Published in final edited form as:

Biochem Biophys Res Commun. 2010 April 9; 394(3): 509–514. doi:10.1016/j.bbrc.2010.02.180.

Bis(monoacylglycerol)phosphate and Ganglioside GM1 Spontaneously Form Small Homogeneous Vesicles at Specific Concentrations

Janetricks N. Chebukati, Philip C. Goff, Thomas E. Frederick, and Gail E. Fanucci*

Department of Chemistry, P.O. Box 117200, University of Florida, Gainesville, FL 32611-7200,
Tel: +1 352 392 2345; Fax: +1 352 392 0872

Gail E. Fanucci: fanucci@chem.ufl.edu

Abstract

The morphology and size of hydrated lipid dispersions of bis(monoacylglycerol)phosphate (BMP) mixed with varying mole percentages of the ganglioside GM1 were investigated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Electron paramagnetic resonance (EPR) spectroscopy of these same mixtures, doped at 0.5 mole percent with doxyl labeled lipids, was used to investigate acyl chain packing. Results show that for 20–30% GM1, hydrated BMP:GM1 mixtures spontaneously form small spherical vesicles with diameters ~ 100 nm and a narrow size distribution profile. For other concentrations of GM1, hydrated dispersions with BMP have nonspherical shapes and heterogeneous size profiles, with average vesicle diameters > 400 nm. All samples were prepared at pH 5.5 to mimic the lumen acidity of the late endosome where BMP is an essential component of intraendosomal vesicle budding, lipid sorting and trafficking. These findings indicate that GM1 and BMP under a limited concentration range spontaneously form small vesicles of homogeneous size in an energy independent manner without the need of protein templating. Because BMP is essential for intraendosomal vesicle formation, these results imply that lipid-lipid interactions may play a critical role in the endosomal process of lipid sorting and trafficking.

Keywords

Bis(monoacylglycerol)phosphate; ganglioside GM1; vesicle morphology; late endosome; membrane biophysics; transmission electron microscopy; biomembranes

Introduction

Bis(monoacylglycerol)phosphate (BMP), also called lysobisphosphatidic acid (LBPA), is a characteristic lipid of the endocytic degradative pathway that is found in the luminal membranes of the late endosome in concentrations of approximately 15 mole percent [1]. The chemical structure of BMP, shown in Figure 1, differs from that of other glycerophospholipids, in that each of the acyl chains in BMP is esterified to two different

*Corresponding author: Gail E. Fanucci, Department of Chemistry, PO BOX 117200, Gainesville FL, 32611-7200. Tel:352-392-2345; Fax:352-392-0872 fanucci@chem.ufl.edu.

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glycerol moieties [2–4]. Additionally, BMP has sn-1-glycerophospho-sn-1'-glycerol (sn1:sn1') stereoconfiguration that differs from the typical sn-3-glycerophosphate stereoconfiguration found in other glycerophospholipids [5–8].

Due to its increased concentration in the late endosomes, BMP is thought to play important structural and functional roles in this organelle [9]. Several *in vivo* investigations have demonstrated that antibodies and chemicals that interact with BMP lead to changes in the sorting and trafficking of proteins and lipids in late endosomes, which result in altered endosome morphology [1,10] and abnormal accumulation of cholesterol [11]. BMP is also essential for lysosomal catabolism processes[2], such as the activator-stimulated hydrolysis of gangliosides GM1 [12] and GM2 [13], and the hydrolysis of ceramide by acid ceramidase [14]. The structures of BMP and GM1 are shown in Figure 1.

Gangliosides are sialic acid-containing glycosphingolipids found in the cell membranes of vertebrates, with high abundance found in the plasma membrane of neuronal cells [15]. Glycosphingolipid degradation proceeds via endocytosis from the plasma membrane and subsequent transportation to the endosomes, followed by intraendosomal vesicle formation, and final trafficking as intralysosomal vesicles for degradation [16]. Deficiencies in the catabolism of gangliosides results in lysosomal glycosphingolipid accumulation, leading to clinical disorders known as sphingolipid storage diseases that mainly affect neuronal cells within the brain [17].

Much insight into the process of membrane recycling and digestion has been realized through the investigation of glycosphingolipid catabolism [2,16,18]. The lysosomal degradation of glycosphingolipids is a sequential pathway of reactions that are catalyzed by exohydrolases under acidic conditions. These enzymes are assisted by small glycoprotein cofactors, known as the sphingolipid activator proteins (SAPs), and lipid composition has been shown to alter the *in vitro* degradation kinetics; where optimum activity is obtained with low cholesterol content and the presence of the negatively charged lipid BMP [2].

Recently, the morphology and molecular organization of sn-3-sn-1'dioleoyl-BMP (BMP) under both acidic and neutral pH conditions was characterized by TEM. It was showed that when hydrated, BMP forms small, stable lamellar vesicles with interior volumes and with acylchain dynamics and packing similar to other glycerophospholipids [19]. Given the unique chemical structure of BMP, this vesicle morphology was surprising as it had been previously assumed that BMP would form either micellar structures, similar to detergents, or inverted hexagonal morphologies, as is seen with phosphatidylethanolamine lipids [20]. It has also been shown that the morphology of the hydrated BMP dispersions varies with pH and ionic strength [19,21], which further suggests a role for BMP in intraendosomal vesicular body formation that is triggered in the late endosome by the biosynthesis of BMP and a drop in endosomal lumen pH [19].

In this report, dynamic light scattering (DLS) and negative staining-transmission electron microscopy (TEM) were utilized to monitor the size, morphology and structure of hydrated dispersions of BMP:GM1 mixtures. All samples were prepared at pH 5.5, which mimics the pH of the late endosome. EPR spectroscopy with 5-doxyl and 7-doxyl phosphatidylcholine was used to investigate the acylchain packing as GM1 was mixed with BMP.

Materials and methods

Materials

BMP_{18:1}, ((S, R Isomer) sn-(3-Oleoyl-2-Hydroxy)-Glycerol-1-Phospho-sn-3'-(1'-Oleoyl-2'-Hydroxy)-Glycerol, ammonium Salt), 5-doxyl PC (1-Palmitoyl-2-Stearoyl-(16-DOXYL)-

sn-Glycero-3-Phosphocholine) and 7-doxyl PC (1-Palmitoyl-2-Stearoyl-(16-DOXYL)-sn-Glycero-3-Phosphocholine) in chloroform and ganglioside GM1 powder, were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, $C_8H_{18}N_2O_4S$); NaOAc, (sodium Acetate); EDTA, (ethylenediamine tetraacetic acid, $C_{10}H_{16}N_2O_8$) and NaCl, (sodium chloride) were purchased from Fisher Biotech (Pittsburgh, PA). CH_3Cl , (chloroform); MeOH, (methanol); NH_4OH , (ammonium hydroxide); HCl, (hydrochloric acid) and NaOH, (sodium hydroxide) were obtained from Fisher Scientific (Pittsburgh, PA). $UO_2(CH_3COO)_2 \cdot 2H_2O$, (uranyl acetate) and 400-mesh Formvar-coated copper grids were purchased from Ted Pella (Redding, CA). Single-sealed 50- to 1000-mL disposable cuvettes (10-mm path length) were obtained from Eppendorf (Westbury, NY). 400-nm polycarbonate extrusion membranes and filter supports were purchased from Avanti Polar Lipids (Alabaster, AL). Silica-coated aluminum thin layer chromatography (TLC) plates were purchased from Whatman (Florham Park, New Jersey).

Preparation of hydrated lipid dispersions and extruded unilamellar vesicles

The desired amount of stock lipids were mixed and then dried under a gentle nitrogen stream until the solvent evaporated, forming a dry, thin lipid film. Stock solutions typically contained 5 mg/mL BMP in chloroform, 10 mg/ml n-doxyl lipid in chloroform, or 1 mg/mL GM1 in chloroform:methanol (2:1 v/v) mixture). The sample was then further dried under vacuum desiccation for at least 12 hours to remove any residual solvent. Dry lipid films were hydrated with 2 mL of either 5 mM NaOAc buffer for pH 4.2, 5.5 and 6.1, or 5 mM HEPES buffer for pH 7.4. All buffers contained 100 mM NaCl and 0.1 mM EDTA, and the final lipid concentration was approximately 0.75 mM. Hydrated lipid mixtures were freeze-thawed in liquid N_2 five times. All hydrated dispersions were incubated at room temperature for approximately 12 hours before measurement or extrusion. To form large unilamellar vesicles (LUVs), hydrated lipid dispersions were extruded by passing 31 times through 400 nm polycarbonate extrusion membranes. Phospholipid integrity was verified by thin layer chromatography (TLC), where approximately 10 μ L of lipid sample was spotted on silica-coated aluminum plates. Plates were placed in a chamber containing a CH_3Cl :MeOH: NH_4OH (65:25:10) mobile phase. The TLC plates were developed in an iodine chamber and visualized by eye.

Dynamic Light Scattering (DLS)

Size distribution measurements of hydrated dispersions and extruded unilamellar lipid vesicles were performed with a Brookhaven 90Plus/BI-MAS ZetaPALS spectrometer operated at a wavelength of 659 nm and at 25° C. The instrument uses a BI-9000AT digital autocorrelator and 9KDLSW data acquisition software. A 100- μ L sample volume in a disposable cuvette was used for each measurement. For each sample, 3 runs were performed with each run lasting 3 minutes. Data and histograms were further analyzed and converted into B-spline plots using OriginPro software. DLS data was reported as an average of 3 runs for each sample, and errors calculated as a standard deviation from the average diameter.

Negative Staining-Transmission Electron Microscopy (TEM)

TEM images were obtained using a Hitachi H-7000 transmission electron microscope operated at 75–100 kV with a Soft-Imaging System MegaViewIII with AnalySIS digital camera (Lakewood, CO). The microscope has a maximum resolution at 0.2 nm with a magnification range of 110 $\times$ to 600,000 $\times$. Prior to TEM measurements, samples were further prepared by negative staining. Briefly, for all samples, using a disposable pipette, a drop of the lipid vesicle sample was spread on a 400-mesh Formvar-coated copper grid held by tweezers and incubated for 2 minutes. Excess lipid sample was gently dabbed away with filter paper, and the grid was allowed to dry for 2 minutes. In some instances, a drop of

deionized water was added to the grid to remove any excess salt from the buffer solution used in vesicle preparation. One drop of 2% uranyl acetate was then added to the grid and allowed to stain for 2 minutes, after which any excess uranyl acetate was wiped away, and the sample was allowed to dry for 2 minutes before being placed in the electron microscope specimen holder for image analysis and collection.

Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR spectroscopy was performed on a modified Bruker ER200 spectrometer with an ER023M signal channel, an ER032M field control unit, and a loop gap resonator (Medical Advances, Milwaukee, WI). A quartz Dewar (Wilma-Labglass) surrounded the loop gap resonator for variable temperature experiments; temperature was controlled by passing nitrogen gas through a copper coil submerged in a recirculating bath (Thermo Scientific) containing 40% ethylene glycol. Typically, a sample volume of 10 μ L was loaded into a 0.60 I.D. $\times$ 0.84 O.D. capillary tube.

Results and discussion

Effects of pH on BMP vesicle morphology

Because the pH varies in different sub compartments of the endocytic pathway, with acidification increasing progressively from the endocytic carrier vesicles and early endosomes to late endosomes to lysosomes [22], BMP hydrated dispersions were prepared at four different pH conditions; pHs 4.2, 5.5, 6.1 and 7.4, indicative of the *in vivo* pH in the lysosome, late endosome, early endosome and cytosol, respectively [2,18]. Negative staining-TEM images (Fig. 1B) show that BMP dispersions exhibit different morphologies as the pH is varied; progressing from fully spherical, non-structured vesicles at acidic pH 4.2 to non-spherical, highly structured vesicle clusters at neutral pH 7.4. In addition, the sizes of the BMP dispersions are found to vary as a function of pH as demonstrated by the dynamic light scattering (DLS) data (Fig. 1C). At pH 4.2, BMP lipid dispersions are homogeneously spherical, non-structured, and have an average size of $\sim$ 1000 nm. This is in contrast to the vesicle morphology of BMP dispersions seen at late endosomal pH 5.5, which although spherical in shape, show some slight structuring and are also significantly smaller, with a size distribution near 300–500 nm. BMP vesicles at pH 6.1 exhibit structural deviations from a spherical shape, appearing like aggregations of a number of smaller vesicles. The vesicles at early endosomal pH 6.1 have a size distribution of 500–800 nm. At pH 7.4, BMP dispersions have a highly budded and protruding non-spherical shape, appearing highly clustered, with an average vesicle size of 500 nm. The effects of pH change the size distributions only slightly, but have a profound impact on the overall morphology of the BMP dispersion. The sizes seen with TEM are consistent with results from DLS measurements. The DLS data shown represent single traces. Typically 3–5 traces were taken to obtain average values. The average values and standard deviations are given in Figure 1D.

Morphology of BMP:GM1 mixed lipid dispersions

Based on their individual molecular geometries and chemical structures, BMP and GM1 independently self-assemble into different polymorphisms when hydrated. The ganglioside GM1 has a large sugar headgroup and forms micelles in aqueous solvent [23]. On the other hand, BMP has been shown both experimentally and computationally to adopt a lamellar bilayer structure in an aqueous environment [9,19]. Here, the macroscopic polymorphisms that result for mixtures of BMP and GM1 at pH 5.5, which mimics late endosomal pH conditions were investigated.

Figures 2A–2F show representative negative staining-TEM images of BMP:GM1 hydrated dispersions where the mol % of GM1 varies from 0 % to 50 % in 10 % increments, with

Figure 2G showing a larger image of the 70:30 BMP:GM1 mixture. Figure 2H shows the corresponding DLS data for these dispersions. The 100 % BMP dispersions (Fig. 4A, 100:0) are spherical in shape with diameters ranging between ~ 200 – 500 nm. Upon addition of 10 % GM1, (Fig. 4B, 90:10), the vesicle morphology changes to non-spherical heterogeneous structures mixed with some small spherical vesicles. The average diameter for this composition is between ~ 400 – 600 nm, with sizes larger than 1000 nm seen in TEM images. For BMP:GM1 mole ratios of 80:20 (Fig. 4C) and 70:30 (Fig. 4D), spherical vesicle structures are obtained. The vesicles formed from the 80:20 BMP:GM1 mixture have a spherical shape, with average diameters ranging from ~ 300–500 nm seen in TEM images. On the other hand, when the composition of GM1 is increased to 30 mol%, a homogeneous size distribution of spherical vesicles of diameter ~100 nm was observed by TEM. From Figure 5, it becomes clear that these smaller structures are aggregated in the TEM images. The results of DLS measurements for the 70:30 sample composition, gray data in Figure 6 and highlighted with an asterisk, reveal a size distribution of 200 – 600 nm, with an average vesicle size of 430 nm. This increased average vesicle size seen with DLS is likely due to the aggregation of the smaller vesicles that are discernable in the TEM images. We interpret the difference to arise from the fact that DLS reports the average spherical shape of the aggregate hydrodynamic diameter of the aggregations, and not that of the individual vesicles.

It is noteworthy that the concentration range, 20–30 mol% GM1, where spontaneous formation of homogeneous vesicles occurs, is consistent with previous work by Lee and coworkers [23]. Using Langmuir-monolayer preparations of GM1 mixtures with dipalmitoylphosphatidylcholine (DPPC) they showed that GM1 has a condensing effect on DPPC lipids over the concentration range of 20 – 30 mol %. Taken together, these findings may indicate a biologically relevant concentration range for GM1 incorporation into lipid rafts for endocytosis or for intraendosomal vesicle budding, where BMP/GM1 enriched vesicles bud off and are trafficked to the lysosome for ganglioside degradation.

For concentrations ≥ 40 mol % GM1, heterogeneous, non-spherical structures are again obtained. At this ratio (Fig. 2E), both small spherical vesicles and irregularly shaped larger structures are observed. This heterogeneity in size and structure gives a distribution of diameters ranging from 400 – 700 nm. The presence of two distinct macroscopic morphologies may indicate immiscibility of the two lipids above the 30 mol % ratio. Similar findings are seen for the 50:50 BMP:GM1 sample (Fig. 2F), where now the size distribution increases to 500 – 800 nm.

Table 1 summarizes the average vesicle sizes seen with both DLS and TEM for the BMP:GM1 concentrations investigated. In general, the vesicle sizes determined from DLS measurements (Fig. 2H) are consistent with results from TEM images. However as mentioned previously, for the 70:30 BMP:GM1 composition, the DLS measurements give a larger size than seen with TEM, likely because of aggregation of the smaller vesicles. DLS data were also collected for the BMP:GM1 mixtures after mechanical extrusion through 400 nm polycarbonate porous membranes (data not shown). As we had seen previously for 100% BMP dispersion, passage through the 400 nm pore results in the formation of vesicles of ~ 200 nm in size. The specific values of diameters obtained from DLS are also given in Table 1.

Effects of GM1 on acylchain packing in BMP dispersions

EPR spectroscopy of 0.5–1 mol% of n-doxyl labeled lipids provides a facile means of investigating local acylchain packing and dynamics of lipid assemblies [24–28]. Here, both the 5-doxylPC and 7-doxylPC spin probes were used to characterize the local packing in the BMP and BMP:GM1 mixtures. Figure 3 shows resultant EPR spectra and the chemical

structures of the n-doxylPC lipids. For 100% BMP dispersions, the EPR spectra of both 5-doxylPC and 7-doxylPC are nearly identical to those obtained when this spin probe is incorporated into POPC liposomes (dashed lines). We recently showed that for all positions along the acylchains, $n = 5, 7, 10, 12, 14$ and 16 , the spectral line shapes obtained in BMP were nearly identical to those in POPC, POPG, DOPC and DOPG [19]. Figure 3C shows how micellization of BMP by the detergent sodium dodecylsulfate (SDS) alters the EPR spectrum. Upon micellization, the organization and dynamics of the lipids are increased, which results in a narrowing of the EPR spectral lines. All EPR spectra in Figure 3 are plotted with normalized area of the integrated absorption spectra, so an increase in intensity results from a narrowing of the EPR line. In addition to the overall narrowing upon addition of SDS, the structural features in the low-field EPR absorption are lost. As can be seen in the spectra of all BMP:GM1 mixtures, these spectral features are not narrowed. In fact, the overall breadth of the spectra of both 5-doxylPC and 7-doxylPC in these mixtures increases, as indicated by the dotted lines in Figure 3. These findings imply that the acylchain packing in BMP:GM1 dispersions are more similar to those seen in liposomes than in mixed lipid:detergent micelles.

Conclusion

The spontaneous formation of small homogeneously shaped vesicles with narrow diameter size distribution suggests an apparent miscibility of GM1 with BMP over the 20–30 mol % ratio, implying that favorable interactions between these two lipids occur to form a lipid complex. This hypothesis is drawn from comparison of our results to those from Langmuir-monolayer studies of GM1 and DPPC mixtures, which show that the pressure-area isotherms of GM1 and DPPC mixtures follow “ideal” mixing behavior when the two species were considered to be a 3:1 DPPC:GM1 complex interacting with excess DPPC [23]. Our results on GM1 interactions with BMP provide morphological and size distribution evidence that GM1 mixes with BMP to form small (~100 nm) spherical shaped vesicles with a narrow size distribution at similar concentrations that were seen to condense DPPC and form a specific complex. This specific mixture of GM1 with BMP may be important for *in vivo* vesicular trafficking and lipid sorting in the endosome/lysosome pathways.

Acknowledgments

The research herein was funded by NIH R01 GM-77232 (GEF). The DLS instrument was purchased with funds from DARPA MODICE to Randolph S. Duran. TEM images were obtained using instrumentation provided by the Interdisciplinary Center for Biotechnology Research facility with the kind assistance of Donna Williams and Karen Kelley. We thank Joanna R Long for helpful discussions.

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Figure 1.

TEM and DLS characterization of BMP hydrated dispersions as a function of pH. (A) Chemical structures of the phospholipid BMP and the ganglioside GM1. (B) Representative negative staining-TEM images of BMP lipid dispersions as a function of pH. Note that the magnification changes for these images. (C) DLS size distributions of BMP hydrated dispersions at varying pH. The traces shown represent single histogram results from a single measurement. Typically, three to five measurements were made for each sample. (D) Average DLS results and errors are summarized with the error calculated as standard deviations of the most probable size obtained from three independent DLS histograms.

Figure 2.

Characterization of BMP:GM1 vesicle morphology by TEM and DLS. (A–F) Representative negative staining-TEM images of hydrated BMP:GM1 dispersions. Note that the magnification is the same in all of these images. (B) Magnified section of the 70:30 BMP:GM1 mixture showing aggregation. (C) DLS histograms of BMP: GM1 hydrated dispersions. The traces shown represent single histogram results from a single measurement. Typically, three to five measurements were made for each sample. Average results and errors are summarized in Table 1.

Figure 3.

EPR characterization of BMP:GM1 hydrated samples. Stack plot of 100G X-band EPR spectra of (A) 5-doxylPC and (B) 7-doxylPC incorporated into BMP:GM1 mixtures. The dashed spectrum show for comparison the spectra obtained when mixed with POPC. The dotted lines show how the breadth increases upon addition of GM1 (C) 100G X-band EPR spectra of 5-doxylPC incorporated into BMP when solubilized to a mixed micelle with SDS. (D) Chemical structures of 5-doxylPC and 7-doxylPC

Table 1

DLS results for BMP:GM1 hydrated lipid dispersions and unilamellar vesicles extruded with 400 nm polycarbonate membranes.

BMP:GM1 concentration	Average Vesicle Size (nm)				
	100:0	90:10	80:20	70:30	50:50
dispersions (TEM) ^b	390 ± 150 ^a (200 – 500)	420 ± 150 (300 – 600)	380 ± 70 (300 – 500)	430 ± 250 (~100)	540 ± 110 (400 – 700)
Extruded	230 ± 40	230 ± 70	180 ± 20	230 ± 30	230 ± 50
					240 ± 50

^aError bars for DLS measurements were calculated as standard deviations of the most probable average vesicle size obtained from three independent DLS histograms.

^bThe numbers in parenthesis indicate the representative vesicle size determined from 8–10 TEM images of each sample.