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Review

Thin-layer chromatography, overlay technique and mass spectrometry: A versatile triad advancing glycosphingolipidomics ☆

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ABSTRACT

Much effort is currently invested in the development of mass spectrometry-based strategies for investigating the entirety of glycosphingolipids (GSLs) of a certain cell type, tissue, organ or body encompassing the respective glycosphingolipidome. As part of the investigation of the vertebrate glycosphingolipidome, GSL analysis is undergoing rapid expansion owing to the application of novel mass spectrometry techniques acting as the linchpin in the network of collaborations challenged to unravel structural and functional aspects of GSLs. Difficulties may arise in the determination of the exact structures of GSLs due to the heterogeneity of the sugar moiety varying in the number and sequence of monosaccharides, and their anomeric configuration and linkage type, which make up the principal items of the glyco code of biologically active carbohydrate chains. The ceramide variability caused by the diversity of the long-chain amino alcohol and the fatty acid, which both may vary in chain length, degree of unsaturation, and type and number of substituents, further contributes to the increasing number of possible GSL species. In view of this heterogeneity, a single-method analytical mass spectrometry (MS) technique without auxiliary tools yields limited data, providing only partial structural information of individual GSLs in complex mixtures. Approaching this challenge, current advances on a triad system matching three complementary methods are described in this review: (i) silica gel based TLC separation of GSLs, (ii) their overlay detection on the TLC plate (mostly based on antibody-mediated recognition), and (iii) direct and indirect MS based structural characterization, i.e. directly on the TLC plate or in lipid extracts from silica gel. We will focus on recent improvements by employing antibodies, AB_5 toxins and bacteria for direct IR-MALDI-o-TOF MS and indirect ESI-QTOF MS analysis of GSLs. We believe that the combinatorial approach using conventional TLC and modern mass spectrometry provides a developmental advance in exploring the glycosphingolipidome of biological material.

1. Introduction

The German clinician Johann Ludwig Wilhelm Thudichum, born in 1829 can be considered the father of (glyco)sphingolipid research [1,2]. The sphingosine backbone of sphingolipids was named by Thudichum in 1884 for its enigmatic ("Sphinx-like") properties. In Greek mythology, the sphinx is a monster that posed a riddle to all it encountered and dispatched those who could not decode the riddle [3]. However, despite tremendous progress in glycosphingolipid (GSL) research, their function has largely remained enigmatic. According to a review published in 1989 [4], more than 250 different GSLs have been reported up to that time and their number is still increasing. GSLs, as cell surface molecules, are complex highly regulated participants in cell recognition and the modulation of function of membrane-associated proteins such as receptors, transducers and transporters [5,6]. The functional role of GSLs and their derivatives as modulators of transmembrane signaling has been further thoroughly reviewed by many authors and the interested reader is referred to some excellent reviews [7–10]. The physiological importance, for instance, of sialylated GSLs (= gangliosides) as

Abbreviations: AB2-3, Neu5Acα3Galβ4GlcNAc-specific antibody; AB2-6, Neu5Acα6Galβ4GlcNAc-specific antibody; AP, Alkaline phosphatase; a-TOF, Axial time-of-flight; BCIP, 5-bromo-4-chloro-3-indolylphosphate; CD, Cluster of differentiation; Cer, Ceramide; CID, Collision-induced dissociation; CTB, Cholera toxin B subunit; d18:1, 4-sphingenine; *E. coli*, *Escherichia coli*; Er:YAG, Erbium:yttrium–aluminum–garnet; ESI, Electrospray ionization; GSL(s), Glycosphingolipid(s); HPLC, High-performance liquid chromatography; *H. pylori*, *Helicobacter pylori*; IR, Infrared; IUPAC-IUB, International Union of Pure and Applied Chemistry–International Union of Biochemistry; LacCer, Lc2Cer, lactosylceramide; MALDI, Matrix-assisted laser desorption/ionization; MS, Mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; o-TOF, Orthogonal time-of-flight; Plexigum, Polyisobutylmethacrylate; Q, Quadrupole; RCL, *Ricinus communis* lectin; rViscumin, Recombinant Viscumin; Stx, Shiga toxin; TLC, Thin-layer chromatography; TOF, Time-of-flight; UV, Ultraviolet

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regulatory elements in the immune and the central nervous system has been impressively shown by Schnaar and colleagues [11–14] and their key roles in the development, but also in the pathogenesis of organs has been demonstrated by Yu and collaborators and others [15–18]. However, further improvements in the isolation and separation technologies as well as the implementation of novel methodologies for GSL structural characterization are required in order to pave the way in particular to solve questions on the involvement of GSLs in cell physiological processes.

2. Glycosphingolipids

2.1. Chemical structure, nomenclature, and isolation techniques

GSLs are built up from a hydrophobic ceramide moiety and a hydrophilic oligosaccharide residue which render GSLs amphipathic molecules [19,20]. The ceramide anchor consists of a sphingoid base [21] that carries a fatty acid linked to the nitrogen atom of the long-chain bivalent aminoalcohol. In vertebrate GSLs the dihydroxylated and singly unsaturated long-chain amino alcohol sphingosine (4-sphingenine, d18:1) and a fatty acid, which may vary in chain length most commonly from C16 to C24 and degree of unsaturation, represent the typical core structures of the ceramide anchor. The oligosaccharide is connected *via* glycosidic linkage to the primary hydroxy group of the ceramide. GSL biosynthesis occurs by stepwise connection of monosaccharides and sialic acids being accomplished by specific glycosyl- and sialyltransferases, respectively [22–24].

Neutral GSLs and sialic acid-containing gangliosides constitute the majority of mammalian GSLs which in most cases belong to one of the four main structural families: the ganglio (Gg)-, globo (Gb)-, lacto (Lc)- or neolacto (nLc)-series (Fig. 1). According to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [25] the name of a certain GSL contains the designation of the structure family, the number of monosaccharide units (indicated by the suffixes “biaosyl”, “triaosyl”, “tetraosyl”, etc.) and the ceramide moiety abbreviated by “Cer”. Examples are globotriaosylceramide (Gb3Cer) or neolactotetraosylceramide (nLc4Cer) (see Fig. 1).

The parent sialic acids of gangliosides are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), interestingly the latter is absent in normal human tissues [26]. Gangliosides are termed by acronyms consisting of the root structure or the name of the GSL family to which the sialic acid is attached and the number of monosaccharides (e.g., Lc2Cer, Gg4Cer, nLc4Cer) as described above (see Fig. 1), a Roman numeral that indicates the monosaccharide residue to which the sialic acid is attached (counted from the monosaccharide proximal to ceramide), and an Arabic numeral superscript that refers to the position within the oligosaccharide core to which the sialic acid is linked (e.g., II^{3}Neu5Ac-Gg4Cer or IV^{6}Neu5Ac-nLc4Cer; see Table 1). All hexoses are in the D-configuration of the pyranose form and all glycosidic linkages originate from the C-1 hydroxyl group. Neu5Ac is always linked to Gal through the C-2 hydroxyl group in α3- or α6-configuration or through the C-8 hydroxyl group for α8-bound Neu5Ac—Neu5Ac. The biosynthesis scheme and the structures of IV^{6}Neu5Ac-nLc4Cer (d18:1, C16:0) and IV^{3}Neu5Ac-nLc4Cer (d18:1, C16:0) are depicted in Fig. 2. The symbolic representation system according to Varki [27] and the Consortium for Functional Glycomics [28] is used in this review.

The extraction of GSLs from biological material is usually performed using chloroform–methanol mixtures with a recommended tissue-to-solvent ratio of 1:20 to 1:40 (weight/volume). Details concerning the isolation and purification procedures have been previously described in classical reviews by Ledeen and Yu [29] and Schnaar [30]. Anion-exchange chromatography using DEAE-linked matrices is convenient to separate crude GSL extracts into neutral and negatively charged GSLs and have become well established standard procedures. Thereafter, the neutral and acidic GSL containing fractions can be further purified by silica gel chromatography or as peracetylated derivatives by Florisil-chromatography. Individual GSLs can then be isolated by preparative high-performance liquid chromatography (HPLC) (for review see [31]).

2.2. Lipid rafts and caveolae

Lipids organize laterally within the lipid bilayer of a biological membrane to form fluid platforms in which certain lipid species are concentrated while others are diminished. These assemblies named as lipid rafts are composed mainly of (glyco)sphingolipids and cholesterol in the outer membrane leaflet [32,33], somehow connected to domains of largely unknown composition in the inner leaflet [34]. GSLs preferentially reside in the outward-facing part of the plasma membrane bilayer (Fig. 3) [35] where they are clustered in a liquid-ordered phase

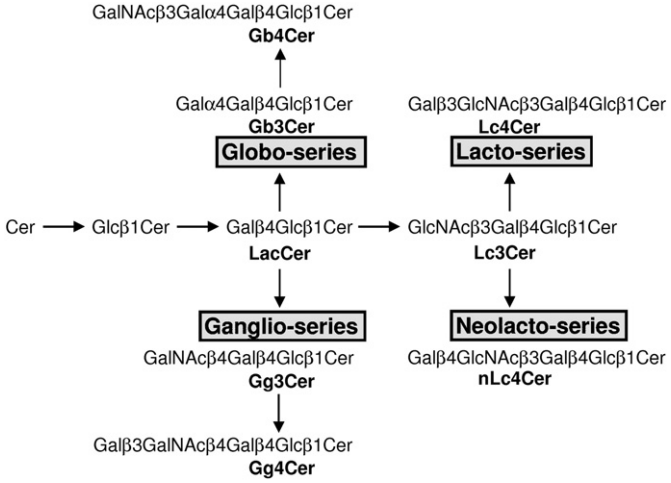

Fig. 1. Biosynthesis flow chart of neutral GSLs with tetrahexosylceramide core of the ganglio-, globo-, lacto-, and neolacto-series. Core structures may be sialylated at different stages of neutral GSL biosynthesis (e.g. LacCer → GM3 or nLc4Cer → IV^{6}Neu5Ac-nLc4Cer, see Table 1) or ganglioside biosynthesis (e.g. GM3 → GD3 or GM1 → GD1a) as reviewed by Lahiri and Futerman [8]. Further prolongation of nLc4Cer by 1, 2 or more Galβ4GlcNAc-repeats results in nLc6Cer, nLc8Cer or even longer nLc-core structures.

Table 1
GM3, GD3, ganglio- and neolacto-series gangliosides.

Ganglioside[a]	Abbreviation[a]	CD classification[b]
II^{3}Neu5Ac-Lc2Cer	GM3	–
II^3(Neu5Ac)$_2$-Lc2Cer	GD3	CD60a
Ganglio-series		
II^{3}Neu5Ac-Gg3Cer	GM2	–
II^{3}Neu5Ac-Gg4Cer	GM1	–
II^3(Neu5Ac)$_2$-Gg3Cer	GD2	–
IV^{3}Neu5Ac,II^{3}Neu5Ac-Gg4Cer	GD1a	–
II^3(Neu5Ac)$_2$-Gg4Cer	GD1b	–
IV^3(Neu5Ac)$_2$,II^{3}Neu5Ac-Gg4Cer	GT1a	–
IV^{3}Neu5Ac,II^3(Neu5Ac)$_2$-Gg4Cer	GT1b	–
IV^3(Neu5Ac)$_2$,II^3(Neu5Ac)$_2$-Gg4Cer	GQ1b	–
Neolacto-series		
IV^{3}Neu5Ac-nLc4Cer	IV^{3}nLc4	iso-CD75s-1
IV^{6}Neu5Ac-nLc4Cer	IV^{6}nLc4	CD75s-1
VI^{3}Neu5Ac-nLc6Cer	VI^{3}nLc6	iso-CD75s-2
VI^{6}Neu5Ac-nLc6Cer	VI^{6}nLc6	CD75s-2

[a] Only gangliosides with Neu5Ac-substitution are displayed. For core sequences of Lc2-and the ganglio- and neolacto-series oligosaccharides see Fig. 1. The nomenclature and the abbreviations follow the recommendations of the IUPAC-IUB and Svennerholm [25].
[b] CD: cluster of differentiation.