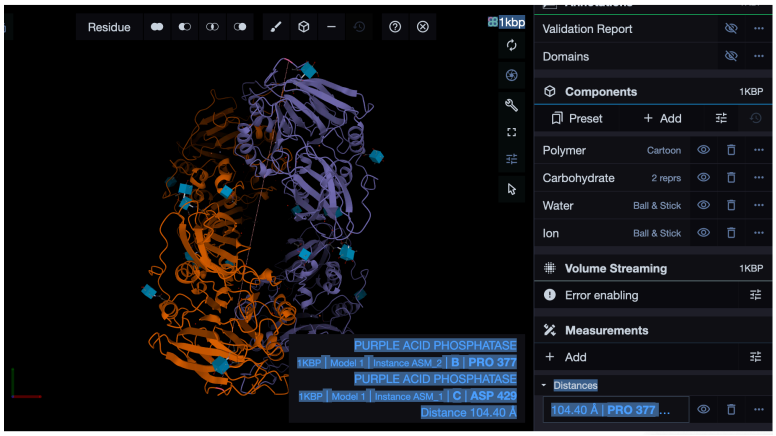
Average vesicle concentration in crude vesicle samples based on encapsulation studies: 1.3×10^{13} vesicles/mL

Vesicles were purified twice via SEC, resulting in a 4-fold dilution.

$1.3 \times 10^{13}/4 = 3.2 \times 10^{12}$ vesicles/mL in purified AP-SV samples

3.2×10^{14} acid phosphatase molecules/mL ÷ 3.2×10^{12} vesicles/mL = 100 acid phosphatase molecules/vesicle

Figure 36: Structure of purple acid phosphatase from PDB with estimated longitudinal diameter.



The longitudinal diameter is estimated to be 104.4 angstroms (10.4 nm).

Protein Data Bank in Europe, 30 July 2021, https://www.ebi.ac.uk/pdbe/entry/pdb/1kbp/

Surface area of vesicle with 140 nm diameter = $4\pi r^2 = 4\pi(70^2) = 62000$ nm^2

Area occupied by acid phosphatase (sphere with r = 5.2 nm) = $\pi r^2 = \pi(5.2^2) = 85$ nm^2

Approximate number of acid phosphatase molecules in a close-packed monolayer = 62000/85 = 730

Percent surface coverage on acid phosphatase functionalized vesicles = (100/730) x 100 = 14%