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Infrared MALDI mass spectrometry imaging of TLC-separated glycosphingolipids with emphasis on Shiga toxin receptors isolated from human colon epithelial cells

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ABSTRACT

Thin-layer chromatography (TLC) combined with infrared MALDI mass spectrometry (IR-MALDI-MS) is a powerful analytical tool for detection and structural characterization of glyco- and phospholipids directly from the TLC plate. Here we coupled a pulsed IR-MALDI laser to a hybrid Synapt G2-S mass spectrometer (Waters) and obtained an effective focal spot size of ~50 × 70 μm² in diameter. We used this new MALDI ion source configuration for TLC-IR-MALDI-MS imaging of neutral glycosphingolipids (GSLs), obtained from human colon epithelial HCT-8 cells, at 100 μm step size. Our analytical focus was on the detection of globo-series GSLs globotriaosylceramide (Gb3Cer) and globotetraosylceramide (Gb4Cer), the main receptors for Shiga toxins (Stxs) produced by enterohemorrhagic *Escherichia coli* (EHEC). The direct TLC-IR-MALDI-MSI analysis allowed precise visualization of the chromatographic separation of the various lipoforms of Gb3Cer and Gb4Cer, with ceramide moieties mainly ranging from Cer (d18:1, C16:0) to Cer (d18:1, C24:0/C24:1) as well as those of their precursor GSLs glucosylceramide (GlcCer) and lactosylceramide (Lc2Cer). Reference TLC overlay immunostaining assays were conducted on parallel developed TLC lanes for confirmation of anomeric sugar configuration of proposed structures. Together, the adopted protocol provided a rapid and near-comprehensive overview of the GSL composition of the investigated cell line of high medical relevance. This possibility could also be highly useful in glycolipidomics studies of complex biological matrices.

1. Introduction

Glycosphingolipids (GSLs) play important roles in a vast variety of biological processes, including cell-cell interactions and modulation of transmembrane signaling [1–3]. Structurally, GSLs comprise a hydrophilic mono- or oligosaccharide facing the extracellular environment and a hydrophobic ceramide moiety anchored in the outer layer of the plasma membrane [4]. The oligosaccharide epitopes of GSLs are abused by numerous pathogens and pathogen-derived toxins as targets for binding and/or subsequent cellular uptake [1,5,6]. For example, Shiga toxins (Stxs) produced by enterohemorrhagic *Escherichia coli* (EHEC) bind to the neutral GSLs globotriaosylceramide (Gb3Cer) and globotetraosylceramide (Gb4Cer). Stxs are the major virulence factors in EHEC pathogenicity and their presence is strongly linked with life-threatening clinical scenarios, including hemolytic-uremic syndrome (HUS) and severe neurological disorders [7]. EHEC outbreaks are happening sporadically worldwide, causing serious health problems and even death of patients [8–10]. For instance, in 2011 a major outbreak was occurring in Germany that resulted in 54 fatalities [11].

A profound knowledge of the distribution of Gb3Cer and Gb4Cer receptors in organs and characterization of their lipoforms provides one basis for unravelling the role of differential cellular susceptibility to Stx and involvement in the development of GSL-related diseases [12]. A classical analytical method for detection of GSLs from cell extracts is silica-based thin-layer chromatography (TLC) [13]. Often the TLC separation is combined with overlay assays to detect individual carbohydrate epitopes using GSL-specific antibodies [14–16]. However, the TLC-based methods generally do

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not allow for discrimination of varying ceramide parts. Previous work suggested that the structural heterogeneity in the ceramide, in particular with regard to variable fatty acid chain length and degree of unsaturation, might have an impact on binding and retrograde transportation of protein toxins [6]. Moreover, GSL-GSL and GSL-cholesterol interactions, which may depend on the fatty acyl composition, have been reported to modulate GSL exposure to pathogenic determinants on the cellular membrane [1,17]. However, all in all these mechanisms are as yet only poorly understood and systematic studies are needed to get insight into multifaceted lipid-lipid interactions. Such investigations would clearly benefit from analytical tools that provide both exact information on the overall GSL compositions and the structures of individual GSLs.

Both infrared (IR) and ultraviolet (UV) lasers have been successfully utilized for direct TLC-MALDI-MS of glyco- and phospholipids [18–20]. For IR-MALDI-MS, the silica gel is typically soaked with a vacuum-stable glycerol matrix and desorption/ionization is achieved by use of pulsed IR lasers that emit at a wavelength of about 2.94 μm, corresponding to the peak absorption of the O–H stretch vibration. For UV-MALDI-MS, TLC plates are typically soaked with a high concentration of a classical UV-MALDI matrix (e.g., 2,5-dihydroxybenzoic acid [20]). Compared to UV-MALDI, the use of an IR laser is generally considered to lead to softer desorption/ionization conditions [21,22]. Moreover, deeper layers of the analyte-containing silica gel are ablated by the IR-laser. Both factors can have advantages for the sensitive analysis of low-concentrated (labile) lipids.

Distler and colleagues were the first to introduce a further powerful extension of TLC-IR-MALDI-MS and coupled it with TLC overlay binding assays. For example, these authors used their method, with which ions can be generated directly from immunostained bands, exemplified for the characterization of tumor-associated GSLs [14] and for identification of neolacto-series gangliosides as tumor markers [23]. Advances of GSL analysis by means of TLC-MALDI-MS have been extensively described in several recent reviews [13,24–26].

Another extension of TLC-MALDI-MS, by which mass spectra are typically acquired from small areas (e.g., single chromatographic bands), is TLC-MALDI-MS imaging (MSI). So far full MSI, by which all positions on a TLC plate (or a selected area) are analyzed pixel-by-pixel – and ideally all analytes from an entire chromatogram are visualized in 2D-intensity plot – has only been reported in combination with UV-MALDI. For example, TLC-UV-MALDI-MSI was used for imaging of the phospholipid content in egg yolk [27], colorectal adenocarcinoma xenografts partially treated with the anti-cancer drug DMXAA [28], as well as for bacterial phospholipids [29]. The lateral resolution obtained in these two works was about 150 and 200 μm, respectively. In a recent study, the method was moreover applied to reveal the presence of various lipoforms of monohexosylceramides, lactosylceramide (Lc2Cer), Gb3Cer, and Gb4Cer, as well as those of sphingomyelins, ceramides, and gangliosides in muscle tissue [30]. With regard to TLC-IR-MALDI-MS, a previous study demonstrated the usefulness of using linescans across the separated bands for distinction of phospholipid lipoforms varying in their fatty acyl chain composition [19] and for obtaining an "1D-image" of TLC-separated milk oligosaccharides [31].

Here we report the first implementation of direct TLC-IR-MALDI-MSI on a state of the art Synapt G2-S mass spectrometer (Waters) (in contrast to the previously used second generation orthogonal extracting time-of-flight, oTOF, instruments). Because of their high medical impact as potential targets for Stxs, we selected human colon epithelial HCT-8 cells as test samples. Our analytical focus was on the determination of the lipoform variability of Stx receptors Gb3Cer and Gb4Ce and their precursor GSLs in this cell line. The MS experiments were complemented by overlay assays performed on parallel TLC lanes for identification of the sugars forming the carbohydrate epitope.

2. Experimental

2.1. Purification of neutral glycosphingolipids

Neutral GSLs were isolated from HCT-8 cells (CCL-244, ATCC, Manassas, VA, USA) as described previously [32]. Briefly, GSLs were extracted with methanol (Roth, Karlsruhe, Germany) and methanol/chloroform mixtures of gradually increasing chloroform (Roth) concentration. Co-extracted phospholipids and triglycerides were degraded by alkaline treatment. Neutral GSLs were isolated by means of anion exchange chromatography using a DEAE Sepharose CL-6B column (GE Healthcare, Buckinghamshire, United Kingdom). The used GSL nomenclature follows the IUPAC-IUB recommendations [33].

2.2. Orcinol stain and TLC overlay assay

Neutral GSLs were applied as several parallel lanes onto glass plates precoated with silica gel 60 (HPTLC plates, size 10 cm × 10 cm, thickness 0.2 mm, cat. no. 1.05633.0001; Merck, Darmstadt, Germany) using a TLC sample applicator (Linomat 5; CAMAG, Muttenz, Switzerland). GSLs were separated in chloroform/methanol/water (120/70/17, each by volume). One lane of the developed TLC plate was stained with orcinol. This destructive protocol specifically stains all sugar-containing analytes. Further parallel lanes were used to identify specific GSLs by immunostaining with anti-GSL antibodies (see Chapter 2.3. *Primary anti-GSL and secondary antibodies*) directly on the plate as described previously [15,32,34]. Polyclonal antibodies for identification of Lc2Cer, Gb3Cer, and Gb4Cer were delivered onto TLC plates in 1:2000 dilutions, each. For GlcCer detection, RAS_0011 antibody was applied at a final concentration of 50 μg/mL.

2.3. Primary anti-GSL and secondary antibodies

Polyclonal rabbit anti-GlcCer antibody was from Glycobiotech (RAS_0011, Research Center Borstel, Germany). Binding specificities of polyclonal chicken anti-Lc2Cer, anti-Gb3Cer, and anti-Gb4Cer antibodies have been previously described [15,16,32,34,35]. Alkaline phosphatase (AP)-conjugated rabbit anti-chicken IgY antibody (code 303-055-033) and goat anti-rabbit IgG antibody (code 111-055-003), both from Dianova (Hamburg, Germany), served as secondary antibodies in TLC overlay assays. Bound antibodies were visualized as blue precipitate using 0.05% (w/v) 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP, Roth) in glycine buffer for color development.

2.4. Preparation of TLC plates for MS analysis

TLC-plates were cut to ~2 cm × 5 cm-wide pieces comprising the entire chromatogram of Stx receptor GSLs Gb3Cer and Gb4Cer as well as their precursor GSLs Lc2Cer and GlcCer. To obtain a uniform coating of the silica gel with the glycerol matrix (Roth), a key prerequisite for high-quality MS imaging, a custom-made setup and coating protocol were developed. To allow the viscous fluid to completely fill all pores of the silica particles, TLC plates were placed in a vacuum chamber at ~10^{-3} mbar for a few minutes. Within this time most air was evacuated from the pores of the silica gel. The plates were succeedingly emerged into a glycerol reservoir (kept in the same vacuum chamber). Upon venting the chamber, the pressure rise pressed the matrix evenly into the airless pores. Excess glycerol on the surface of the silica gel was carefully removed using a stream of dry nitrogen until no visible film of liquid was apparent. After