Colloids and Surfaces B: Biointerfaces 94 (2012) 374–377

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Short communication

Use of attenuated total reflectance Fourier transform infrared spectroscopy to study lactosylceramide and GD3 DMPC bilayers

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ARTICLE INFO

Article history:
Received 22 September 2011
Received in revised form
31 December 2011
Accepted 23 January 2012
Available online 10 February 2012

Keywords:
Attenuated total reflection
Fourier transform infrared
Lactosylceramide
GD3
DMPC
Lipid bilayer

ABSTRACT

Attenuated total reflection Fourier transform infrared spectroscopy was used to monitor the formation of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), DMPC: lactosylceramide, and DMPC: GD3 lipid bilayers onto a zinc selenide surface. Infrared absorption peak position, bandwidth, and intensity were all used to monitor the formation, acyl chain ordering, and chemical environment within each bilayer. The results from this study indicate that the addition of glycosphingolipids into a DMPC lipid bilayer introduces decreases in both, acyl chain ordering, and homogeneity within the bilayer. GD3:DMPC lipid bilayers possess lipid chain characteristics that are indiscernible from those present in the lactosylceramide:DMPC bilayer, while possessing different structural head groups, indicating that the head group has little influence on the underlying lipid structure. Differences in the phosphate hydration are, however, evident between the three types of bilayer, with phosphate hydration decreasing in the order LacCer:DMPC (1223.4 cm^{-1}) > DMPC only (1226 cm^{-1}) > GD3:DMPC (1229.6 cm^{-1}).

1. Introduction

Glycosphingolipids, ubiquitous components of cell membranes, are known to be involved in diverse biological processes, including cellular recognition and cell–cell communication. These complex molecular conjugates have characteristic amine-containing ceramide lipid backbones, in which a fatty acid is linked to a sphingoid base. A carbohydrate head group is attached at the primary hydroxyl of the lipid backbone [1]. The ceramide imbeds in the membrane and operates as a component of the functional architecture and an intracellular regulator, while the oligosaccharide interacts with the environment and serves as an identification tag. Glycosphingolipids are often found with high structural heterogeneity arising from variations in the ceramide tails and carbohydrate moieties [2]. Studies have shown that, during the progression of specific diseases, glycosphingolipid expression undergoes specific structural changes, and when ingested may contribute prevention of infection [3]. The variety of monosaccharide types and linkages has been shown to be related to specific recognition for environmental factors such as toxins and bacteria.

Modern techniques used to investigate the interaction between cell membrane glycosphingolipids and the environments have several limitations. For example, TLC-based toxin binding assays may provide improper orientation of membrane-incorporated glycosphingolipids. This molecular disorientation is due to the glycosphingolipid's hydrophilic carbohydrate group potentially interacting with the polar silica TLC plate [4]. FRET-based assays have been performed to assess toxin-receptor binding [5] providing a method for characterizing toxin-receptor interactions. These methods, however, lack the ability to provide further information on the molecular environments found at or within the lipid membrane during toxin-receptor interactions.

In order to provide an analytical technique conveying information on biological molecule-glycosphingolipid interactions within a biomimetic system, while monitoring chemical environments within the biomimetic system, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) is evaluated for its potential utility. This study demonstrates the use of ATR-FTIR to study two different glycosphingolipids, lactosylceramide and ganglioside GD3, incorporated within a continuous well-formed lipid bilayer. GD3 is a sialylated derivative of LacCer, increasing the size

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doi:10.1016/j.colsurfb.2012.01.034

of the oligosaccharide head group by two saccharides and producing an anionic species, whereas LacCer is neutral. These findings demonstrate manufacturing a lipid bilayer with properly incorporated glycosphingolipids yields a vastly different transmembrane environment compared to one lacking in glycosphingolipids. The effects on lipid tail ordering of further conjugation to the head group of a bilayer-incorporated glycosphingolipid have been studied and are presented here. This study highlights the importance of understanding the chemical environments in which glycosphingolipids are incorporated into when manufacturing cell-like environments.

2. Materials and methods

A 45° single-reflection zinc selenide (ZnSe) internal reflection element (IRE) was purchased from Pike Technologies (Madison, WI). Ultra-pure water (UPW) was purified from a Millipore Synergy water system purchased from Fisher Scientific (Pittsburgh, PA). Tris[hydroxymethyl]aminomethane (Tris), sodium chloride (NaCl), and other solvents used in IRE substrate preparation were purchased from Sigma–Aldrich (St. Louis, MO). 1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), a Mini-Extruder, 100 nm polycarbonate membranes, filters, filter supports, and heating block were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Bovine buttermilk lactosylceramide (Galβ1-4Glcβ-Cer) and ganglioside GD3 (NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ-Cer) were purchased from Matreya (Pleasant Gap, PA).

2.1. ATR-FTIR-measurements and substrate preparation

In order to prepare the single-reflection internal reflection element (IRE) ZnSe crystal was polished with aluminum oxide and colloidal silica, and then subjected to ozone cleaning. The crystal was then mounted in a crystal plate along with a flow cell.

IR spectra were recorded using an overlayer enhanced attenuated total reflectance Fourier transform infrared spectroscopy (OE-ATR-FTIR) system (Mattson Galaxy 5020) equipped with a narrowband liquid nitrogen cooled mercury–cadmium–telluride (MCT) detector. Each spectrum was recorded at a resolution of 4 cm^{-1} in the range of 750–4000 cm^{-1} with 512 scans averaged for each spectrum. The spectrometer was purged for at least 24 h with dry air to reduce absorption due to carbon dioxide and water vapor.

Absorption spectroscopy requires that a sample spectrum be ratioed against that of a blank. Blank spectra consisting of 512 scans were obtained while flowing a buffer solution consisting of 10 mM Tris and 100 mM NaCl, pH 7.4, prior to vesicle solution injection. At least two trials of each injected vesicle solution were carried out for these experiments.

The C—H stretching region is analyzed from 2800 cm^{-1} to 3000 cm^{-1}. Third and fourth derivative analyses are performed on the selected region to identify the number of characteristic bands present within each spectrum. These bands are then simultaneously curve-fit by a weighted sum of Gaussian and Lorentzian functions. The specific band for this study is located near 2850 cm^{-1}, and is assigned to the CH_2 symmetric stretching vibration. Adsorbed lipid concentrations and characteristics of the lipid layer are determined by integration, band position, and full width at half maximum (FWHM) of the curve-fitted band near 2850 cm^{-1}. In previous work in our group [13], we have shown that the band intensities, positions, and widths can be used to determine whether the adsorbed lipids are present as monolayers, bilayers, or intact vesicles. For this study, the lipid films were determined to be present as bilayers.

The region between 1100 and 1300 cm^{-1} is selected to analyze the phosphate region. Third and fourth derivative analyses are performed on the selected region to identify the number of characteristic bands present within each spectrum. These bands are then simultaneously curve-fit by a weighted sum of Gaussian and Lorentzian functions. The specific band important for this study is located near 1226 cm^{-1}, and is assigned to the PO_2^- asymmetric stretching vibration. Relative hydration of the bilayer interface is determined from the band position and full width at half maximum (FWHM) of the curve-fitted band near 1226 cm^{-1}.

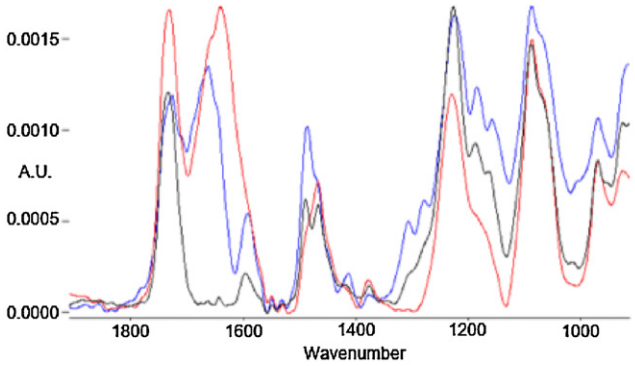

Fig. 1. Shows the superimposed IR fingerprint region for DMPC (black), DMPC:LacCer (blue), and DMPC:GD3 (red) bilayers. The IR region displays an increase in the Amide absorption band for both of the DMPC:LacCer and DMPC:GD3 bilayer, and absent for the DMPC bilayer. The region also shows an increase in the carbonyl absorption for the DMPC:GD3 bilayer presumably due to the additional carbonyls found within the sialic acid residue, compared to the DMPC and DMPC:LacCer bilayers that are equivalent to one another. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Vesicle preparation and injection

DMPC lipids, DMPC lipids and lactosylceramide (DMPC:LacCer) 85:15 mol%, and DMPC lipids and GD3 ganglioside (DMPC:GD3) 85:15 mol% dissolved in chloroform were dried under a steady stream of nitrogen and then placed under vacuum for at least 12 h. The glycosphingolipids have ceramide lipid moieties with 18-carbon sphingosines (d18:1) in amide linkage to C16–24 fatty acids. Dried lipid film was re-suspended in a solution of 10 mM Tris and 100 mM NaCl buffer at a pH of 7.4 to give a concentration of 1.0 mg/ml. A Mini-Extruder, polycarbonate membrane with a mean size pore diameter of 100 nm, and a heating mantle (Avanti Polar Lipids Inc.) were used to prepare vesicles. New vesicle solutions were prepared prior to each trial. Injections of vesicle solutions onto ZnSe were carried out separately nominally under room temperature (21 °C) conditions. A syringe pump held at a constant rate of 1.5 ml/h controlled all injected solutions. The continuous injection above the prepared surfaces involved 1 ml of vesicle solution for 40 min, after which buffer solution would flow for several hours.

3. Results and discussion

The superimposed IR spectra fingerprint regions are obtained after each separate exposure of 110 nm DMPC (black), DMPC:LacCer (blue), and DMPC:GD3 (red) vesicle solution to ZnSe are shown in Fig. 1. These spectra are obtained 120 min after the initial injection of the vesicle solution; thus 80 min after vesicle solution has been flushed from the solution by pure buffer. As Fig. 2 demonstrates these spectra represent stable surface-adsorbed thin films. Fig. 1 shows an amide (~1633 cm^{-1}) absorption band present in both of the DMPC:LacCer and DMPC:GD3 bilayer, and is absent in the DMPC lipid bilayer. The absence of this functional group within DMPC's molecular structure is the cause for no absorption occurring at the observed frequency attributed to amides; however, the LacCer and GD3 both posses amide functional groups (1: LacCer and 3:

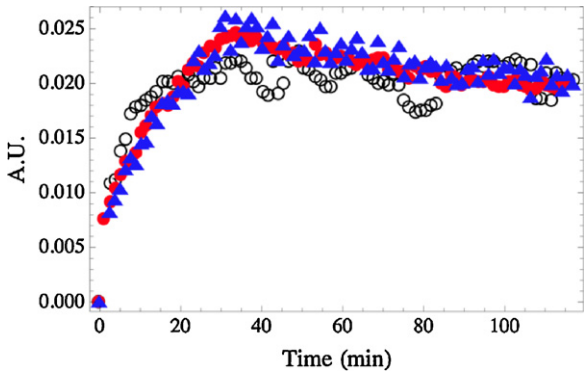

Fig. 2. Represents the integrated absorbance of the symmetric methylene groups found within a bilayer of DMPC (black), DMPC:LacCer (blue), and DMPC:GD3 (red) adsorbed to ZnSe. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GD3) [6]. The inequality in the number of amide functional groups present within DMPC:GD3 and DMPC:LacCer bilayers are presumed to cause the differences in the observed absorption. This region also shows an increase in the carbonyl absorption band (~1776 cm^{-1}) for the DMPC:GD3 bilayer [7]. This increase is presumably due to the additional carbonyls located within the DMPC:GD3 bilayer owing to the molecular structure of GD3; compared to both the DMPC and DMPC:LacCer bilayers that are approximately equivalent to one another.

The adsorption kinetics for each vesicle solution is monitored by the time dependent integrated absorbance of the ν_{sym}—CH_2 (~2850 cm^{-1}) absorption band as shown in Fig. 2. The time dependent absorbance signal for the lipid vesicles and lipid-mixture vesicles interacting with the ZnSe surface show a monotonic increase in the absorbance, asymptotically approaching ~0.020 a.u. The initial rise in integrated methylene intensities is attributed to initial vesicle adsorption to the surface of ZnSe. 80 min after completion of vesicle injection and bilayer formation, the three different well-formed lipid bilayers possess similar asymptotic absorbance values, suggesting similar surface densities within each bilayer.

3.1. Bilayer characteristics

Studying the relative ordering and homogeneity within the lipid bilayer's hydrophobic acyl chain core for each well-formed bilayer is accomplished by monitoring the time dependent maximum observed frequency and FWHM of the ν_{sym}—CH_2. Fig. 3

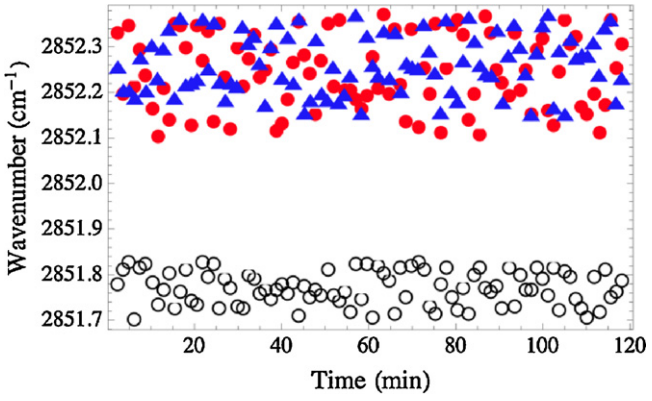

Fig. 3. Maximum absorbance band position of the symmetric methylene stretch for the bilayers of DMPC (black), DMPC:LacCer (blue), and DMPC:GD3 (red) adsorbed to ZnSe. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

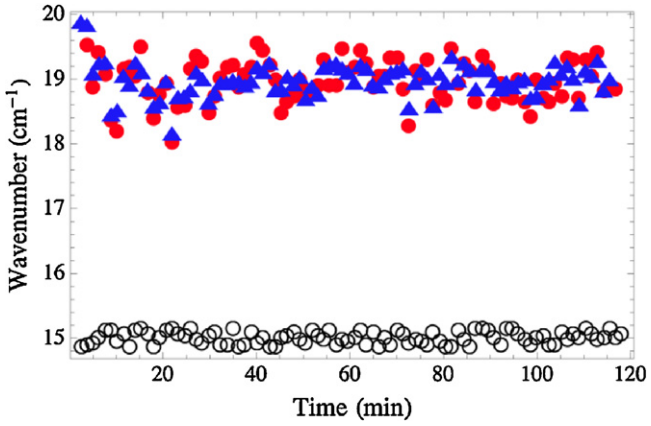

Fig. 4. FWHM of the symmetric stretching mode for the methylene groups present in the bilayers of DMPC (black), DMPC:LacCer (blue), and DMPC:GD3 (red) adsorbed to ZnSe. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shows the time dependent maximum observed frequency for the three different bilayers, with values ranging between 2851.7 cm^{-1} and 2852.3 cm^{-1}. The DMPC bilayers containing GD3 and LacCer show overlapping maximum frequencies with a mean observed frequency centered at 2852.25 $cm^{-1} \pm 0.05$ cm^{-1}, while the homogeneous DMPC lipid bilayer maintains a mean observed frequency at 2851.75 $cm^{-1} \pm 0.05$ cm^{-1}. It has been previously reported that the observed maximum frequency decreases as the ratio of gauche to trans conformers within the acyl chains decreases [8]. Therefore, the acyl chain ordering within the homogeneous DMPC lipid bilayer shows increased ordering compared to the DMPC bilayers containing GD3 and LacCer. The decrease in transmembrane domain ordering is in good agreement with previous studies investigating DMPC:ganglioside bilayers [9]. The hydrophobic mismatch between ceramide, present in both GD3 and LacCer, and the phospholipid tail group within DMPC presumably cause the decrease in acyl chain ordering [10]. It is proposed that the reason for overlapping frequencies for the DMPC bilayers containing GD3 or LacCer is due to their molecular structure introducing very similar, if not the same, amount of disorder within the hydrophobic core. It is likely that they should introduce similar amounts of disorder into the DMPC bilayer, because the molecular structure participating in hydrophobic interactions is the same in both GD3 and LacCer [11].

Investigations in probing homogeneity for each lipid bilayer were accomplished by monitoring the values for the time dependent FWHM during and subsequent to the generation of each well-formed bilayer. The FWHM values for the ν_{sym}—CH_2 absorption band are representative of the homogeneity of chemical environment it is located in, therefore, providing information on the transmembrane domain within the lipid bilayer. Fig. 4 shows the observed time dependent FWHM values for the 3 different bilayers ranging between 15 cm^{-1} and 20 cm^{-1}. The DMPC bilayers containing GD3 or LacCer exhibit overlapping FWHM values which center about 19 $cm^{-1} \pm 0.5$ cm^{-1}, while the homogeneous DMPC lipid bilayer maintains FWHM values that center about 15 $cm^{-1} \pm 0.2$ cm^{-1}. These results are in agreement with each of the hydrophobic core bilayer environments; in comparing a binary component bilayer to a homogeneous bilayer one anticipates an increased variation in the number of chemical environments that are to be present within the inhomogeneous bilayer. The overlap in observed FWHM values for DMPC bilayers containing GD3 or LacCer is again attributed to the molecular structure participating hydrophobic interactions is the same in both GD3 and LacCer.

The relative hydration of the bilayer interface is analyzed through the asymmetric phosphate band characteristics centered about 1225 cm^{-1}. Previous studies have shown that the location of this absorbance band increases with decreasing relative hydration of the phosphates found within the phospholipid head groups [12]. Data presented here shows the band position increasing from LacCer:DMPC (1223.4 cm^{-1}) < DMPC only (1226 cm^{-1}) < GD3:DMPC (1229.6 cm^{-1}) lipid bilayers. This data suggests that phosphates within the LacCer:DMPC lipid bilayer are more hydrated compared to the other two bilayers. The differences in hydration within the three systems are likely due to availability of phosphates at the aqueous interface. The LacCer content within the bilayer may allow for an increased amount of water to coordinate with the phosphate's polar head group. Contrasting this idea, the GD3 content within the bilayer may restrict the phosphate availability to coordinate with water. This may be due to lateral geometric constraints present at the bilayer interface from the bulkier GD3 head group.

For this study, the lipid films were determined to be present as bilayers, as clearly evidenced by the integrated absorbance intensities of 0.22 a.u. for the methylene symmetric stretch in Fig. 2. Monolayers would give an intensity near 0.11 a.u. (half that of the bilayer) and vesicle intensities would be ~0.45 a.u., as determined by numerous previous studies in our laboratory (not shown). The degree to which substrate interactions influence the effects observed is unknown. However, others have shown that an adsorbed bilayer is typically supported on a layer of water on the substrate. The relatively small size of the LacCer and GD3 head groups would place them inside of that layer. Thus, it is likely that substrate effects contribute only minimally to the observed differences.

4. Conclusions

This study has shown that ATR-FTIR is valuable for investigating glycosphingolipid–phospholipid bilayers, particularly in monitoring the formation of biomimetic systems, and segregating chemical environments due to variations in component composition. Results presented here show independent behavior of the lipids' acyl chain relative ordering on further conjugation of the glycosphingolipid head-group, even while differences are observed in phosphate head group structures. This independent lipid behavior provides a basis for future studies concerning biomolecule-glycosphingolipid interactions that are studied within a biomimetic system. This study extends the information about influences that glycosphingolipid's have on biomimetic lipid systems, and aids in tailoring experiments designed to study biomolecule-glycosphingolipid head group interactions.

Acknowledgments

The authors also acknowledge funding via grant number T32-GM008799 (Mateo Hernandez) from NIGMS-NIH and NSF CHE-08-0991. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIGMS or NIH.

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