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Time-Resolved Loading of Monomers into Bilayers with Different Curvature.

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Abstract

Directed assembly of nanostructures within temporary and recyclable self-assembled scaffolds is emerging as an attractive method for the synthesis of nanomaterials with programmed properties. Understanding interactions of building blocks with amphiphilic scaffolds is critical for rational design of new nanostructures and nanodevices. Here we examine loading of hydrophobic monomers into bilayers with different curvatures. Time-resolved loading was studied by high performance liquid chromatography and dynamic light scattering. Despite differences in initial bilayer geometry, loading rates and maximum bilayer capacity are the same for liposomes with radii ranging from 25 to 100 nm. When using divinylbenzene (DVB) and dimyristoylphosphatidylcholine (DMPC), monomer/lipid loading ratio of 1.2 was achieved within 12 hours. While accommodation of a large amount of monomers is likely to be accompanied with significant changes in bilayer structure, all liposomes in this study including those with smallest size and higher bilayer curvature retain encapsulated content and show no evidence of fusion during monomer loading. These results contribute to our understanding of interactions between hydrophobic molecules and lipid bilayers and expand the scope of the directed assembly method.

Introduction

Application of self-assembled amphiphilic scaffolds for directed assembly of functional nanomaterials has been gaining considerable interest in the recent years.¹⁻¹³ We have used lipid bilayers for the synthesis of rigid organic nanodisks and nanothin membranes containing nanopores with programmed size and chemical environment.¹⁻³ Multiple groups have used liposomes previously to form polymer nanocapsules.⁴⁻¹¹ Ryoo used surfactants to direct the assembly of zeolite nanosheets.¹² Understanding interactions between building blocks and temporary scaffolds is critical for gaining control over the structure of supramolecular assemblies and, ultimately, for creating nanomaterials and nanodevices with programmable properties.

We have previously reported controlled loading of monomers into bilayers made of saturated phospholipids.¹³ We found that the loading of acrylates and styrene derivatives occurred on a timescale of several hours and the initial loading rate is inversely proportional to the aqueous solubility of monomers. Interestingly, while the monomer/lipid molar ratio in fully loaded liposomes varied among different monomers, the total volume of monomers was constant for each lipid.

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Supporting Information Available DLS data for loading experiments at 12 °C, calibration curve of calcein emission vs. concentration.

These studies help us gain control over critical parameters necessary to produce nanocapsules consistently for practical applications, such as drug delivery. However, many questions remain to be answered for complete understanding of interactions between lipids and monomers. In particular, little is known about influence of bilayer curvature on loading. Both fundamental and applied aspects of this relationship are important. Higher curvature is associated with higher strain in the bilayer.¹⁴⁻¹⁶ Are highly curved bilayers capable of accommodating monomers without collapse? Is bilayer capacity affected by its curvature? One can hypothesize that more curved bilayers would accommodate less monomers. Is this hypothesis valid? Do hydrophobic molecules enter a curved bilayer faster? Is there a measurable relationship between bilayer curvature and loading kinetics? On a more practical note, most reports so far used liposomes with 100 nm diameter to template nanocapsules. What if one needed to reduce the capsule size two-fold to increase the transcellular mobility or increase the size two-fold to increase the encapsulation capacity?

This work aims at answering these questions. To perform a time-resolved study, we need a rapid and accurate method for the determination of the amount of monomers incorporated in the bilayer. We also need to examine the integrity of liposomes during monomer loading.

We selected liposomes with radius ranging from 25 to 100 nm (Figure 1). There are several reasons for this selection: the curvature changes dramatically with radius change in this region; this size range is especially intriguing for nanocapsule applications; and unilamellar liposomes with a narrow size distribution can be reliably prepared by conventional techniques.¹⁷

We used divinylbenzene (DVB) as a representative non-volatile hydrophobic monomer and dimyristoylphosphatidylcholine (DMPC) as a representative saturated phospholipid.

Experimental Details

Chemicals

All solvents used were HPLC grade. The phospholipid (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids, Inc. as a dry powder. Calcein, *p*-divinylbenzene (DVB), methanol and Sephadex G-50 (medium) were purchased from Sigma-Aldrich. DVB was passed through alumina column to remove the inhibitor. Sephadex G-50 (10g) was swollen in 120 ml of water in a glass screw-capped bottle for at least 5h at room temperature and stored at 4°C until required for use. All other chemicals were not further purified before use.

Small Unilamellar Liposome Preparation

Unilamellar liposomes were prepared by the extrusion method.¹⁷ DMPC (100 mg) was hydrated with 5 mL of deionized water to produce a solution of multilamellar liposomes. The solution was then extruded through a polycarbonate membrane with 0.05, 0.1, or 0.2 μ m pores using an Avanti Mini-Extruder (Avanti Polar Lipids).

Liposome Loading

5 mL of liposomes of each size were transferred into a 20 mL glass vial containing a 5×2 mm PTFE coated stir bar. Vials were placed in a water bath at 12 °C or 30 °C. The solution of liposomes was allowed approximately 30 minutes to equilibrate with its temperature-controlled environment. After temperature equilibration, 500 μ L of DVB was added each vial. The vial was sealed with the cap (polypropylene with pulp-backed metal foil liner). All experiments were shielded from light during the duration of the loading, except for taking aliquots for analysis. Excess DVB was clearly visible floating atop the aqueous liposome

solution throughout every experiment. Each sample was stirred gently (60-90 rpm) to prevent the formation of a monomer emulsion in the liposome solution. Temperature was checked every hour throughout the procedure.

High Performance Liquid Chromatography (HPLC)

50 μ L of sample was carefully taken from the bottom of the vial to avoid contamination with excess monomer and mixed with 950 μ L of methanol to lyse liposomes. 20 μ L of the resulting mixture sample was injected into a split-mode injector. Analytical HPLC was performed with a Waters 600 pump and Waters 2487 dual wavelength UV-vis detector. The detection wavelengths used were 250 and 270 nm. The column was a Nova-Pak C18, 3.9mm diameter $\times$ 150mm length. HPLC grade methanol was used as the mobile phase. The flow rate was 2 mL/min. Samples and concentration standards were run at least 5 times for each measurement, and the data were averaged. When necessary, the experimental samples were diluted further so that the signals would fall within a range of concentrations where the detector response was highly reproducible and the relationship between area and concentration could be modeled with a simple polynomial. Standard samples of known concentrations of DVB were prepared by serial dilution of a stock DVB solution in methanol and were analyzed by HPLC as described above. A calibration curve was produced by fitting HPLC data from DVB solutions in methanol at different concentrations via the least-squares fitting method, and was used to interpolate between the points.

Dynamic Light Scattering (DLS)

Hydrodynamic diameter and polydispersity index (PDI) measurements were performed on a Malvern Nano-ZS zetasizer (Malvern Instruments Ltd., Worcestershire, U.K.). The Helium-Neon laser, 4mW, operates at 633 nm, with the scatter angle fixed at 173°, and the temperature at 25°C (for experiments above the transition temperature of DMPC). 80 μ L samples were taken from the reaction vials with a pipette and were placed into disposable cuvettes without dilution (70 μ L, 8.5mm center height Brand UV-Cuvette micro). Each data point was an average of 10 scans.

Fluorescent analysis

Liposomes were prepared as described above in the presence of calcein (50 mM). Non-entrapped calcein was removed by size-exclusion chromatography.¹⁷ A column was prepared by filling a disposable 3 ml syringe with 3 ml of hydrated Sephadex G-50. Column was placed in a culture tube and was spun in a bench centrifuge (VWR Clinical 50) at 1500 rpm for 3 min to remove excess of water. Liposome suspension (0.6 ml) was added dropwise onto column. Column was then spun in a bench centrifuge at 1500 rpm for 5 min. The size-exclusion separation was repeated twice with freshly prepared columns to ensure complete removal of the untrapped dye from the liposome solution. Steady-state fluorescence measurements were performed with SLM 8000 (SLM/Aminco, Urbana, IL) spectrometer, which was upgraded and interfaced to computer by OLIS, Inc. (Jefferson, GA). Emission spectra were recorded in the 450 to 670 nm region with 490 nm excitation. Emission and excitation slits were 2 nm.

Results and Discussion

Measurement of monomers

We have previously described a method for measurement of monomers in bilayers based on the GC analysis of monomers extracted from liposomes.¹³ The GC-based method is accurate and well suited for monitoring polymerization. However, it requires extraction of monomers with a non-polar organic solvent, such as hexane, to avoid fouling the GC column with lipids and buffer salts. The extraction typically takes a few hours.

To enable a time-resolved study of monomer loading with adequate resolution, we developed and validated a rapid HPLC-based method for measurements of the amount of monomers. In this method, an aliquot of liposome solution is taken from the bottom of the vial to avoid contamination with free monomers and is diluted with methanol (approximately 1:20). The solution is analyzed on an HPLC instrument equipped with a UV detector. Separation is performed on a reverse-phase column (C18, 3.9×150 mm) using methanol as mobile phase. When the flow rate is set to 2 ml/min, the retention time for divinylbenzene is approximately 1.5 min (Figure 2).

The amount of monomers is determined from the UV absorption of monomers using a calibration curve obtained from a dilution series of divinylbenzene in methanol (Figure 2, inset). The entire measurement from taking an aliquot to obtaining the result and preparing the column for next injection takes less than 5 min. We chose to sample the liposome solution every 15 min. and perform repeated HPLC measurements in real time. On the time scale of several hours 15 min resolution is adequate for a detailed time-resolved study. In practice, the sampling rate could even be higher since samples diluted with methanol can be stored for extended time before HPLC analysis.

We believe the rapid HPLC method described here will be useful for other studies involving temporary self-assembled scaffolds for directed assembly of nanostructures. Since the sample handling includes dilution in methanol, adjusting the dilution factor and injection volume will ensure the accuracy of measurement and enable detection in a broad range of concentrations. Loading kinetics and bilayer capacity depend on the combination of monomers and lipids. Previously reported difficulties with reproducibility of nanocapsule synthesis are likely to arise from inconsistent monomer loading.^{10,13} Optimal conditions for monomer loading can be easily established using the analytical method described here.

Loading kinetics and bilayer capacity

In all loading experiments, excess of DVB was added to a freshly prepared solution of liposomes extruded through polycarbonate membranes with 0.05 μm , 0.1 μm , or 0.2 μm pores. The solution was gently stirred (60-90 rpm), and aliquots were taken every 15 min for HPLC and DLS analysis. Figure 3 shows the aggregate time-resolved loading data for three sizes of liposomes performed at 30 °C, which is above the phase transition temperature of DMPC (23 °C).¹⁸

Neither kinetics nor maximum loading capacity is affected by the bilayer curvature. It appears that the rate-limiting step in the loading process is the reorganization of the bilayer for monomer accommodation. In all likelihood, this is accompanied by the translocation of lipids. Accommodating hydrophobic monomers within the bilayer interior increases the energy barrier for the translocation. This process gradually lowers the rate of monomer loading until the loading effectively stops and the bilayer reaches its full capacity.

Loading experiments were also performed at 12 °C, which is below the phase transition temperature of DMPC. The loading occurs approximately 1.5-2 times slower than at 30 °C, which is quite reasonable considering the 18 °C temperature difference. In fact, the difference in kinetics can be explained by the temperature difference alone, and there seems to be no noticeable contribution from the change in aggregate state of the bilayer. We and others have reported a lowering of phase transition temperature of lipids due to accommodation of non-polar molecules.^{13,19} In this experiment, it is likely that enough monomers were quickly loaded into the bilayer to cause the transition from gel phase to the fluid phase. The remaining monomers were then loaded into the fluid-state bilayer. Interestingly, there is a difference in the initial loading rates (within the first six hours) between small and large liposomes in this study (Figure 4). This may be indicative of

curvature effect on the phase transition temperature of lipids. Detailed study of phase transition temperature changes due to monomer loading is outside the scope of this work but merits further investigation,

At both 12 °C and 30 °C, there is little difference in the overall loading rate and bilayer capacity among liposomes with different sizes. However, the maximum loading capacity at 12 °C was lower than at 30 °C, which confirmed our preliminary data reported earlier.¹³ This observation supports the hypothesis that the bilayer capacity is limited by the hydrophobic barrier for the translocation of lipids.

Loading curves can be fitted to a first-order kinetics equation. Loading rate constants calculated at 12 °C and 30 °C yield approximate values for the energy barrier of monomer loading in the 43-64 kJ/mol range.

In practical terms, when using DMPC and DVB or similar monomers, overnight loading at ambient temperature appears to be sufficient to achieve maximum loading. We found that after the first 12 hours there was little variation in the amount of monomers within the bilayer.

The fact that the loading capacity is independent of bilayer curvature has important implications for the synthesis of nanothin materials and nanocapsules. In particular, one can expect that the properties of polymer nanocapsules prepared using liposomes with a broad range of sizes will be identical. These findings validate and expand the scope of the nanocapsule synthesis method published previously.^{2,3}

Liposome size and integrity

Throughout each experiment, every sample was subjected to DLS analysis, providing a value for the average size and the polydispersity index (PDI) of the liposomes in the sample. For the purpose of determining the stability of the liposome population during the experiment, we monitored how the average size changed over time. In a control experiment, blank liposomes were measured at the same time intervals. Loading of monomers was associated with the slight increase in liposome size, consistent with bilayer reorganization necessary to accommodate monomers. The timescale for the size increase parallels that of the loading curves on Figure 3. Virtually all of the size increase occurred in the first 10 hours, after which the size of the loaded liposome sample remained constant. There was no noticeable change in size of blank liposomes over time (Figure 5b). Again, liposomes with different sizes and bilayer curvatures exhibited identical behavior.

The size distribution of liposomes was not affected by monomer loading. Figure 6 shows DLS data of DMPC before and after loading with DVB and blank DMPC liposomes incubated at 30 °C. While there are slight variations from one batch to another, interactions with monomers did not have noticeable effect on the size distribution of the liposomes. DMPC liposomes before (Figure 6a, i-iii) and after incubation at 30 °C for 24 hours with DVB (Figure 6a, iv-vi) exhibit virtually identical size distribution. Control samples (Figure 6b, i-iii) were kept at 30 °C for 24 hours in the absence of DVB (Figure 6b, iv-vi).

The polydispersity index (PDI), the measure of the breadth of the dispersion, remained within the 0.05 – 0.17 range (Figure 7). PDI values below 0.2 have been traditionally associated with narrow monodisperse distributions.²⁰⁻²³ The polydispersity index did not change noticeably during the loading experiment (Figure 7a) or in control samples (Figure 7b).

A slight increase in liposome size but not in size distribution strongly suggests that monomer loading does not cause liposome fusion, regardless of bilayer curvature. To confirm that the liposomes remain intact during the loading, we monitored the fluorescence of liposome samples containing self-quenching dye calcein. In these experiments, calcein was encapsulated in liposomes of different sizes at 50 mM. At this concentration, more than 95% of fluorescence is quenched.²⁴ Non-entrapped calcein was removed on a size-exclusion column. In this setup, calcein released into solution from liposomes would be diluted and would immediately reveal itself from increased emission.^{17,24} Release of 3% of the encapsulated calcein would increase the emission intensity approximately two-fold, as calculated using a calibration curve (see Supporting Information). Fluorescence was monitored during loading or incubation in the absence of monomers for 15 hours (Figure 8). Here, meaningful comparison is between samples being loaded and control samples of the same size. Within each pair (e.g. 100 nm liposomes with or without DVB), no noticeable difference in emission was observed. These findings suggest that liposomes did not release their content during monomer loading.

Lack of monomer-induced fusion, retention of liposome content and identical behavior exhibited by liposomes with different curvatures confirms that there is no induced fusion of the liposomes, different curvatures do not affect loading, and that the method for nanocapsule synthesis is robust and applicable for liposomes in broad size range. Directed assembly approach is likely to produce new nanostructures with enhanced performance.²⁵

Conclusions

Liposomes with radii ranging from 25 to 100 nm exhibited similar loading kinetics and bilayer capacity for accommodation of hydrophobic monomers. Monomers with similar loading characteristics to divinylbenzene can be fully loaded into bilayers within 10-12 hours at ambient conditions (e.g., overnight). Data acquired in this work are consistent with the hypothesis that the loading capacity of the bilayer is limited by lipid translocation that slows down as the hydrophobic barrier increases due to monomer loading. Despite significant changes in the bilayer structure that accompany accommodation of monomers, no evidence of monomer-induced fusion or bilayer rupturing was observed. A newly developed rapid HPLC-based method enabled a detailed time-resolved study of monomer loading into bilayers with different curvatures. This method can be useful for other studies of using amphiphilic scaffolds for directed assembly of nanostructures from hydrophobic building blocks.

It is reasonable to expect that the identical monomer/lipid ratios obtained in liposomes with varying curvatures will result in nanocapsules with similar properties of the shells. This study contributes to understanding of interactions between amphiphilic scaffolds and hydrophobic building blocks and broadens the scope of directed assembly of nanomaterials within temporary self-assembled scaffolds. In particular, it validates and expands the methodology for nanocapsule synthesis in the arguably the most interesting and useful size range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure1.
Schematic representation of different bilayer curvatures corresponding to different sizes of liposomes sizes used in this study.

Figure 2. HPLC chromatograms of DMPC liposomes being loaded with DVB taken at different intervals. Inset: calibration curve of DVB in methanol.

Figure 3.

Time-resolved loading of DVB into DMPC at 30 °C. Each series is labeled with the pore size of the filter used extrusion in liposome preparation. Most of the error bars have been omitted for clarity. Remaining error bars are representative of the scale of those omitted. In the absence of liposomes, concentration of DVB is normalized to the standard concentration of lipids used in these experiments (0.03 mM).

Figure 4.
Time-resolved loading of DVB in DMPC liposomes at 12 °C.

Figure 5. (a) Average diameter of the liposomes being loaded with DVB. (b) Control experiment, blank DMPC liposomes without loading DVB.

Figure 6.

Size distribution of DMPC liposomes extruded through 0.05 μm , 0.1 μm , and 0.2 μm filters obtained by dynamic light scattering (a) before (i-iii) and after (iv-vi) loading with DVB for 24 hours and (b) control experiment, blank DMPC liposomes freshly prepared (i-iii) and incubated for 24 h. at 30 $^{\circ}\text{C}$ (iv-vi) in the absence of DVB,.

Figure 7. Polydispersity index (PDI) of (a) DMPC liposomes being loaded with DVB and (b) blank DMPC liposomes as a function of time. Each data point is an average of three independent measurements, each of which was an aggregate of ten scans.

Figure 8.

Change in calcein fluorescence as function of time. In all experiments, calcein (50 mM) was encapsulated in liposomes, and untrapped calcein was separated by size-exclusion chromatography. Lack of increase in fluorescence indicates no release of calcein into solution.