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APPLICATION NOTE

Direct Analysis of Triacylglycerols from Crude Lipid Mixtures by Gold Nanoparticle-Assisted Laser Desorption/Ionization Mass Spectrometry

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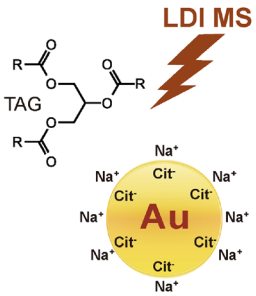

Abstract. Triacylglycerols (TAGs), essential energy storage lipids, are easily detected by conventional MALDI MS when occurring on their own. However, their signals are easily overwhelmed by other lipids, mainly phosphatidylcholines (PCs) and, therefore, require purification. In order to profile TAGs from crude lipid mixtures without prefractionation, we investigated alternative matrixes that can suppress phospholipid ion signals and enhance cationization of TAGs. We found that an aqueous solution of citrate-capped gold nanoparticles (AuNPs) with a diameter of 12 nm is a superior matrix for the laser desorption/ionization mass spectrometry (LDI MS) of TAGs in crude lipid mixtures. The AuNP matrix effectively suppressed other lipid signals such as phospholipids and also provided 100 times lower detection limit for TAGs than 2,5-dihydroxybenzoic acid (DHB), the best conventional MALDI matrix for TAGs. The AuNP-assisted LDI MS enabled us to obtain detailed TAG profiles including minor species directly from crude beef lipid extracts without phospholipid interference. In addition, we could detect TAGs at a trace level from a total brain lipid extract.

Key words: Laser desorption/ionization, Triacylglycerol, Phophatidylcholine, Gold nanoparticle, Graphene oxide

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Introduction

Ion suppression is a well-known phenomenon in matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) of complex mixtures. It depends on the mixture composition, the matrix of choice, the sample-to-matrix ratio, and laser fluence [1]. For example, when analyzing brain lipid extracts enriched with phosphatidylcholines (PCs) containing quaternary ammonia groups, PCs are preferentially detected under conventional MALDI conditions while signals from other less polar lipids are suppressed [2].

In order to detect target molecules subject to suppression, two strategies have been generally employed. One strategy aims to concentrate target molecules by liquid–liquid extraction [3], thin layer chromatography (TLC) [4], or solid phase extraction (SPE) [5]. However, this approach requires extra sample preparation steps and cannot be used for in situ lipid analysis such as MALDI MS imaging. The other strategy is the so called "matrix engineering," in which the LDI environment is altered by employing an alternative matrix, and the ion signals for target molecules are, therefore, selectively enhanced. For example, cerebrosides are hard to detect under conventional MALDI conditions directly from brain tissues or crude lipid extracts although cerebrosides are highly abundant in these samples. However, when using colloidal graphite [6], graphene oxide (GO) [7], or gold nanoparticle (AuNP) [8] as a matrix, cerebrosides are selectively detected while signals from PCs are relatively suppressed.

Triacylglycerols (TAGs), esters of three fatty acids with glycerol, are important energy storage lipids and major dietary fats. Also, TAGs are routinely checked in human blood for evaluating risks for heart disease and stroke in medical care. TAGs are easily detected by conventional MALDI MS when they are predominantly present in samples such as edible oils [9]. The best known MALDI matrix for TAGs is 2,5-dihydroxybenzoic acid (DHB) in acetone or methanol with which the detection limit for a pure TAG is about 800 fmol per sample spot [10,11]. However, signals from TAGs are also easily suppressed in the presence of PCs [10,12]. Therefore, simple chromatographic purification by TLC or SPE is generally required for MALDI MS of TAGs in crude lipid extracts [3,5]. So far, an alternative

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matrix for direct detection of TAGs from complex mixtures has been scarcely reported [13].

In this note, we report that an aqueous solution of citrate-capped AuNP is an efficient LDI matrix for the selective detection of TAGs from complex lipid mixtures with a 100-fold improved sensitivity. First, we compared TAG-detecting performance of a selected set of candidate LDI matrixes in comparison to that of methanolic DHB. With the candidate and the reference matrixes, we analyzed a simple mixture containing PC and TAG standards. Then, the selected matrixes and DHB were tested against crude beef lipid extracts and total brain lipid extracts, which represent entirely different lipid compositions [14].

Experimental

Porcine brain total lipid extract (25 mg/mL in chloroform) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 1,2-Dipalmitoyl-*rac*-glycero-3-phosphocholine [PC(16:0/16:0)] and 1,2-Di(cis-9-octadecenoyl)-3-octadecanoyl-*rac*-glycerol [TAG (18:0/18:1/18:1)] were obtained from Sigma-Aldrich (St. Louise, MO, USA). All solvents except 18 MΩ-cm deionized water were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Aqueous graphene oxide (GO) solution was obtained from UniNanoTech Co. (Yongin, Korea) and diluted to 0.05 mg/mL in aqueous 10 mM $NaNO_3$ for LDI MS applications. Aqueous solution of citrate-capped AuNPs (12±1.3 nm in diameter, 0.05 mg/mL, and Supplementary Figure S1) was purchased from CNVision Co. (Seoul, Korea). The AuNP solution was directly used for LDI MS analysis without further treatment.

In order to evaluate the performance of the matrixes, standard lipid mixtures were prepared in chloroform. The mixtures were composed of a fixed concentration of PC(16:0/16:0), (0.5 μg/μL), and various concentrations of TAG(18:0/18:1/18:1) (from 10 pg/μL to 0.5 μg/μL). Crude beef lipid extracts were prepared from eye-of-round ground beef (0.25 g) by simple extraction with 5 mL hexane/2-propanol (1:1, vol/vol) [5]. The obtained beef lipid extract was directly applied onto a sample plate. For a brain lipid extract, stock solution (25 mg/mL) was diluted to 1 mg/mL in chloroform for LDI MS analysis.

The lipid samples (0.5 μL) were first spotted onto a MALDI target plate (ASTA Inc., Suwon, Korea) followed by a 0.5 μL DHB matrix (25 mg/mL in methanol; Protea Biosciences, Morgantown, WV, USA) or by 2.0 μL of GO or AuNP solution. The plate was then dried in a moderate vacuum (~50 Torr). LDI time-of-flight (TOF) and TOF/TOF mass spectra were recorded using an ABI 4800 Plus MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA). The mass spectrometer was operated in the positive reflectron mode with a 20 kV acceleration voltage. For a given sample spot, sub-spectra were collected from 40 different locations in the spot and then averaged to generate a final mass spectrum (1,200 laser shots/spectrum). For nanomaterial matrixes (AuNP and GO), the spot-to-spot reproducibility of signal intensities was high (±14 % relative standard deviation for five replicates). In case of TOF/TOF experiments, collision induced dissociation was performed using air as the collision gas at 1 kV of collision energy. The mass spectra obtained were processed using a DataExplorer v4.8 (Applied Biosystems) and a mMass software (http://www.mmass.org).

Results and Discussion

Detection of TAG in the Presence of PC

Our criteria for choosing candidate matrixes for selective TAG analysis are described in the Supplementary Information. Selected matrixes, AuNP and GO, were first tested against simple mixtures of PC(16:0/16:0) and TAG(18:0/18:1/18:1). Figure 1 illustrates the selective TAG signal enhancement with the AuNP matrix in the presence of PC. With methanolic DHB, PC was dominantly detected as protonated, sodium-adduct, potassium-adduct, and DHB-adduct ions, whereas TAG

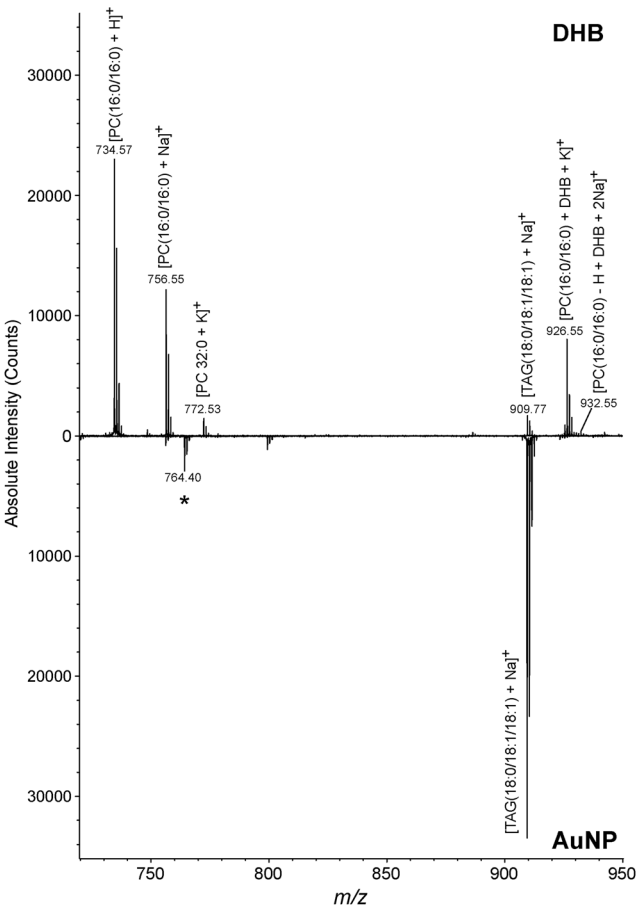

Figure 1. LDI mass spectra of a mixture of PC(16:0/16:0) and TAG(18:0/18:1/18:1) (250 ng and 25 ng per spot, respectively) with DHB (top) or citrate-capped AuNP (bottom) as a matrix. The peak at *m/z* 764.40 was due to an impurity present in the PC standard

produced a suppressed sodiated ion at *m/z* 909.77 (Figure 1, top). However, when using the citrate-capped AuNP matrix, a dominant sodiated ion of TAG was exclusively detected, whereas the signal of $[PC(16:0/16:0)+Na]^+$ at *m/z* 756.55 are almost completely suppressed (Figure 1, bottom). We also observed such selective detection of TAG with a GO matrix. But, AuNP always gave five to six times higher signal-to-noise (S/N) ratios than GO for the same TAG samples. In the presence of 340 pmol/spot PC(16:0/16:0), practical detection limits (i.e., S/N ratio of 10) for TAG(18:0/18:1/18:1) with AuNP and GO matrixes were 30 fmol/spot and 170 fmol/spot, respectively. In contrast, DHB gave about a 100 times poorer practical detection limit (2.8 pmol/spot).

Detection of TAGs from Crude Beef Lipid Extracts and Total Brain Lipid Extracts

The performance of matrixes was further evaluated in detecting TAGs directly from crude lipid extracts. A crude beef lipid extract in which TAGs are dominant species (75 % to 90 % of total lipids) [5] and a total brain lipid extract that contains trace amounts of TAGs (~0.2 % of total lipids) [14] were chosen as model crude samples. Figure 2 shows lipid fingerprints directly obtained from a crude beef extract with DHB and AuNP matrixes. Although phospholipids are minor components in beef lipids (<20 %), both phospholipids and major TAGs were detected with comparable intensities by using DHB and minor TAGs could not be identified because of interference by phospholipid signals (Figure 2a). In contrast, the AuNP matrix produced clear TAG profiles including minor TAGs directly from the crude extract (Figure 2b). In fact, the direct profiles of beef TAGs analyzed with the AuNP matrix was almost identical to the previously reported profile obtained with DHB from a SPE-purified fraction of beef TAGs [5]. Therefore, the AuNP matrix can eliminate fractionation steps such as TLC or SPE in LDI MS analysis of TAGs from beef lipid extracts.

A total brain lipid mixture is one of the most challenging samples in the direct detection of TAGs. This sample contains high concentrations of phospholipids and trace amounts of TAGs. Therefore, conventional MALDI MS is subject to severe ion suppression for TAGs. In addition, even

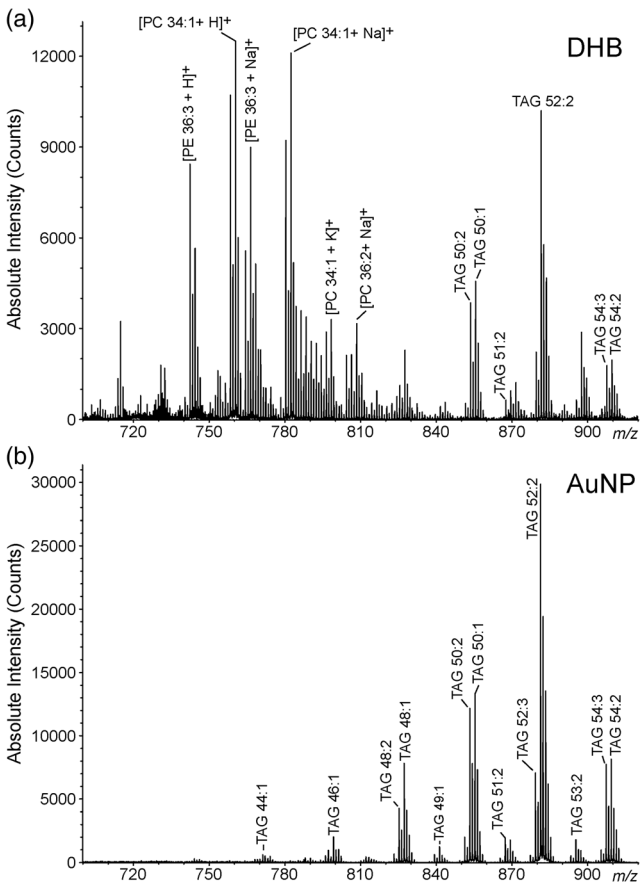

Figure 2. LDI mass spectra of a crude beef lipid extract with **(a)** DHB or **(b)** citrate-capped AuNP as a matrix. All peaks assigned as TAG species had forms of sodiated ions, $[TAG+Na]^+$. See Supplementary Table S1 for the detailed peak assignments

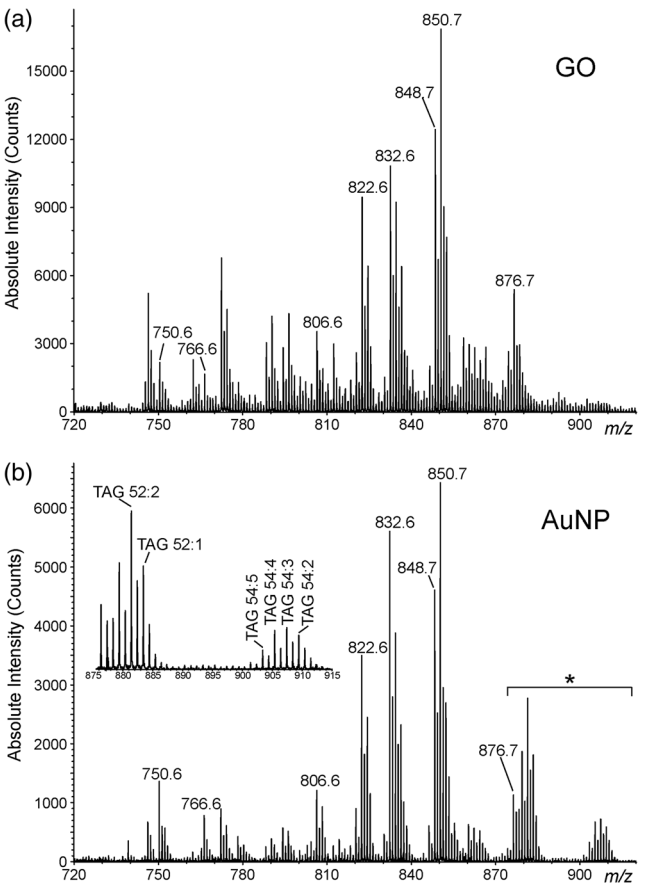

Figure 3. LDI mass spectra of a total brain lipid extract (500 ng per spot) with **(a)** GO or **(b)** AuNP as a matrix. The inset in **(b)** shows a magnified view of *m/z* 875–915 marked with an asterisk (*) in the main spectrum. All peaks with *m/z* values correspond to major cerebroside species. See Supplementary Table S2 for the detailed peak assignments

though an alternative matrix that enhances cationization is employed, TAGs should compete with abundant cerebrosides [6] for cationization. Therefore, a very sensitive and selective matrix is required for detecting TAGs from complex brain lipids. Figure 3 and Supplementary Figure S2 show lipid profiles directly obtained from a total brain lipid extract with various matrixes. DHB produced a phospholipid-focused profile with minor cerebroside peaks (Supplementary Figure S2), whereas GO gave a cerebroside-focused profile (Figure 3a) similar to the previous study [7]. Besides, both matrixes could not produce a detectable TAG signal. In striking contrast, AuNP not only produced a cerebroside profile similar to GO but also generated a clear TAG profile (*m/z* 880–915) directly from crude brain lipids (Figure 3b). The identities of tentatively-assigned TAG peaks were further confirmed by observing characteristic fatty acid loss in their product ion spectra (Supplementary Figure S3) [15,16]. This suggests that AuNP-assisted LDI MS possesses enough sensitivity and selectivity to detect TAGs directly from crude extracts even when TAGs are present as minor species and other cationizable lipids are abundant in a given mixture. Factors driving differences in TAG ionization between GO and AuNP need further investigation.

Conclusions

In this note, we demonstrated an aqueous citrate-capped AuNP solution as a superior matrix for the analysis of TAGs directly from crude lipid mixtures with no pretreatment. First, AuNP effectively suppresses other lipid signals, mainly phospholipids, and thus can eliminate lipid fractionation steps before LDI MS analysis. Second, AuNP-assisted LDI MS provides 100 times better sensitivity compared with conventional MALDI MS, which enables the detection of TAGs even when they are minor species in a given sample. Third, citrate-capped AuNPs are commercially available and, therefore, easily accessible.

Acknowledgments

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