

Polymeric Crowding Agents Improve Passive Biomacromolecule Encapsulation in Lipid Vesicles

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Large solutes such as high molecular weight proteins can be difficult to encapsulate in lipid vesicles. Passive trapping of these macromolecular solutes during vesicle formation typically results in concentrations inside the vesicles that are much lower than in the external solution. Here, we investigated the effect of macromolecular crowding on passive encapsulation of biological macromolecules with molecular weights ranging from 52 kDa to 660 kDa within both individual giant lipid vesicles (GVs, $>3\ \mu\text{m}$ diameter) and populations of 200 nm diameter large unilamellar vesicles (LUVs). Fluorescently labeled biomacromolecules were encapsulated during vesicle formation in the presence or absence of three weight percent poly(ethylene glycol) (PEG; 8 kDa) or dextran 500 kDa, which served as crowding agents. Encapsulation efficiency of the labeled biomolecules was higher for the lower molecular weight solutes, with internal concentrations essentially equal to external concentrations for labeled biomacromolecules with hydrodynamic radii (r_h) less than 10 nm. In contrast, internal concentrations were reduced markedly for larger solutes with $r_h \geq 10$ nm. Addition of PEG or dextran during vesicle formation improved encapsulation of these larger proteins up to the same levels as observed for the smaller proteins, such that internal and external concentrations were equal. This observation is consistent with PEG and dextran acting as volume excluders, reducing the hydrodynamic radius of the biomacromolecules and increasing their encapsulation. This work demonstrates a simple and general route to improved encapsulation of otherwise poorly encapsulated macromolecular solutes in both GV and LUVs up to their concentration in the solution present during vesicle formation.

Introduction

Encapsulation of biological macromolecules within lipid vesicles is important for biophysical research and for a variety of applications such as pharmaceuticals, foods, and cosmetics.^{1–6} Although attractive interactions between solutes and the lipids used to encapsulate them can lead to high encapsulation efficiencies, such methods are only effective for certain solute/lipid combinations. In contrast, passive encapsulation (i.e., without attractive interactions) is more general but leads to internalization of solutes at or below their concentration in the vesicle formation solution. Solute size is a key determinant in the efficiency of passive encapsulation, with larger solutes excluded from the

vesicle interior such that the internal solute concentration is lower than the external concentration during vesicle formation.^{1,7–9}

Knowledge of solute concentrations in the vesicle interior is important for understanding processes as varied as enzymatic turnover, polymerization, crystallization, or phase separation occurring in this volume.^{4,10,11} It is also of interest for applications in which vesicles serve as carriers for solute delivery, such as in food formulations or therapeutics.^{5,6} Quantification of encapsulated solute is accomplished by different methods depending on the size of the vesicles. Encapsulation in large unilamellar vesicles (LUVs) and other submicrometer vesicles (SVs, which include both LUVs and small unilamellar vesicles, SUVs) is quantified in terms of the bulk encapsulation efficiency (BEE). BEE is the percentage of solute trapped within a batch of submicrometer vesicles as compared to the total amount of solute added. It is typically determined by removing all of the unencapsulated solute (e.g., via centrifugation or dialysis) and then lysing the vesicles to quantify the remaining (encapsulated) solute.^{1,9} This method does not provide information on variability in solute encapsulation between individual vesicles. Giant vesicles (GVs, $1\text{--}100\ \mu\text{m}$),¹² on the other hand, are not generally studied via bulk methods due to

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their limited stability, but are large enough to be analyzed individually.^{11,13–15} For example, the concentration of a fluorescently tagged solute encapsulated within an individual giant vesicle can be quantified by comparing the internal solute fluorescence to a calibration curve.¹³ The ability to determine encapsulation efficiency for individual GVs (EE_{ind}) also makes it possible to compare encapsulation between vesicles within a batch, which can vary widely.^{11,13–15} Recently, approaches to determination of EE_{ind} for submicrometer vesicles have been reported; these powerful new tools have not yet become routine.^{16,17}

The most common GV formation techniques, electroformation¹⁸ and gentle hydration,¹⁹ passively encapsulate solutes, such that the expected concentration inside (C_{in}) is equal to the external concentration during vesicle formation (C_{out}). For solutes with $r_h < 10$ nm, mean C_{in} does tend to be equal to C_{out} ; however, we and others have measured considerable variability in C_{in} for individual GVs in the same batch.^{13–15} Larger solutes are poorly encapsulated. For example, mean C_{in} was found to be much less than C_{out} for dextran polymers with hydrodynamic radius, $r_h > 10$ nm.¹⁴

Solute size is also an important determinant of entrapment in submicrometer vesicles.^{1,7–9} For example, Adrian and Huang compared the encapsulation of proteins of increasing molecular weight to that of a small marker protein (lysozyme, 14.1 kDa) in ca. 50 nm diameter vesicles and found decreasing trapping efficiency with increasing MW. The 97 kDa α -amylase was encapsulated only 50% as well as the lysozyme.⁷ The same authors reported that proteins up to 120 kDa could be encapsulated in ca. 200 nm diameter vesicles with the same trapping efficiency as sucrose.⁷ BEE is often quite low for passive uptake because it is defined in terms of the total encapsulated volume (capture volume, CV), which can be $< 1\%$ of the total volume, depending on the amount of lipid present and the morphology of the vesicles. Although CV can be increased up to ca. 50% by using more concentrated lipid,²⁰ this is not always possible for a given system. Indeed, most papers report BEEs much less than 50%.^{21–24} To increase BEE beyond what is possible by passive encapsulation, attractive interactions between solutes and the lipids themselves are commonly employed.^{20,22,25–27} For example, cationic lipids are routinely used to package nucleic acid drugs, despite concerns about their toxicity.^{27,28} Solute encapsulation based on electrostatic attractions can be essentially complete, with nearly 100% of all solute molecules added to the solution being associated with liposomes. Despite excellent loading, attractive interactions with encapsulating lipids

can lead to more complex liposome morphologies, prevent activity of the encapsulated solute until released, and inhibit effective release of solutes upon vesicle rupture.^{1,27,29} While these factors are often considered acceptable in drug delivery applications, it is preferable to have free encapsulated solute of known concentration for biophysical studies, e.g., to evaluate the effects of confinement.³⁰

Encapsulation of $r_h > 10$ nm fluorescently labeled dextran polymers in GV has been reported to improve markedly in the presence of several weight percent additional polymeric solutes.¹⁵ The additional polymers acted as volume excluders, resulting in condensation of the labeled solutes and allowing them to be more easily encapsulated during vesicle formation.¹⁵ Macromolecular crowding is an attractive means for improving macromolecule encapsulation because of its generality.³¹ This approach for improving encapsulation has not previously been tested for biomacromolecules such as proteins, which in many cases already exist in globular conformations and hence may not undergo as large a r_h change in response to volume excluders, nor has it been tested for encapsulation in submicrometer vesicles. Herein, we report the use of PEG as a macromolecular crowding agent to increase the encapsulation efficiency of fluorescently labeled biomacromolecules within GVs and 200 nm diameter LUVs. We find in general that the addition of PEG increases the encapsulation efficiency of high molecular weight (MW) biomolecules compared to those encapsulated in DI water alone. We conclude that volume exclusion in the presence of a crowding agent such as PEG or dextran can be used as a general route to increase encapsulation efficiency of biomacromolecules in both giant and submicrometer lipid vesicles, up to levels expected for passive encapsulation of lower MW solutes (i.e., $C_{in} = C_{out}$).

Results and Discussion

Volume exclusion induced condensation is expected to have the greatest impact on large, extended molecular conformations.^{31,32} Therefore, the macromolecules used in this work were chosen to give a range of molecular sizes and shapes. We first describe the effect of polymeric volume excluders on encapsulation in GVs formed by gentle hydration and then discuss encapsulation in LUVs prepared by a freeze–thaw/extrusion protocol.

Effect of PEG on Biomacromolecule Encapsulation Efficiency within Individual GVs (EE_{ind}). We began by encapsulating fibrinogen, a 340 kDa protein with a hydrodynamic radius (r_h) of ~ 11 nm, which is important in coagulation and thrombosis and is often administered to patients who have suffered severe blood loss or have lost the ability to clot blood on their own.^{33–35}

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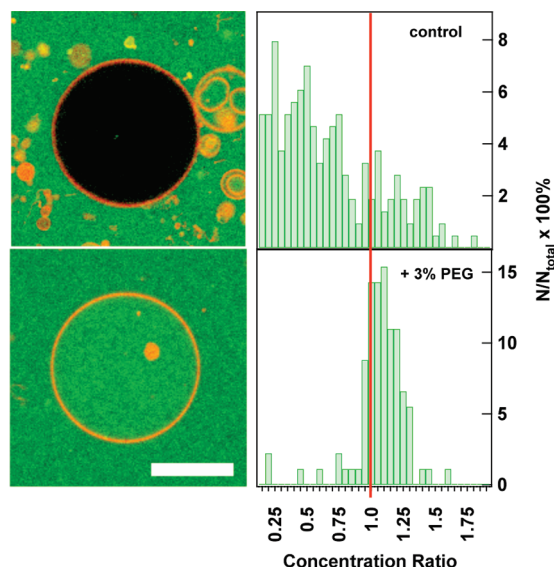


Figure 1. (Left) Confocal fluorescence images of vesicles prepared in the presence of AF488-labeled fibrinogen without (top) and with (bottom) 3 wt % PEG 8 kDa. Membrane composition was 3:7 egg PC:DOPG with 0.05 mol % DOPE-rhodamine. Red represents rhodamine fluorescence, and green represents AF488 fluorescence. The scale bar is 20 μm . (Right) Histograms of the concentration ratio ($C_{\text{in}}/C_{\text{out}}$) for encapsulation of labeled fibrinogen in individual GVs without (top) and with (bottom) 3 wt % PEG 8 kDa ($C_0 = 33 \text{ nM}$).

Fibrinogen has been reported to condense to approximately one-half of its original length during polymerization.^{34,36}

Fibrinogen labeled with Alexa Fluor 488 (AF488) was added during GV formation at a concentration of 33 nM, either in DI water or in a 3 wt % PEG 8 kDa solution. GVs were imaged without removing them from the hydration solution, such that the external fibrinogen concentration remained 33 nM. Figure 1 shows representative confocal fluorescence images and histograms of encapsulation efficiency for individual GVs formed in the presence of fibrinogen alone and coencapsulated with 3 wt % PEG. Here, encapsulation efficiency for the individual vesicles (EE_{ind}) is defined as the concentration ratio between the vesicle interior (C_i) and the external solution (C_o) used during vesicle formation. The concentration ratio (C_i/C_o) for each vesicle was plotted against the number of vesicles at each ratio. A vertical line is included at $C_i/C_o = 1$ to facilitate comparison to the external fibrinogen-AF488 concentration. In the absence of added PEG, encapsulation efficiency is quite poor; the GV interiors appear dark, and fibrinogen-AF488 concentration varied considerably from vesicle to vesicle, ranging from 1 to 54 nM ($0.04\text{--}1.64 \times C_o$) with a mean of 21 nM ($0.64 \times C_o$). In contrast, vesicles formed in the presence of 3 wt % PEG contained fibrinogen-AF488 concentrations similar to that of the external solution, with 65% of the GVs encapsulating internal concentrations between 28 and 38 nM and a mean of 34 nM.

We repeated this experiment with two other proteins having relatively large r_h to test the generality of the method of increasing EE_{ind} via macromolecular crowding. Thyroglobulin is a 660 kDa protein with a pI of 4.6 that is important in the synthesis, storage,

and secretion of thyroid hormones.^{37,38} The native structure has two slightly elongated subunits, with an r_h of 10 nm.³⁹ In vivo, thyroglobulin structure is known to condense in order to increase the storage capacity of the thyroid.⁴⁰ Encapsulation of thyroglobulin-AF488 (18 nM) in GVs in the absence and presence of 3 wt % PEG 8 kDa produced results similar to those for fibrinogen-AF488 (Supporting Information Figure 1), which had similar r_h and ability to compact. We then repeated the experiment with catalase-AF488, a 250 kDa enzyme with a pI of 5.4 and an r_h of 10.4 nm.^{41,42} This protein catalyzes the decomposition of hydrogen peroxide to water and oxygen,⁴³ and has been encapsulated in submicrometer liposomes along with superoxide dismutase and injected to protect against oxygen toxicity in rats.⁴⁴ Images and a histogram for C_{in} are shown in Supporting Information Figure 2. Table 1 summarizes encapsulation results for each of the proteins. EE_{ind} for was significantly increased vs control by addition of PEG 8 kDa during encapsulation of all three of these relatively large proteins (Student's t test, $p < 0.001$). These results are consistent with the role of PEG 8 kDa as a volume excluder, driving the condensation of macromolecular structures.^{45–53} Proteins, nucleic acids, and enzymes are all known to adopt condensed structures in macromolecularly crowded solutions.^{45–55} Thus, we interpret the improved encapsulation of these high molecular weight proteins in 3 wt % PEG 8 kDa as the result of their condensation to give reduced hydrodynamic radii, which in turn caused them to be encapsulated as effectively as lower MW biomolecules.

We next evaluated the effect of PEG 8 kDa on encapsulation of two smaller proteins and a DNA oligomer, all of which had hydrodynamic radii less than 10 nm. α -1-antitrypsin (AAT) is a 52 kDa protein with an r_h of 3.8 nm and a pI between 4.9 and 5.1.^{57,58} It is responsible for inhibiting elastase in vivo, which destroys elastin on connective tissues.⁵⁷ AAT deficiency often leads to lung diseases, and clinical trials have been reported where patients are given AAT infusions, which showed beneficial effects.⁵⁸ It has also been delivered to mice via encapsulation in submicrometer liposomes.⁵⁹ We began by encapsulating 1.9 μM

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Table 1. Effect of Crowding Agents on Encapsulation of Biomolecules in Giant Vesicles

solute (concentration)	hydrodynamic radius (nm)	crowding agent	Percent theoretical EE_{ind} (%) ^d
Fibrinogen ^c ; 340 kDa (33 nM)	11 ^{33–35}	none	64 ± 39
		3% PEG ^a	104 ± 21
		3% dextran ^b	101 ± 15
Thyroglobulin ^c ; 660 kDa (18 nM)	10 ^{38,39}	none	67 ± 33
		3% PEG ^a	100 ± 26
		3% dextran ^b	53 ± 30
Catalase ^c ; 250 kDa (0.13 μM)	10.4 ^{41,42}	none	106 ± 15
		3% PEG ^a	103 ± 4
		3% dextran ^b	102 ± 4
α-1-Antitrypsin ^c ; 52 kDa (1.9 μM)	3.8 ^{56,57}	none	103 ± 4
		3% PEG ^a	107 ± 9
		3% dextran ^b	98 ± 3
Human Serum Albumin ^c ; 67 kDa (3.3 μM)	3.5 ^{56,57}	none	103 ± 10
		3% PEG ^a	104 ± 5
		3% dextran ^b	104 ± 5
173 nt ssDNA ^f ; 53 kDa (0.24 μM)	< 7.5 ^g	none	103 ± 10
		3% PEG ^a	104 ± 5
		3% dextran ^b	104 ± 5

^a 8 kDa. ^b 500 kDa. ^c Alexa Fluor 488 – labeled. ^d Theoretical EE_{ind} defined as the solute concentration in the external solution during vesicle preparation, which is given in parentheses for each solute in the first column. ^e FITC – labeled; ^f fluorescein – labeled; ^g Hydrodynamic radius of 7.5 nm for 280 nt ssDNA was reported in ref 66 under conditions where excluded volume effects can be neglected; extrapolation of length vs r_h data in ref 66 suggests ~5 nm hydrodynamic radius for our 173 nt sequence.

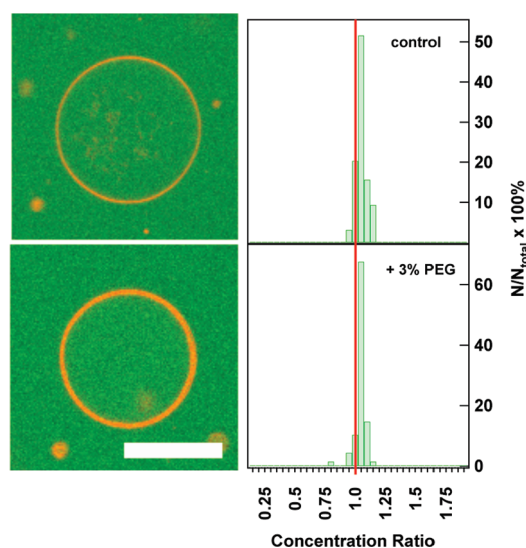


Figure 2. (Left) Confocal fluorescence images of vesicles prepared in the presence of 1.9 μM labeled α-1-antitrypsin without (top) and with (bottom) 3 wt % PEG 8 kDa. Membrane composition was 3:7 egg PC:DOPG with 0.05 mol % DOPE-rhodamine. Red represents rhodamine fluorescence, and green represents AF488 fluorescence. The scale bar is 20 μm. (Right) Histograms of the concentration ratio (C_{in}/C_{out}) for encapsulation of labeled α-1-antitrypsin in individual GVs without (top) and with (bottom) 3 wt % PEG 8 kDa ($C_o = 1.9 μM$).

AAT-AF488 in GVs formed in with the presence and absence of 3 wt % PEG 8 kDa. AAT-AF488 was encapsulated at essentially $C_{in} = C_{out}$ with or without 3% PEG (Figure 2).

Similar results were observed for encapsulation of labeled human serum albumin (HSA) in PEG solution as compared to DI water (Supporting Information Figure 3). HSA has similar characteristics as AAT (i.e., HSA has a MW of 67 kDa, pI of 4.9–5.1, and r_h of 3.51).^{56,57} We also studied the encapsulation of a single-stranded 173 base pair oligomer (53 kDa) labeled with fluorescein (FAM). Nucleic acids are often encapsulated in submicrometer vesicles for gene delivery and in GVs to study biochemical reactions.^{27,28,60} DNA is known to undergo condensation in the presence of divalent cations, polycations, or even neutral polymers such as PEG.^{54,55} This oligonucleotide was

sufficiently small to be effectively encapsulated in the GV even without addition of a crowding agent (Supporting Information Figure 4).

Table 1 compares encapsulation for each of the biomacromolecular solutes with and without added PEG. In addition to the increase in C_{in} for the larger proteins when encapsulated in the presence of 3 wt % PEG, decreased heterogeneity in C_{in} across a population of vesicles in a batch is suggested by the standard deviations in C_{in} (Table 1) and the histograms for individual solutes (Figures 1 and 2 and Supporting Information Figures 1–4). An F-test⁶¹ was performed to evaluate whether differences in the variance between the control and + PEG populations were statistically significant. We found statistically significant changes in variance for the fibrinogen ($p < 0.001$), catalase ($p < 0.001$), and AAT ($p < 0.05$), but not for the other solutes. The increase in encapsulation homogeneity when fibrinogen and catalase were coencapsulated with a crowding agent arises largely from the shift to higher encapsulated concentrations. Decreased variance for AAT may also be due in part to condensation, which has been reported for AAT and other small polymers,^{62,63} but is more likely due to PEG increasing the viscosity and osmotic pressure of the solutions, as has been discussed previously.^{15,64} For example, increased viscosity could improve encapsulation heterogeneity by facilitating pore formation in the membrane, allowing more time for solutes to equilibrate between internal and external solutions.⁶⁵

We note that the proteins were not found to be associated with the lipid membrane of the GVs, but rather to be uniformly dispersed in the exterior and/or interior of the vesicles (see, for example, Figures 1 and 2, left panels). Polymerization or aggregation on microscopically visible length scales was also not observed for any of the biomacromolecular solutes. Preparation of the vesicles in 3% PEG did not inhibit their formation or change their size or morphology in any obvious way as compared to preparation in PEG-free solution, consistent with our previous findings of

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Table 2. Bulk Encapsulation Efficiency in Submicrometer Vesicles

solute (concentration)	hydrodynamic radius (nm)	crowding agent	% of theoretical BEE based on capture volume ^{a,b}
Carboxyfluorescein; 376 Da (53 μ M)	0.5 ⁶⁸	none	72 \pm 3
		3% PEG 8 kDa	64 \pm 3
FITC-dextran; 500 kDa (0.82 μ M)	15 ^{9,15}	none	31 \pm 18
		3% PEG 8 kDa	118 \pm 46
thyroglobulin-AF488; 660 kDa (0.14 μ M)	10 ^{38,39}	none	50 \pm 5
		3% PEG 8 kDa	89 \pm 21

^a Theoretical BEE based on capture volume = 0.38%. ^b Errors represent the standard deviation of BEE measurements taken for three separate batches of vesicles on three different days.

decreased average vesicle diameter at 10 and 20 wt % PEG but no noticeable difference between 0 and 3 wt % PEG in terms of GV formation, size, or morphology.¹⁵

The improvements in EE_{ind} observed in the presence of PEG should be applicable to other crowding agents.¹⁵ We therefore coencapsulated fluorescently labeled fibrinogen and α -1-antitrypsin with 3 wt % dextran 500 kDa for comparison. The 3 wt % dextran 500 kDa had the same effect on encapsulation as PEG 8 kDa: the higher MW protein, fibrinogen, showed an increase in EE_{ind} magnitude and homogeneity with the introduction of either PEG or dextran; and the lower MW protein, AAT, showed essentially no change in mean EE_{ind} , but a decreased variance (F-test, $p < 0.05$) when dextran was added (Table 1).

Effect of PEG on Bulk Encapsulation Efficiency (BEE) in Submicrometer Vesicles. Internal solute concentrations in SV cannot be determined via simple confocal imaging as for the GV described above. Therefore, we estimated C_{in} and the effectiveness of solute encapsulation by comparing the percentage of total solute that has been encapsulated within a batch of vesicles (bulk encapsulation efficiency, BEE) to the capture volume. Capture volume was estimated based on the moles of lipid present and the size of the vesicles, assuming unilamellarity. LUVs 200 nm in diameter were prepared by a freeze–thaw/extrusion protocol. BEE was determined by removing the unencapsulated solute from a batch of vesicles, then lysing the vesicles and quantifying the remaining (encapsulated) solute. For effective passive encapsulation, one can expect to achieve the same concentration inside the vesicles as is present outside ($C_{in} = C_{out}$). However, for high MW solutes, BEE is often less than the capture volume (i.e., interior concentrations < exterior concentrations).^{1,7,9}

Table 2 shows the average BEE for three encapsulated solutes (carboxyfluorescein (CF), 500 kDa dextran-FITC, and thyroglobulin-AF488) with and without added PEG in 200 nm diameter vesicles. The theoretical BEE for passive encapsulation in this system is 0.38%, calculated by estimating the number of vesicles formed based on the amount of lipid added to the system (500 μ M) and then calculating the volume encapsulated by those vesicles as compared to the initial volume. We estimated that there were 3.4×10^5 lipid molecules per 200 nm diameter vesicle, for 4.5×10^{12} vesicles that collectively encapsulated 19 μ L, or 0.38% of the total solution volume (5 mL), based on calculations for similar systems.^{1,67} Dynamic light scattering verified that vesicle diameter remained constant whether in dilute or crowded solution, and the addition of a fluorescent lipid in the membrane verified that a negligible amount of lipid was lost to the filter during extrusion.

Encapsulation of the small fluorescent molecule, carboxyfluorescein (CF), was performed as a control. It was expected that the effect of the coencapsulation of PEG would be negligible, since the addition of PEG did not significantly impact the encapsulation efficiency of CF in GVs.¹⁵ The change in experimental CF BEE observed between the solutions with and without PEG was not statistically significant based on a Student's t test ($p = 0.63$) (Table 2).

Encapsulation of FITC-dextran 500 kDa and thyroglobulin-AF488 did show improvement for LUVs prepared in solutions containing PEG 8 kDa as compared to solutions without PEG (Student's t test, $p < 0.05$). Experimentally determined BEE for FITC-dextran 500 kDa and thyroglobulin-AF488 in water were 31% and 50% of the theoretically predicted value, respectively – these low BEEs are most likely due to the large size of dextran 500 kDa ($r_h \sim 15$ nm) and thyroglobulin ($r_h \sim 10$ nm).^{9,15,38,39} Addition of 3 wt % PEG 8 kDa significantly increased the BEE of FITC-dextran and thyroglobulin-AF488 in these vesicles, consistent with condensation of the dextran and thyroglobulin structures via macromolecular crowding.¹⁵ Although the error is large, it appears that with PEG 3%, the LUVs are essentially encapsulating even these very large solutes at $C_{in} = C_{out}$. BEE was increased without chemically modifying the vesicle or solute to utilize specific attractive interactions. In contrast to the GV encapsulation data, improvements in standard deviation were not observed in the LUV experiments. This underscores the difference in the two measurements: the error in GV C_{in} measurements indicates vesicle-to-vesicle variability within a batch, while variability in LUV BEE experiments reports on differences between entire batches prepared separately and as such may reflect variability in capture volume. CF BEE was less than 100% despite its small size. We interpret this as the result of some CF loss from the vesicle interior during centrifugation and/or other handling, which is commonly reported in submicrometer vesicles.⁶⁹

These data are exciting, because while the majority of reports published on BEE utilize specific interactions between liposome/solute, this method is quite general and should apply to a variety of solutes that can be condensed under conditions of macromolecular crowding. Although the absolute BEE in these experiments was low, we have demonstrated that addition of 3 wt % PEG provides a means of improving encapsulation for large solutes such that $C_{in} = C_{out}$ for LUVs prepared by freeze–thaw/extrusion. Increasing the amount of lipid used in a batch has been shown by others to increase CV from <1% to as high as 50%; which would lead to an increase in BEE, as previously reported.²⁰

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Conclusions

Our results indicate that the addition of the polymeric coencapsulants PEG 8 kDa and dextran 500 kDa as macromolecular crowding agents during passive encapsulation of biomacromolecular solutes can substantially increase mean encapsulated concentration and in some cases homogeneity for otherwise poorly encapsulated high MW proteins in giant vesicles. The addition of a crowding agent also reduced vesicle-to-vesicle encapsulation variability for the lower MW AAT, which was already encapsulated efficiently (i.e., with $C_{in} = C_{out}$) in dilute solution. The mechanism for increased encapsulation is general, such that crowding agents need not be varied with the identity of the solute. This work is important to those using GV's as bioreactors, where knowledge of encapsulated concentration is critical to quantifying the progression of products formed during a reaction. Measurements of bulk encapsulation efficiency (BEE) in LUVs in the presence of PEG suggest that this approach works similarly in these smaller vesicles as for GV's, bringing the internal solute concentration closer to the external concentration such that the BEE is determined by capture volume. The approach used here should be effective for a wide range of membrane/macromolecular solute combinations.

Materials and Methods

Chemicals and Materials. L- α -Phosphatidylcholine (egg PC), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)](sodium salt) (DOPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-dioleoyl-*sn*-glycero-3(phosphoethanolamine-*N*-lissamine Rhodamine b sulfonyle) (ammonium salt) (Rhodamine-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Carboxyfluorescein, fluorescein isothiocyanate (FITC)-labeled and unlabeled dextrans and poly(ethylene glycol) (PEG) were obtained from Sigma. Alexa Fluor 488 and FITC labeling kits along with fibrinogen-Alexa Fluor 488 were purchased from Molecular Probes, Inc. (Eugene, OR). All other proteins were from Calbiochem (San Diego, CA). A fluorescein nucleic acid labeling kit was purchased from Mirus Bio (Madison, WI). A 173 nucleotide single-stranded oligomer with the sequence for influenza B was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Water used in these experiments was deionized to a resistance of 18 M Ω with a NANOpure Diamond water system from Barnstead Int. (Dubuque, IA).

Preparation of Giant Lipid Vesicles. We used the gentle hydration method to form lipid vesicles, as previously described.^{14,15,19} Silanized borosilicate glass culture tubes, 12 \times 75 mm, were used to prevent biomolecules from sticking to the glass surface during formation (Kimble Chase, Vineland, NJ). We first prepared a chloroform solution of lipids containing a 3:7 molar ratio of egg PC:DOPG at a concentration of 0.26 mg/mL with 0.05 mol % Rhodamine-DOPE. Lipid solutions were dried under Ar (g) to form a thin, uniform lipid film, and then, the vials were vacuum-desiccated for \sim 2 h to remove any residual organic solvent. Then, an aqueous solution containing a fluorescent biomolecule with or without 3 wt % unlabeled PEG or dextran heated to 37 $^{\circ}$ C was added along the wall of the tube, and the lipids were hydrated 48 h at this temperature. We chose 3 wt % PEG 8 kDa based on prior work with dextran 500 kDa condensation, in which we observed increased encapsulation efficiency in GV's up to 10 wt % PEG, with the bulk of the increase achieved by 3% and decreased average GV diameter for \geq 10 wt % PEG.¹⁵ Following incubation, the solutions were allowed to cool to room temperature. The contents of the vials, which remained undiluted, were removed with a micropipet and transferred to a microscope slide silanized with *N*-(triethoxysilylpropyl)-*o*-polyethylene oxide urethane (Gelest, Inc., Morrisville, PA) for analysis. Coverslips were also coated with *N*-(triethoxysilylpropyl)-*o*-polyethylene oxide urethane to prevent the adhesion of fluorescently labeled biomolecules to the glass surface.

The concentrations of crowding agents are referred to in wt %, since this is the common notation used for crowding agents in the macromolecular crowding literature; the 3% PEG 8 kDa used here corresponds to 3.75 mM. Concentrations for fluorescently labeled solutes, which are considerably lower than for the crowding agents, are given as molarities.

Determination of Encapsulated Solute Concentrations in Giant Vesicles. A fluorescence line scan was taken across each vesicle to determine the fluorescence intensity of the Alexa Fluor 488, FITC, or fluorescein inside the vesicle (I_i) and in the undiluted bulk solution (I_o , outside the vesicle), as reported previously.^{14,15} The concentration of biomolecule inside (C_i) and outside (C_o) the GV was determined directly from the intensities (I_i , I_o) by a calibration curve of the labeled biomolecule free in solution. From the calibration curves, it was determined that the bulk concentration (C_o) was equal to the initial concentration used to hydrate the vesicles. A ratio of C_i/C_o was taken to determine the EE_{ind} .

Microscopy. Imaging was performed using an LSM-5 Pascal laser scanning confocal microscope from Carl Zeiss, Inc. (Oberkochen, Germany) with a Plan-Apochromat 63 \times oil immersion objective (1.4 NA), and Pascal software as previously reported.^{14,15} Scan time was 1.97 s for a 512 \times 512 pixel image. Only vesicles that were greater than 3 μ m in diameter and appeared to be uni- or oligolamellar were analyzed.

Preparation of Submicrometer Lipid Vesicles. DPPC/cholesterol vesicles (80:20 mol ratio) were prepared using a modified technique provided by the distributor to yield large unilamellar vesicles by extrusion (LUVET) as previously reported.^{16,70} Briefly, DPPC and cholesterol were combined with chloroform in a round-bottom flask and evaporated for 4 h. Following this, 5 mL of the solute to be encapsulated was added in either water or 3 wt % PEG 8 kDa to yield a final lipid concentration of 0.34 mg/mL. The lipid mixture was allowed to incubate in this solution at room temperature for 30 min. Then, the flask was subjected to five freeze/thaw cycles to promote entrapment of water-soluble compounds into the vesicles.⁷¹ The mixture was then extruded through a 0.2 μ m polycarbonate membrane for a total of 20 passes (Mini Extruder Kit, Avanti Polar Lipids, Inc., Alabaster, AL) to yield a monodisperse suspension of unilamellar vesicles. Excess solute was removed from the vesicle suspension by washing with a 10 000 MW dialysis cassette in DI water for 48 h (Slide-A-Lyzer, Thermo Scientific, Rockford, IL) and then centrifuging at 16 100 \times g, removing the supernatant, and replacing it with a solution free of fluorescent molecules. Vesicle diameter was confirmed using dynamic light scattering (Zetasizer Nano S, Malvern Instruments, Worcestershire, U.K.).

Determination of Bulk Encapsulation Efficiency in Submicrometer Vesicles. After centrifugation, the vesicles were lysed in 0.5% Triton-X 100, and the encapsulated concentration was determined via fluorescence measurements on a Jobin Yvon Horiba FL3-21 fluorimeter.

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Supporting Information Available: Confocal images and histograms of EE_{ind} data for thyroglobulin, catalase, human serum albumin, and the 173 nt oligomer. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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