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Lipid-assisted formation and dispersion of aqueous and bilayer-embedded nano-C₆₀

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Abstract

Lipid assemblies provide a biocompatible approach for preparing aqueous nanoparticles. In this work, dipalmitoylphosphatidylcholine (DPPC) was used to assist in the formation and dispersion of C₆₀ and nano-C₆₀ aggregates using a modified reverse phase evaporation (REV) method. This method led to the rapid formation of aqueous nano-C₆₀ at DPPC/C₆₀ molar ratios from 500:1 to 100:1 (12–38 nm; verified by cryogenic transmission electron microscopy), which were present in the bulk phase and encapsulated within vesicles. In addition to forming nanoparticles, C₆₀ was trapped within the vesicle bilayer and led to a reduction in the lipid melting temperature. Solvent extraction was used to isolate nano-C₆₀ from the lipids and bilayer-embedded C₆₀. Our results suggest that bilayer-embedded C₆₀ was present as molecular C₆₀ and as small amorphous nano-C₆₀ (2.3±0.4 nm), which clustered in the aqueous phase after the lipids were extracted. In addition to developing a new technique for nano-C₆₀ formation, our results suggest that the lipid bilayer may be used as a hydrophobic region for dispersing and assembling small nano-C₆₀.

Introduction

Aqueous dispersions of C₆₀ fullerene nanoparticles (nano-C₆₀) have been explored as candidates in the areas of biomedicine, materials, and cosmetics.^{1–7} Given their size and ability to persist in nature, studies have also been conducted to examine nano-C₆₀ environmental fate and biocompatibility.^{8–11} While C₆₀ exhibits poor solubility in organic solvents and is insoluble in water, solvent-exchange and ultrasonication processes can be used to form aqueous nano-C₆₀. A common technique involves dissolving C₆₀ in a solvent (e.g. tetrahydrofuran, 9 mg/L C₆₀ solubility¹²), mixing this solution with an aqueous phase, and gradually evaporating the solvent.^{13–15} During this process, which can be very time and solvent intensive, C₆₀ aggregates into nano-C₆₀ particles. It has been shown that nano-C₆₀ has a negative surface charge, which aids its dispersion through electrostatic repulsion.¹⁵ The dispersibility decreases greatly in the presence of salts as cations adsorb onto nano-C₆₀, screen the surface charge, and cause aggregation.¹⁶ Therefore, for biomedical or toxicological studies, they are commonly stabilized chemically, through surface modification (e.g. hydroxylation¹⁷), or physically, through the addition of surface-active molecules.

Surfactant and protein¹⁸ molecules have been used to increase the stability and concentration limit of aqueous nano-C₆₀. A recent study by Lee and Kim¹⁹ examined how the aggregation and photocatalytic activity of nano-C₆₀ was affected by the addition of anionic, cationic, or nonionic surfactants. Anionic surfactants repelled nano-C₆₀ and prevented it from entering the micelles. Alternatively, cationic surfactants caused nano-C₆₀ to adsorb at the headgroups of the micelles and led to charge neutralization and agglomeration. The zwitterionic surfactant

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Triton X-100 showed the greatest inhibition of nano- C_{60} clustering in water and the greatest photocatalytic activity, determined by the generation of singlet oxygen (1O_2). Their results indicate that nonionic surfactants with small hydrophilic headgroups are most effective for dispersing nano- C_{60} .¹⁹

Lipid bilayer vesicles, which are used as model cell membranes, provide an interesting platform for aiding the formation and dispersion of nano- C_{60} . A unique advantage of this approach is that nano- C_{60} may be formed in the aqueous phase and encapsulated within the vesicles, and molecular C_{60} and/or small C_{60} aggregates may be embedded within the hydrophobic bilayer. Furthermore, lipids are inherently biocompatible, and hybrid lipid/nanoparticle assemblies have received considerable interest as multifunctional agents for biomedical and pharmaceutical application.²⁰

The concept of fullerene-embedded lipid bilayers, as dispersed vesicles or as planar supported membranes, has been explored for photocatalytic reactions,²¹ as laser-triggered therapeutic agents (referred to as fullerenosomes),²² and as a means of facilitating charge transfer across bilayers for sensing applications.²³⁻²⁷ Charge transfer is greatly enhanced as C_{60} aggregates and forms clusters within the bilayer.²⁵ Additional effects of C_{60} embedding and aggregating within lipid bilayers include disruption of lipid packing and phase behavior, bilayer thinning, and ripple-like in-plane ordering.²⁸ It is unclear how these factors affect vesicle function or stability, or what role they may play in bioaccumulation, cell membrane disruption, or cytotoxicity.

In this study, a modified reverse phase evaporation (REV) procedure was used to prepare dipalmitoylphosphatidylcholine (DPPC) vesicles that provided a dual hydrophilic-hydrophobic environment for the formation and dispersion aqueous and bilayer-embedded nano- C_{60} . In addition to forming nano- C_{60} , this approach provides a potential model system for studying membrane disruption. Zwitterionic DPPC was employed to minimize electrostatic interactions with aqueous nano- C_{60} , which has been shown to aid the inclusion of C_{60} in the hydrophobic regions of amphiphile assemblies.¹⁹ We examined, as a function of the DPPC/ C_{60} molar ratio, (i) the preference of molecular C_{60} to reside within the vesicle bilayer or form aqueous nano- C_{60} , (ii) the formation of small nano- C_{60} aggregates within the bilayer and their affect on lipid phase behavior, and (iii) the ability to separate the lipids and bilayer-embedded C_{60} from aqueous nano- C_{60} . Structure and morphology of the DPPC/ C_{60} vesicles and nano- C_{60} were examined by cryogenic transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS) before and after lipid extraction. The effects of C_{60} -lipid interactions on bilayer phase behavior were examined by differential scanning calorimetry (DSC) and fluorescence anisotropy. In addition to aiding the rapid formation of small aqueous nano- C_{60} , this work also provides insight into the ability of C_{60} to partition into biological membranes and its effect on membrane structure.

Materials and Methods

Chemicals

C_{60} (99.9%) and the membrane probe diphenylhexatriene (DPH) were purchased from ACROS Organic. DPPC was purchased from Avanti Polar Lipids. Chloroform ($CHCl_3$), carbon disulfide (CS_2), methanol (CH_3OH), and tetrahydrofuran (THF), and anhydrous magnesium perchlorate ($Mg(ClO_4)_2$), were purchased from Fisher Scientific. All solvents were HPLC grade. Deionized water was used from a Millipore Direct-Q3 UV purification system.

Preparation of DPPC/C₆₀ vesicles

An REV²⁹ method was used to prepare the DPPC/C₆₀ vesicles and nano-C₆₀ (Figure 1). DPPC dissolved in chloroform and C₆₀ dissolved in carbon disulfide were placed in round-bottom flask. For fluorescence anisotropy measurements, an aliquot of DPH in THF was added at a DPPC/DPH molar ratio of 500:1. Deionized water with 71 mM Mg(ClO₄)₂ was then added to form an emulsion. The solvents were removed by rotary evaporation at 50°C (above the DPPC melting temperature, T_m , of ca. 41°C) and 300 mbar for 15 minutes, at which point the pressure was reduced to 100 mbar for 10 minutes. The samples were then ultrasonicated for 2 hours. Samples were prepared at 20 mM DPPC (14.7 mg/ml) with DPPC/C₆₀ molar ratios of 500:1, 200:1, and 100:1, which correspond to 29, 72, and 144 mg/L C₆₀.

Extraction of lipids from DPPC/C₆₀ vesicles

The Folch Method was used to extract DPPC from the DPPC/C₆₀ vesicles and recover nano-C₆₀.³⁰ In this method, 4 parts of a chloroform-methanol (2:1 by volume) mixture were added to 1 part of a DPPC/C₆₀ assembly solution. The extract was shaken and allowed to phase separate. The bottom extract phase was composed of chloroform-methanol-water at 86:14:1 (by volume) and contained the lipids, while the top phase was composed of chloroform-methanol-water at 3:48:47 (Figure 1).

Cryo-TEM imaging

DPPC/C₆₀ samples were prepared for cryo-TEM (JEOL JEM2100) using a Vitrobot (FEI), which is a PC-controlled robot for sample vitrification. Quantifoil grids were used with 2 μ m carbon holes on 200 square mesh copper grids (Electron Microscopy Sciences). A small sample (ca. 10 μ l) was placed on the grid, blotted to reduce film thickness, and vitrified in liquid ethane. The sample was then transferred to liquid nitrogen for storage.

To image the top methanol/water-rich phase of the extraction solution, a small sample drop was placed on a grid and dried under vacuum overnight. Particle size analysis was performed using ImageJ software.³¹

Differential scanning calorimetry (DSC)

Lipid bilayer phase behavior in the DPPC/C₆₀ vesicles was analyzed by DSC (TA Instruments Q10). Samples were equilibrated at 25°C and sequential heat/cool cycles were performed from 25-50°C at scan rate of 1°C min⁻¹.

Fluorescence Anisotropy

Lipid bilayer fluidity was examined by fluorescence anisotropy (Perkin Elmer LS 55) of DPH at 0.01 mM DPPC from 30 to 50°C at a rate of 1°C/min under continuous mixing. Steady-state DPH anisotropy was determined at λ_{ex} = 350 nm and λ_{em} = 452 nm using the expression $\langle r \rangle = (I_{VV} - I_{VH}) / (I_{VV} + GI_{VH})$ where I represents the fluorescence emission intensity, V and H represent the vertical and horizontal orientation of the excitation and emission polarizers, and $G = I_{HV} / I_{HH}$ accounts for the sensitivity of the instrument towards vertically and horizontally polarized light.

Dynamic Light Scattering (DLS)

Size distribution measurements of aqueous nano-C₆₀ in the top methanol/water-rich phase after lipid extraction were performed by DLS (Model BI-9000AT, Brookhaven Instrument). The digital correlator was calibrated with nanosphere size standards (20 nm, Duke Scientific). For measurements, 2 ml of top aqueous phase after extracting the lipids was transferred into a glass vial, which had been rinsed with deionized water under dust-free condition. All DLS measurements were carried out at 25°C at a wavelength of 532 nm. Size distributions were

obtained using a continuous non-negative least squares (NNLS) fit of the autocorrelation function.

Ultraviolet-Visible Spectroscopy

To confirm the presence of C_{60} and/or nano- C_{60} within the bottom chloroform-rich phase after extraction, the absorbance of C_{60} was measured by UV-vis spectroscopy (Varian Cary 50). For calibration, we measured a set of C_{60} /chloroform solutions and determined the linear relationship between the area of the adsorption peak and the concentration of C_{60} . The concentrations of C_{60} in chloroform were 0, 5, 10, and 25 mM.

Results and Discussion

Nano- C_{60} formation during DPPC/ C_{60} assembly

The DPPC/ C_{60} samples are shown in Figure 2. With increasing C_{60} concentration, i.e. decreasing DPPC/ C_{60} ratio, the sample changed from pale yellow at 500:1 to dark orange at 100:1. The samples remained dispersed for 3 days when stored at 50°C. Cryo-TEM revealed the formation of large and small unilamellar vesicles, and the size, morphology, and location of nano- C_{60} formed during the REV process. In Figure 3, which is shown at a DPPC/ C_{60} ratio of 200:1 as representative images, the C_{60} nanoparticles are observed in DPPC/ C_{60} vesicles. From the observation, the size of the nano- C_{60} ranged from ca. 12 to 38 nm. For comparison, aqueous nano- C_{60} diameters of ca. 56 and 100 nm have been reported when prepared by solvent exchange from toluene¹⁶ and THF,⁸ respectively. Moreover, the images show that some of the nano- C_{60} was encapsulated within vesicles, and others existed outside vesicles in the bulk phase. Visual inspection did not reveal changes in color or the presence of precipitated aggregates after formation.

Using the REV procedure, we were unable to prepare dispersed nano- C_{60} in the absence of DPPC. In this case, C_{60} precipitated and the color of the aqueous phase did not turn yellow, which is indicative of nano- C_{60} , but remained clear. This demonstrates that the presence of DPPC was needed to stabilize and form aqueous nano- C_{60} .

Lipid phase behavior in DPPC/ C_{60} vesicles

Based on cryo-TEM, the presence of lipid assisted in the rapid formation of aqueous nano- C_{60} during vesicle formation; however, C_{60} is hydrophobic and has also been shown to partition into the acyl region of lipid bilayers.³² This may occur as molecular C_{60} or small nano- C_{60} . To detect the presence of C_{60} within the bilayers, we have examined the effects of C_{60} on the lipid bilayer phase behavior using DSC. Figure 4 shows the DSC transition curves of the samples at different DPPC/ C_{60} molar ratios. The presence of C_{60} reduced the lipid melting temperature (T_m) by nearly 1°C. However, changes in the width of the melting region were negligible and the cooperative melting unit, defined as the van't Hoff enthalpy divided by the calorimetric enthalpy, varied little from that of DPPC vesicles (171 lipid molecules). The pre-transition of DPPC/ C_{60} vesicles, which was observed at 36°C for the control, was not observed when C_{60} was present. Elimination of the pretransition (gel to rippled-gel) is consistent with the ability of C_{60} to trigger ripple-like in-plane ordering in DPPC bilayers from 30 to 70°C based on SANS data.²⁸ Hence, ripples may have been present at 30°C, which corresponds to a gel phase for DPPC in the absence of C_{60} . The DSC results were repeatable between replicate samples, and for single samples over a three-week timeframe (results not shown). This repeatability suggests that the amount of C_{60} within the bilayer and the nature of the DPPC/ C_{60} interaction did not change during this timeframe. We do not attribute bilayer disruption to the adsorption of nano- C_{60} at the lipid/water interface, which is consistent with studies by Spurlin and Gerwith³³ where aqueous nano- C_{60} was shown to adhere to zwitterionic dipalmitoylphosphatidylcholine (DMPC) bilayers, but does not affect lipid chain packing.

Fluorescence anisotropy results indicate that bilayer-embedded C₆₀ increased the degree of lipid ordering at temperatures corresponding to gel (30°C), rippled-gel (37°C), and fluid (50°C) DPPC phases. A greater DPH anisotropy was observed in the DPPC/C₆₀ vesicles relative to DPPC vesicles (Table 1); however, there was no clear relationship between anisotropy and the DPPC/C₆₀ ratio. A reduction in DPPC melting temperature was also observed, in agreement with the DSC results.

Theoretical work by Qiao et al.³⁴ suggests that pristine C₆₀ resides 1.1 nm from the center of the bilayer. Molecules that intercalate between lipid tails tend to inhibit lipid motion and increase ordering. This is well known for long-chain n-alkanes that align parallel to the lipid tails. In contrast, short-chain n-alkanes accumulate in the bilayer center perpendicular to the tails.³⁵⁻³⁷ Ordering results based on DPH anisotropy support the concept of C₆₀ mixing between lipid tails. However, an increase in lipid ordering is commonly associated with an increase in melting temperature. Both DSC and fluorescence anisotropy results indicate a reduction in T_m . While we cannot explain the results at this time, the conflict between ordering and T_m may be attributed to the shape and size of C₆₀ relative to linear hydrophobic molecules. C₆₀ has a van der Waals diameter of ~1.1 nm,³⁴ which is similar to the acyl tail length of DPPC in a fluid bilayer (~1.4 nm).³⁸ C₆₀ may intercalate between lipids and reduce their mobility, but it is a rigid sphere and may not pack well, which could also give rise to bilayer disruption.

Isolating C₆₀ from DPPC/C₆₀ vesicles

If present within the bilayers, C₆₀ can exist as molecules or as molecular aggregates (i.e. small nano-C₆₀). As bilayer aggregates, they cannot exceed the thickness of a lipid bilayer (ca. 5 nm) without compromising vesicle structure. If present, small nano-C₆₀ could not be detected by cryo-TEM as the beam energy needed to provide high magnification would have destroyed the samples. Therefore, lipid extraction was performed with a chloroform/methanol mixture to isolate bilayer-embedded C₆₀ from aqueous nano-C₆₀. It is been reported that aqueous nano-C₆₀ cannot be extracted into toluene³⁹ which has shown to be a better solvent for molecular C₆₀ than chloroform (4.2×10^{-4} mole fraction solubility in THF verses 1.8×10^{-5} in CHCl₃).⁴⁰

According to the Folch method, 99% of the lipid was extracted into the bottom chloroform-rich phase. The picture inset in Figure 5 shows the color change of DPPC/C₆₀ sample after extraction in the two-phase system. Both the top and bottom phases were transparent, and bottom phase of samples 2, 3, and 4 were weakly pink due to dissolved C₆₀. With ratio of DPPC/C₆₀ decreasing from 500:1 to 100:1, the pink color deepened as more C₆₀ was dissolved.

The concentration of C₆₀ extracted with the lipids into the bottom chloroform-rich phase was analyzed by UV-vis spectroscopy (Figure 5). The spectra show absorbance peaks at 260 and 330 nm, consistent with our C₆₀/chloroform calibration solutions and results obtained by Deguchi et al.¹⁵ for C₆₀ dissolved in THF. Furthermore, broad absorbance peaks characteristic of nano-C₆₀ (400-500 nm) were not observed.¹⁵ C₆₀ concentrations extracted from the original samples were 16, 36, and 68 μ M. The original C₆₀ concentrations were 40, 100, and 200 μ M when ratios of DPPC/C₆₀ are 500:1, 200:1 and 100:1, respectively. From this analysis, 40% of the C₆₀ was extracted at 500:1, 36% at 200:1, and 34% at 100:1. Assuming that this extracted C₆₀ was originally embedded within the DPPC bilayers leads to C₆₀ bilayer mole fractions of 0.8, 1.8, and 3.4 ($\times 10^{-3}$), respectively.

Previous attempts to extract from C₆₀ from lipid bilayers using toluene required that KCl be added to the suspensions to disrupt the bilayer.⁴¹ While toluene is a better solvent for C₆₀ than chloroform, it is a poor solvent for DPPC. By using chloroform, molecular C₆₀ could be recovered from the lipid bilayers and nano-C₆₀ from DPPC/C₆₀ suspensions.

DLS was used to obtain a particle size distribution of nano-C₆₀ in the top methanol/water-rich phases, which contained the Mg(ClO₄)₂ used in the vesicle formation process (Figure 6). For the control with no C₆₀ (sample 1), there were no measurable structures, confirming that the lipid was extracted into the bottom chloroform-rich bottom phase. The measured size distribution of nano-C₆₀ ranged from 140 to 170 nm at 500:1 DPPC/C₆₀, 75 to 140 nm at 200:1, and 80 to 130 nm at 100:1. This result shows that nano-C₆₀ aggregates were retained in the top phase. The sizes of these aggregates were larger than that observed by cryo-TEM before extraction, which suggests that by removing the lipids from solution the nano-C₆₀ (12 to 38 nm) aggregated during recovery. Aqueous nano-C₆₀ has been shown to be negatively charged, and removing the lipids would allowed charge screening by Mg²⁺ to cause aggregation, which has been shown by Mchedlov-Petrosyan et al.⁴² and Chen and Elimelech¹⁶ for divalent cations.

Figures 7 shows TEM images of nano-C₆₀ and nano-C₆₀ aggregates that remained in the top methanol/water-rich phase after extracting the lipids (shown for DPPC/C₆₀ at 200:1 as representative images). In Figure 7C, we observe nano-C₆₀ aggregates (ca. 10-30 nm) composed of small individual C₆₀ nanoparticles with an average diameter of 2.3±0.4 nm. The C₆₀ nanoparticles have diameters smaller than the thickness of a lipid bilayer (4-5 nm), which suggests that they may have formed within the bilayer acyl tail region. The amorphous nature of these particles was similar to nano-C₆₀ formed using polyvinylpyrrolidone (PVP) as a stabilizing agent.⁴³ In this case, nano-C₆₀ was not crystalline as is typical. It should be pointed out that specimen preparation for these images involved drying a 10 ml drop of the sample a TEM grid. This procedure, along with the presence of Mg(ClO₄)₂ or methanol, may have altered the size and structure of the nano-C₆₀ aggregates.

Conclusions

Nano-C₆₀ was successfully formed in both aqueous solution and within zwitterionic lipid bilayers during vesicle formation. We propose that the large nano-C₆₀ formed in the bulk phase during DPPC/C₆₀ vesicle assembly. The vesicles prevented nano-C₆₀ aggregation by encapsulation and physical stabilization. Molecular C₆₀ within the bilayer caused lipid disordering and clustered to form small nano-C₆₀. The molecular C₆₀ was extracted from the bilayer, along with the lipids, while the small nano-C₆₀ was retained in the aqueous phase. Compared to the more common solvent exchange methods, the lipid-assisted REV method allows for a higher aqueous C₆₀ concentrations and rapid formation of nano-C₆₀ with less solvent consumption. Future studies will focus on tailoring nano-C₆₀ size distribution, elucidating bilayer-C₆₀ interaction mechanisms, and evaluating factors that influence the structural stability of DPPC/C₆₀ assemblies, such as sonication, pH, and salt concentration.

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Figure 1.
Schematic illustration for preparation of DPPC/C₆₀ vesicles and lipid extraction

Figure 2.
The concentration of DPPC is 10 mM, and the ratios of DPPC/C₆₀, from sample 2 to sample 4, are 500:1, 200:1 and 100:1, respectively.

Figure 3. Cryo-TEM images of the DPPC vesicle control (A, without C_{60}) and samples at a DPPC/ C_{60} ratio of 200:1 containing DPPC vesicles and nano- C_{60} (B and C).

Figure 4. DSC thermographs of (1) DPPC and DPPC/C₆₀ vesicles at DPPC/C₆₀ ratios of (2) 500:1, (3) 200:1, and (4) 100:1.

Figure 5.

UV/Vis analysis of C₆₀ dissolved in the bottom chloroform-rich phase after extraction for (1) DPPC and DPPC/C₆₀ vesicles at DPPC/C₆₀ ratios of (2) 500:1, (3) 200:1, and (4) 100:1. A calibration solution at (5) 25 μ M C₆₀ is shown for comparison.

Figure 6. Size distribution of top methanol/water phase of samples 2 (black), 3 (gray), and 4 (white) are shown in Figure 6. Samples 2-4 correspond to DPPC/C₆₀ ratios in the original samples of 500:1, 200:1, and 100:1.

Figure 7. TEM images of the aqueous phase after extracting lipids from DPPC/C₆₀ at a ratio of 200:1 (C is a magnification of the selected region in B).

Table 1

DPPC bilayer fluidity and melting temperature determined by fluorescence anisotropy of diphenylhexatriene (DPH).

DPPC/C ₆₀ (mol/mol)	30°C	$\langle r^2 \rangle$ 37°C	50°C	T_m (°C)
control	0.323	0.302	0.116	40.9
500:1	0.341	0.326	0.120	40.3
200:1	0.343	0.322	0.124	40.1
100:1	0.337	0.319	0.125	39.9