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Quantitative Analysis of Phospholipids Using Nanostructured Laser Desorption Ionization Targets[§]

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Abstract

Since its introduction as an ionization technique in mass spectrometry, matrix assisted laser desorption ionization (MALDI) has been applied to a wide range of applications. Quantitative small molecule analysis by MALDI, however, is limited due to the presence of intense signals from the matrix coupled with non-homogeneous surfaces. The surface used in nanostructured laser desorption ionization (NALDI) eliminates the need for a matrix and the resulting interferences, and allows for quantitative analysis of small molecules. This study was designed to analyze and quantitate phospholipid components of liposomes.

Here we have developed an assay to quantitate the DPPC and DC_{8,9}PC in liposomes by NALDI following various treatments. To test our method we chose to analyze a liposome system composed of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and DC_{8,9}PC (1,2-bis(tricoso-10,12-diynoyl)-*sn*-glycero-3-phosphocholine), as DC_{8,9}PC is known to undergo cross-linking upon treatment with UV (254 nm) and this reaction converts the monomer into a polymer.

First, calibration curves for pure lipids (DPPC and DC_{8,9}PC) were created using DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) as an internal standard. The calibration curve for both DPPC and DC_{8,9}PC showed an R² of 0.992, obtained using the intensity ratio of analyte and internal standard. Next, DPPC:DC_{8,9}PC liposomes were treated with UV radiation (254 nm). Following this treatment, lipids were extracted from the liposomes and analyzed. The analysis of the lipids before and after UV exposure confirmed a decrease in the signal of DC_{8,9}PC of about 90%. In contrast, there was no reduction in DPPC signal.

Keywords

NALDI; MALDI; mass spectrometry; lipids; quantitation; drug delivery

[§]List of abbreviation terms: DC_{8,9}PC: (1,2-bis(tricoso-10,12-diynoyl)-*sn*-glycero-3-phosphocholine), DHB: 2,5 - dihydroxybenzoic acid, DIOS: desorption ionization on silica, DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, ESI: Electrospray ionization, GC: Gas chromatography, HPLC: High-performance liquid chromatography, LC: Liquid chromatography, LDI: laser desorption ionization, MALDI: Matrix-assisted laser desorption/ionization, MS: Mass Spectrometry, NALDI: Nano-Assisted Laser Desorption/Ionization, TOF: Time-of-flight

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Introduction

Chromatography has been the traditional technique of choice for the analysis of lipids. Thin-layer chromatography [1] and liquid chromatography [2] have been the most widely used methods in the field, especially in combination with UV detection [3]. Those techniques are well-established and robust but they are also time-consuming, because they often involve steps of derivatization and long separation times [4]. Spectroscopic methods, such as IR and NMR, have been applied in the lipid analysis field as well [3]. The first use of mass spectrometry for the analysis of lipids was reported by Gohlke in 1959 [5] using gas chromatography mass spectrometry (GC-MS). Mass spectrometry provides information on the molecular weight of the species of interest that can be useful for identification purposes. Since then GC-MS has been applied to many analytical questions regarding the biology and chemistry of lipids [6], however GC-MS may require derivatization when the analyte is not volatile and thermally stable [7]. While derivatization may help, in some cases it results in additional steps that can complicate the entire analysis [8].

In recent years with the introduction of the so-called “soft ionization” techniques, electrospray ionization and matrix-assisted laser desorption ionization, [9] that allow for the analysis of intact lipids [10], mass spectrometry has gained importance in the analysis of lipid mixtures [11] and in the rapidly growing field of mass spectrometry tissue imaging [12]. Electrospray ionization (ESI) is the most frequently used mass spectrometry technique for lipids analysis, but matrix-assisted laser desorption ionization (MALDI) represents a powerful tool in this field, because the time necessary for the analysis can be reduced to less than one minute per sample [10]. ESI mass spectrometry in association with liquid chromatography (LC) allows for separation and identification of molecular species, however analysis times can be long and the buffers used for LC/MS are often incompatible with lipid solubility.

In MALDI, the sample is mixed with a solution of matrix that absorbs the laser energy. The mixture is allowed to dry to form co-crystals on the surface of target. The homogeneity of the solid crystals on the plate is essential for the reproducibility of the analysis, therefore accurate sample preparation is necessary [10]. In most cases, the matrix is soluble in the same organic solvents as the lipid analytes, so very homogenous co-crystals are produced facilitating the analysis.

The choice of the most suitable matrix is often quite challenging. While water soluble analytes have a wide range of useful matrices [13], only a few matrices have been utilized for lipid analysis due to their lipophilic nature. Recently Teuber et al. [14] compared a series of matrices for lipids. Along with the most commonly used DHB (2,5 - dihydroxybenzoic acid), other matrices showed interesting characteristics, especially a newly synthesized compound, α -cyano-2,4-difluorocinnamic acid, for analysis of charged phospholipids, and 9-aminoacridine for negative ion analysis. For small molecule analysis it is necessary to choose a matrix that does not generate polymers by photochemical reactions (e.g. sinapinic acid), because the polymerization products will saturate the detector [4] and suppress the signal from the analytes. In addition the presence of interfering peaks due to matrix adducts (e.g. DHB with Na^+), [15] in the lower m/z range is a crucial issue in the analysis of small molecules, therefore other approaches have been taken into consideration. Some studies suggested the use of high molecular weight matrices such as meso-tetrakis(pentafluorophenyl)porphyrin [16–18] for the analysis and quantification of fatty acids. In this case the signals due to molecular ions of the matrix or its impurities, adducts or clusters are in a higher mass range and do not overlap with the analyte signal.

Recently matrix-free laser desorption ionization (LDI) has become a promising approach in the field of small molecules. In this case the sample is applied onto a surface that absorbs the laser energy without the use of additional compounds. The direct deposition of the sample involves minimal sample preparation and practically no signal from the support surface [15]. Silicon has been one of the first materials to be used as a suitable support for matrix-free LDI [19]. Its surface can be easily oxidized and modified to trap the analyte and it has high absorptivity in the UV, therefore it can act like the matrix itself. This technique, termed desorption ionization on silica (DIOS), has been applied to a number of small molecule analytes [20]. The use of silicon as a LDI mass spectrometry support has evolved with the recent introduction of silicon nanowires [21]. Muck et al. provided evidence of the superiority of this approach compared with other traditional techniques that use energy absorbing matrices of questionable purity and stability [22]. Other nanostructures have interesting characteristics. Carbon nanotubes have shown low signal background and good capability to transfer energy to the analytes [23–25]. Carbon nanotubes often have solubility issues, however. Ren et al. used oxidization to improve solubility and produced a more homogeneous phase with reproducible analysis [25].

An issue with the use of nano-structured materials is that they are produced by a random process [20] that cannot always guarantee a homogeneous surface where the sample should be applied. In contrast, ZnO nanowires can be selectively grown on a substrate, are easy to synthesize, and their orientation can be controlled, so that on a flat substrate they can be organized as vertical wire arrays with defined dimension and structure [26]. ZnO presents characteristics that are particularly suitable for many industrial applications [27, 28]. It is a semi-conductive material that can absorb energy from the laser in wavelengths typically used in MALDI and transfer it to the analyte. ZnO nanoparticles have been successfully applied to the desorption/ionization of several small molecules, from drugs to lipids [29]. Kang et al. compared a suspension of analyte/ZnO nanowires and the direct deposition of the analyte onto the surface of a nanowire chip [28]. The nanowire chip showed better performance and a potential applicability to quantitative analysis.

In this study we demonstrate how a NALDI™ chip (Bruker Daltonics) can be used as a support for desorption/ionization applied for small molecule quantitative analysis. We have examined two phospholipids, DPPC and DC_{8,9}PC, that have been recently used for development of photo-triggerable liposomes [30–32]. Our system represents a novel and elegant method to determinate the concentrations of the two phospholipids of interest in the liposomes. This methodology bears the potential for future applications for the analysis of molecules with similar chemical and physical properties.

Methods and Materials

Liposome preparation and treatment

Liposome Reagents—DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) as solutions in chloroform. DC_{8,9}PC (1,2-bis (tricoso-10,12-diynoyl)-*sn*-glycero-3-phosphocholine) was synthesized by Dr. Alok Singh at the Naval Research Laboratory (Washington, DC) using a previously published procedure [30]. Methanol HPLC grade was purchased from OmniSolv (EMD Chemicals, Inc., Gibbstown, NJ), while Chloroform ACS was from Fischer Scientific (Pittsburg, PA).

Preparation of Liposomes—Liposomes were prepared using the probe sonication essentially as described [30]. Briefly, DPPC:DC_{8,9}PC (80:20 mol%) lipid mixtures were mixed in a glass tube. The lipid film was formed by removing the solvent under nitrogen and any residual chloroform was removed by placing the film overnight in a vacuum desiccator.

To encapsulate calcein, the lipid film was reconstituted in HEPES buffer (HBS, 10 mM HEPES, 140 mM NaCl, pH 7.5) containing self-quenched concentration of calcein (0.1 M at pH=7.2–7.6). Unilamellar Vesicles were formed by sonication at 4°C for 5–10 min (1 min pulses and 1 min rest) using a Probe Sonicator (W-375 Heat Systems-Ultrasonics, New York, USA). The samples were centrifuged to remove any titanium particles and larger aggregates. Solute-loaded liposomes were separated from untrapped calcein using a size exclusion gel chromatography column (Bio Gel A0.5m, 1 × 40 cm, 40 ml bed volume).

UV Treatment—Liposomes were placed in a 96-well plate (total volume of 0.2 ml) as described [23], and irradiated with a UV lamp (UVP, short wave assembly, 115V, 60Hz) at 254 nm in a distance of 1 inch for 45 minutes. We routinely examined calcein leakage and DC_{8,9}PC photo-crosslinking to confirm the modification in our liposome preparations as previously described [30, 31]. For mass spectrometry analysis (see below) lipids were extracted according to the Bligh/Dyer method [33].

Mass Spectrometry Analysis

Reagents—Methanol was HPLC grade (OmniSolv, EMD Chemicals, Inc., Gibbstown, NJ). Chloroform was HPLC grade from Chromasolv (Sigma-Aldrich, St. Louis, MO). Water was LC-MS grade from J.T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ). Cesium chloride (CsCl, 99.9%) was purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparation—Sample preparation used only glassware and Wiretrol disposable glass micropipettes (Drummond Scientific Company, Broomall, PA). The lipid films obtained from Bligh-Dyer extraction were reconstituted in 200 µL of chloroform, then diluted 1:10 in a mixture methanol/chloroform, 1/1, v/v. Standard solutions for each lipid were prepared in methanol/chloroform, 1/1, v/v. DMPC was used as an internal standard at a concentration of 25 ng/µL in methanol/chloroform, 1/1, v/v. A CsCl solution, 50 mM in methanol, was used as a matrix additive to promote formation of [M+Cs] pseudo molecular ions for the lipids studied. Calibration curves were created for the phospholipid combination by mixing DC_{8,9}PC in equal volume (5 µL) with the corresponding dilution of DPPC, followed by addition of 5 µL of DMPC 25 ng/µL and 15 µL of CsCl 50mM. Concentration ranges for DPPC and DC_{8,9}PC were 10–200 ng/µL and 2.5–100 ng/µL, respectively. We washed the NALDI target twice with HPLC grade chloroform and allowed to dry prior to use. The standard solutions were mixed and 3 µL spotted onto the surface of a NALDI chip (Bruker Daltonics, Billerica, MA). Standards were spotted in triplicate. In a similar fashion, 10 µL of the diluted sample solutions were mixed with DMPC 25 ng/µL (5 µL) and CsCl 50 mM (15 µL) and 3 µL of each solution spotted in triplicate onto the surface of the same target where the corresponding standard solutions were spotted.

Mass Spectrometry Analysis—All spectra were acquired on a Bruker Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Spectra of intact lipids were acquired in positive ion reflector mode, with no ion extraction delay. Ion source voltage 1 was 25kV, ion source voltage 2 was 21.30 kV and lens voltage was 9.52 kV. Reflectron and reflectron 2 were 29.50 kV and 13.84 kV, respectively. Monoisotopic masses were determined using FlexAnalysis 3.0 (Bruker Daltonics) with the SNAP peak picking algorithm. The instrument was calibrated in reflector mode using peptide standards of the Bruker peptide calibration kit (Bruker Daltonics, Billerica, MA). Data acquisition and peak analysis were completely automated with an accumulation of 1200 shots using a random walk pattern. To create the calibration curves, intensity ratios of the analyte signal relative to the intensity of the internal standard were calculated and averaged from the three measurements at each concentration.

Results and Discussion

Phospholipids are the basis of cellular structures and because of their amphiphilic properties, they have the natural tendency to form a bilayers in an aqueous system [3]. These unique properties allowed A.D. Bangham to formulate the first preparation of liposomes with encapsulated materials in 1965 [34]. Since then, scientists have concentrated their efforts to take advantage of the characteristics of liposomes to understand the biophysical properties of membranes, study lipid interactions and to create efficient drug delivery systems [35–38]. The evolution of this concept led Yavlovich et al. [30] to design an innovative liposome formulation for potential use as an efficient drug release system in appropriate organs and at the appropriate times. They were able to co-assemble several phospholipids (e.g. DPPC and EggPC) and DC_{8,9}PC in the liposome bilayer. DC_{8,9}PC, a phospholipid with reactive diacetylenic groups, is known to undergo a photo cross-linking reaction when treated with UV radiation (254nm) [39, 40]. Similar photo-crosslinking was observed when DC_{8,9}PC was embedded in DPPC bilayers at mole ratios 1:9 and above leading to release of entrapped liposome contents (30). It was hypothesized that photo cross-linking generates local defects in the liposome membrane that can promote drug release.

The detailed analysis of the individual lipids in these liposomes is critical to understand the nature of molecular interactions that result in photo-crosslinking and such information may improve design of these formulations. Initially, we set out to develop a quantitative method for the analysis of these lipids by MALDI mass spectrometry. MALDI-TOF MS has not been considered the first choice for quantitative analysis of lipids or other small molecular weight molecules for two main reasons: first the presence of matrix peaks and adducts in the low molecular range can interfere with analyte peak detection and second, inhomogeneity of the matrix/sample crystallization process reduces shot-to-shot reproducibility. Therefore, a matrix-free approach was chosen to avoid the disadvantages of the matrix and selected nanowire targets which had been shown to be applicable to quantitative analysis of small molecules [28, 41].

In our initial method development we observed in the lipid spectra the presence of multiple peaks related to the analyte of interest – protonated and sodiated adducts – and realized this could present difficulties in data analysis and reproducibility of the assay. Considering the work of Shiller et al. [42], we decided to use CsCl as a cationization agent. Using CsCl, the MS signal of the phospholipids was shifted by 133 Da corresponding to the atomic mass of cesium. Other groups chose cesium acetate [18] and they were also able to obtain dominant cesiated adducts. Our analytes, dissolved in a mixture of chloroform/methanol, were mixed with a methanolic solution of cesium chloride prior to spotting on the NALDI target. Cesium chloride is soluble in water but not in chloroform/methanol; therefore we prepared a stock solution of 1M cesium chloride in water and diluted to our working concentration – 50mM – in methanol, so it would be miscible with our analyte dilution buffer (chloroform/methanol, 50/50, v/v). We tried to use other, less volatile solvents to facilitate the sample dilution procedure, but a decrease in the quality of the spots was observed, and consequently of the analysis, probably due to solubility issues related to the lipophilic nature of the analytes.

The choice of organic solvents such as chloroform and methanol necessitated the use of glassware and glass micropipettors for the sample preparation. We observed an advantage in the use of volatile solvent systems: the rapid evaporation of the solvent mixture promoted an efficient co-crystallization of lipids and cesium chloride, resulting in homogeneous spots, as previously observed by other investigators [43]. Homogenous spots, along with triplicate analyses, allowed us to control variability issues, such as shot-to-shot, region-to-region and sample-to-sample [43, 44] variability. The possibility of bias acquisition was considered and therefore we opted for a completely automated acquisition of data collecting 1200 shots, in a

random walk pattern and with an intensity of laser between 15% and 60% in order to avoid saturation of the instrument. A better reproducibility was observed with the average of 1200 spectra, rather than with a smaller number of acquisitions for each spot.

Release of contents from DPPC: DC_{8,9}PC 4:1 liposomes occurs as a result of UV-triggered DC_{8,9}PC polymerization in the bilayer [30]. Therefore the liposomes were loaded with 50 mM calcein and its release was monitored after treatment with UV (254 nm) for 45 minutes using a fluorescent micro plate reader (Methods section). Before and after UV treatment liposome lipids were extracted and analyzed by the NALDI mass spectrometry method.

MALDI signals for DPPC and DC_{8,9}PC were linear over the selected ranges and the calibration curves resulted in coefficient of determinations (R^2) = 0.99, as shown in Figure 1. Figure 2 shows two examples of spectra obtained from the lipids extracted and treated as described above. The decrease in intensity of the signal of the pseudo molecular ion for DC_{8,9}PC after UV treatment is evident. We were able to determine the concentration for both DPPC and DC_{8,9}PC using the equation from the linear regression for each compound. Figure 3 compares the concentration, expressed in μ M, for both species before and after UV treatment. The concentration of DPPC remained constant (260 μ M) while DC_{8,9}PC decreased from 120 μ M in the starting liposome to 12 μ M in the liposome treated with 254 nm irradiation.

It is important to mention that the possibility of ion suppression and ionization efficiencies of the individual lipids we analyzed was considered. This is a common drawback in any mass spectrometry assay and we attempted to overcome this issue with the use of DMPC as the internal standard as it is structurally similar to the analytes of interest. The use of internal standards for quantitative analysis is standard practice [18, 45–47] and it is of crucial importance in any MALDI approach to these types of analyses. However in some cases a suitable internal standard may not be available, but reasonable quantitation may still be possible [48]. For optimal results, the use of a stable isotope analog of the analyte of interest would be the primary choice [47] for an internal standard, but in our case the available stable label analog of DPPC was not of sufficient purity for use as an internal standard so we choose a slightly smaller saturated PC as our internal standard.

By application of this novel MS method to the analysis of the phospholipids extracted from liposomes prepared and treated as described by Yavlovich et al. [30], we were able to confirm the decrease of DC_{8,9}PC from 120 μ M to 12 μ M, before and after the UV treatment (Figure 3). As expected DPPC concentration did not change. It has been demonstrated that DC_{8,9}PC liposomal photo-triggering by UV light occurs via direct photopolymerization mechanism [30].

Conclusion

The NALDI-based analysis of the phospholipids presented here provides a fast and reproducible approach for the quantitative analysis of lipids in relatively complex mixtures. We have demonstrated that our method is sufficiently specific for the lipids of interest because it was not necessary to use tandem mass spectrometry as suggested by other investigators [15, 46, 49]. The use of the NALDI targets allows for greatly simplified sample preparation and eliminates the matrix-associated issues of inhomogeneous crystals and matrix adducts. This approach should also be possible to for a wide range of lipids and structurally similar molecules.

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Figure 1. Calibration curves for DPPC and DC_{8,9}PC. Concentration ranges for DPPC and DC_{8,9}PC were respectively 10–200 ng/μL and 2.5–100 ng/μL.

Figure 2. Mass spectra of phospholipids from liposome before (A) and after (B) UV treatment (254 nm).

Figure 3.

Quantitative comparison of DPPC and DC_{8,9}PC in liposome before and after UV treatment. A decrease of DC_{8,9}PC from 120 μM to 12 μM, before and after the UV treatment, was observed, while the concentration of DPPC remained unchanged following exposure of the intact liposome at 254 nm irradiation.