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Title	An Efficient Glycoblotting-Based Analysis of Oxidized Lipids in Liposomes and a Lipoprotein
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Citation	Chembiochem, 18(19), 1903-1909 https://doi.org/10.1002/cbic.201700384
Issue Date	2017-10-05
Doc URL	https://hdl.handle.net/2115/71638
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Type	journal article
File Information	ChemBioChem_18(19)_1903-1909.pdf



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An efficient glycoblotting-based analysis of oxidized lipids in liposomes and a lipoprotein

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Abstract: Even though the widely occurring lipid oxidation, which is triggered by reactive oxygen species (ROS), produces a variety of oxidized lipids, practical methods to efficiently analyze oxidized lipids remain elusive. Herein, we show that the glycoblotting platform can be used to analyze oxidized lipids. The analysis is based on the principle that lipid aldehydes, one of the oxidized lipid species, can be captured selectively, enriched, and detected. Moreover, we discovered that 3-methyl-1-*p*-tolyltriazene (MTT) methylates phosphoric and carboxylic acids, and that MTT-mediated methylation is, in combination with conventional tandem mass spectrometry (MS/MS) analysis, an effective method for the structural analysis of oxidized lipids. Using three classes of standards, liposomes, and a lipoprotein, we demonstrate that glycoblotting represents a powerful approach for focused lipidomics even in complex macromolecules.

Lipid oxidation, a ubiquitous process triggered by reactive oxygen species (ROS), produces a diverse variety of oxidized lipids such as hydroperoxides, alcohols, epoxides, aldehydes, ketones, and carboxylic acids.[1a-d] Oxidized lipids have been associated with several pathological (e.g. atherosclerosis, neurodegenerative diseases, sclerosis, diabetes, and cancer) and other biological processes (e.g. inflammation, signaling, immune responses, and infections).[2a,b] Combatting these often deleterious phenomena requires the development of efficient analytical systems. Our and other groups have previously reported protocols to analyze oxidized lipids by liquid chromatography/mass spectrometry (LC/MS) techniques,[3a-i] and carbon-nanotube sensors.[4a-b] Even though the LC/MS-based approach is a useful method for untargeted lipidomics, high-throughput methods are preferable for clinical or medicinal applications.[5]

In 2004, we reported a novel glycan analysis platform, which was termed glycoblotting.[6] This method exhibits several advantages relative to conventional methods: 1) chemoselective capture and enrichment of glycans,[7] 2) release and labeling of glycans in a stable form (oxymes),[7] 3) high efficiency: chromatographic separation and parallel/automatic operations (SweetBlot) are not required.[8] The versatility of this concept has been demonstrated in numerous clinical,[9a-d], biological[10a-d], and nutritional[11a-c] studies.

The basis of glycoblotting is the reaction between molecules that contain an aldehyde group or glycans and BlotGlyco® H beads bearing hydrazide groups.[7] By extension, we speculated that glycoblotting may also be applicable to lipidomic analysis, which focuses on lipid aldehydes. A schematic illustration of this concept is shown in Figure 1.

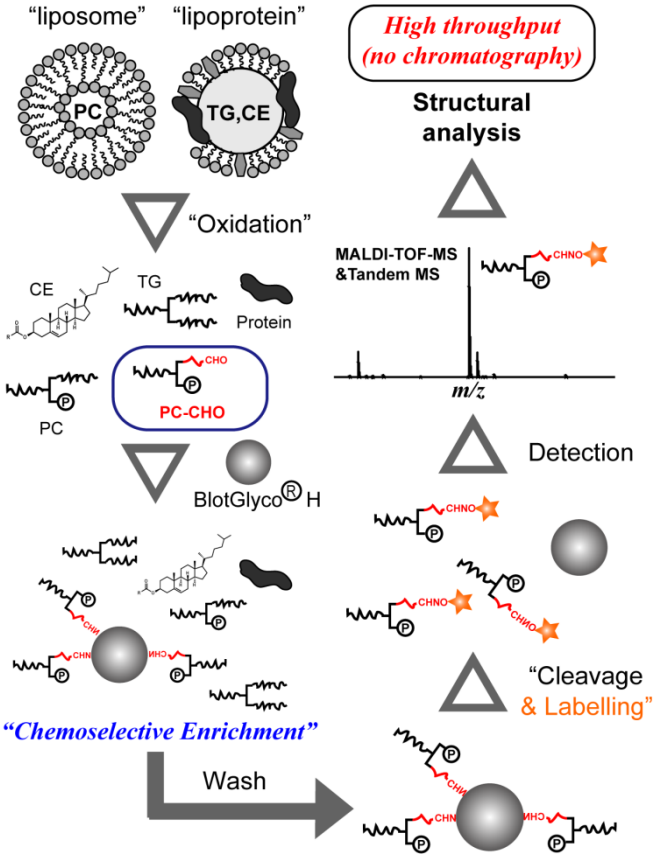
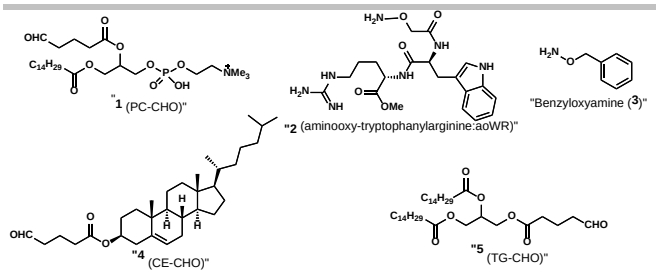


Figure 1. Conceptual overview of the present study.

Small aldehydes generated by lipid oxidation, e.g. malondialdehyde (MDA), acrolein (ACR), or 4-hydroxynonenal (4-HNE) are harmful substances upon formation of imines or other, more stable structures. [12a-b] Although several studies on small aldehydes have been reported, knowledge on lipid core aldehydes, i.e., aldehydes still bound to the parent skeleton, remained elusive.[13] It has become increasingly clear in recent years that various inflammation conditions generate lipid core aldehydes that contribute to changes in cell behavior during their pathology.[1a] Therefore, we decided to investigate lipid core aldehydes using the glycoblotting platform.

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Scheme 1. Representative compounds examined in the present study.

Initially, we established the optimal conditions for a lipid aldehyde analysis protocol using chemically synthesized phosphocholine core aldehyde (PC-CHO) **1** as a standard (Scheme 1). As expected, **1** was observed as an aminooxy-tryptophanylarginine (aoWR)-labeled form (**2**) (Figure 2). In addition, we discovered that lipid aldehydes can be quantified by glycoblotting, which is demonstrated on the example of glycan analysis, as the signal intensities of a dilution series exhibited a linear correlation (Figure S1). The detection limit is comparable to that of the glycan analysis.[10d]

Also, *in situ* NMR measurements of model reactions between **1** and benzyloxyamine **3**, which is another labeling reagent, showed that this reaction proceeds very quickly and quantitatively, indicating efficient capture by and cleavage from the beads (Figure S2). Furthermore, we discovered that MS/MS analysis in positive ion mode provides information on the polar head group (neutral loss -59 Da, -183 Da; Figure S3), while an MS/MS analysis in negative ion mode allows characterizing the fatty acid composition (*m/z* 201 for $C_{14}H_{29}CO_2^-$; Figure S4)

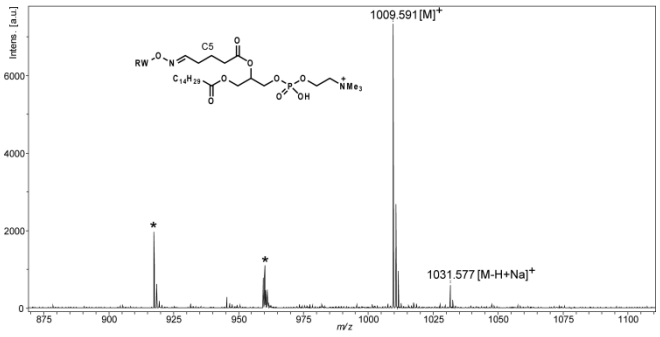


Figure 2. MS detection of the synthetic standard **1**, whereby "*" indicates signals from the matrix and the residual aoWR reagent.

Encouraged by this proof-of-concept, we attempted to analyze the oxidation products in 1-palmitoyl-2-linoleoyl-PC (PLPC) and 1,2-dilinoleoyl-PC (DLPC) liposomes. The mass spectrum obtained from the PLPC system revealed three main peaks for the oxidized products at *m/z* = 1051, 1065, and 1079 (Figure 3). These molecular species were assigned to C16:0-C7:0CHO-PC, C16:0-C8:0CHO-PC, and C16:0-C9:0CHO-PC, respectively, and this assignment was confirmed by an LC-HRMS analysis (Figure S5-7).

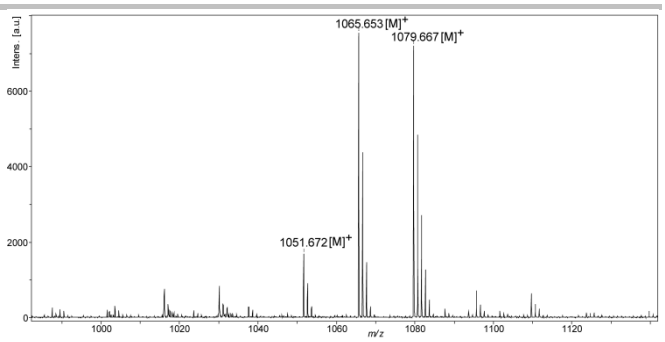


Figure 3. MS analysis of the aldehyde products in an oxidized PLPC liposome.

The DLPC system afforded a more complicated mass spectrum than the PLPC system (Figure 4A). In positive ion mode, we were able to assign the major signal of this system (*m/z* = 1011). According to the MS/MS analysis in both positive and negative ion mode, this species contains a phosphocholine head group and a C10:0COOH fatty acid (Figure S8). Therefore, this peak was assigned to C10:0COOH-C8:0CHO-PC. The observation that the signal at *m/z* = 1009 becomes much stronger in negative ion mode supports this assignment, as this peak should contain a carboxylic acid functionality.

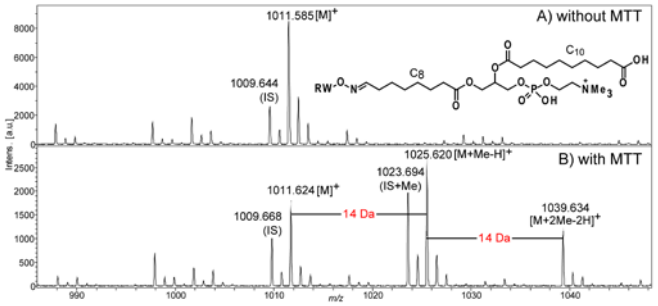
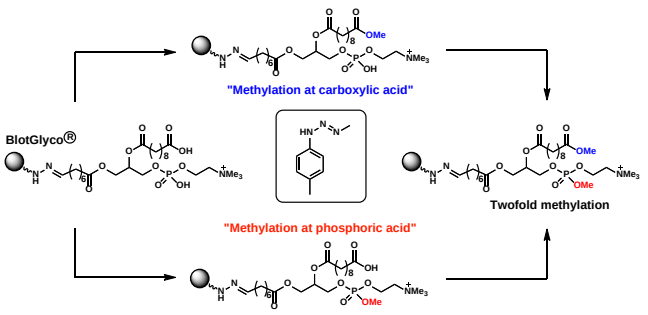


Figure 4. MS analysis of the aldehyde products in a DLPC liposome in the presence or absence of MTT (*m/z* = 1009 is derived from IS depicted in Figure 2). A) Mass spectrum obtained using the original protocol. B) Mass spectrum obtained from a protocol containing an additional MTT treatment step.

It is interesting that even though the oxidation occurred at the same linoleoyl chain in PLPC and DLPC, it afforded oxidation products with different chain lengths. While it is well known that oxidation of the linoleic acid chain provides a C9-oxidized product,[1d-e] our results include unusual C7-, C8-, and C10-oxidized products.[19] This may result from the relatively strong oxidation conditions applied, but shows that our protocol should be able to detect not only common but also uncommon molecular species.

In the glycoblotting platform, 3-methyl-1-*p*-tolyltriazene (MTT) is an essential methylating reagent.[14] In particular, methylation at sialic acid residues is crucial in terms of 1) the suppression of undesired fragmentation in the MS analysis, and 2) the enhancement of the sensitivity in positive ion mode. Apart from these advantages in the glycan analysis and the semi-quantitative nature of glycoblotting, we introduced this methylation step simply to obtain more information on the carboxylic acid functionality. As expected, the peak at *m/z* = 1011 shifted to *m/z* = 1025 upon methylation (Figure 4B), suggesting that *m/z* = 1011 contains a carboxylic acid group.

More interestingly, this methylation afforded an additional signal at *m/z* = 1039 (Figure 4B). An MS/MS analysis of the peaks at *m/z* = 1025 and 1039 in positive ion mode showed that an *m/z* = 198 fragment resulted from the polar head group instead of an *m/z* = 184 fragment (Figure S9), which indicates that MTT methylates not only carboxylic, but also phosphoric acid group (Scheme 2). To the best of our knowledge, the methylation of phosphoric acid by MTT has not yet been reported.[15] This unexpected reaction was reproduced for **1** (Figure S10), suggesting that MTT treatment can be useful for the quick discrimination between phospholipids and other classes of lipids without MS/MS analysis.



Scheme 2. Reaction pathway of the twofold methylation of phospholipids by MTT.

Having established the fundamental basics of this method, we subsequently proceeded to analyze a low-density lipoprotein (LDL) as a representative lipoprotein. LDLs include triglycerides (TG), cholesterylesters (CE), phospholipids, and apolipoproteins.[16] In general, lipoproteins are important biomarkers for the diagnosis of metabolic disorders.[17a-c] In particular, oxidized LDLs (oxLDLs) have been identified as a risk factor,[17a-c] and their characterization is thus of paramount importance. However, such an analysis should not be trivial, given that lipoproteins include several species. It is therefore not surprising that the obtained spectrum for oxLDLs in the glycoblotting platform was much more complex, but luckily two identical signals, which appeared in the PLPC liposome experiment (*m/z* = 1065, 1079), were observed (Figure 5A). As the MTT treatment afforded a new signal at *m/z* = 1121 and a different intensity pattern (Figure 5B), we thus assigned these signals to PC-CHO species. As PC contains a cation, it should probably be detected more easily than other species. Possible PC-CHOs were examined further using LC-ESI-Orbitrap, and probable fatty acid compositions are summarized in Table 1.

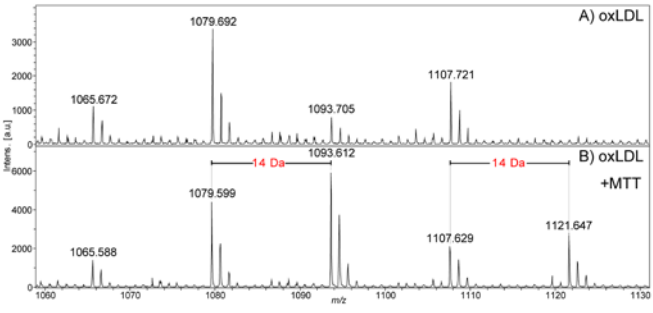


Figure 5. MS analysis of the aldehyde products of oxLDL. A) MS spectrum obtained from the original protocol. B) MS spectrum obtained from a protocol containing an additional MTT treatment step.

Table 1. *m/z* values and presumable fatty acid compositions in the oxLDL analysis

#	Saturated chain or skeleton	Aldehyde chain	MALDI-TOF		LC-ESI-Orbitrap	
			m/z	Δppm	*m/z*	Δppm
1	C16:0	C8:0	1065.643	+6.6	1065.6359	-0.1
2	C16:0	C9:0	1079.670	+16.7	1079.6616	+0.0
3	C18:0	C8:0	1093.684	+11.9	1093.6672	+0.1
4	C18:0	C9:0	1107.693	+9.0	1107.6821	-0.7
5*	Cholesterol	C5:0	914.618	+7.7	914.6113	-0.1

* *m/z* value did not shift after MTT treatment

In order to investigate class compatibility, chemically synthesized CE-CHO **4** and TG-CHO **5** (for details, see SI) were tested using the glycoblotting method (Figure 6), and detected at *m/z* = 914.658 and 1068.732, respectively. These results suggest that all major classes of lipid core aldehydes can be analyzed using the glycoblotting platform.

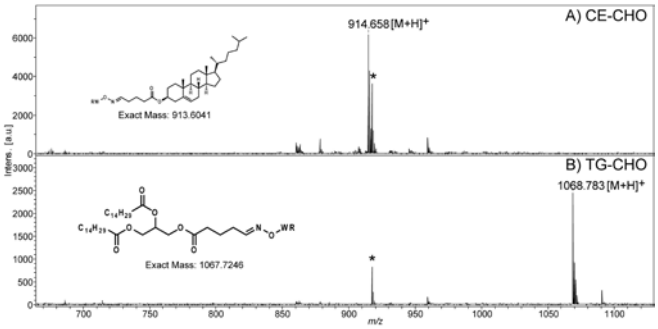


Figure 6. Glycoblotting of A) CE-CHO **4** and B) TG-CHO **5**, whereby "*" indicates signals from residual aoWR reagent.

In conclusion, we have demonstrated that glycoblotting, an innovative method for glycan analysis, is also a powerful tool for the analysis of oxidized lipids, especially when focusing on lipid core aldehydes. For the DLPC liposome, we observed the rare and twofold oxidized species C10:0COOH-C8:0CHO-PC. During the analysis of LDL, several PC core aldehyde species were detected and most of them showed *m/z* values that were identical to those of the PLPC liposome experiment. Moreover, a CE core aldehyde was identified in the LDL experiment, and this assignment was further corroborated by an LC-HRMS measurement. The versatility of this approach is evident from the successful analysis of synthetic TG and CE standards. Furthermore, we showed that an MTT treatment in combination with an MS/MS analysis can be useful for a quick discrimination of lipids and offer additional information for a structural characterization. In particular, the MTT-mediated methylation of the phosphoric acid moiety is unprecedented. Currently, we are trying to overcome the challenges associated with clinical

applications in order to examine lipid core aldehydes as biomarkers, because we were unable to get any meaningful signals in those samples. The reason should be simple: the sensitivity is insufficient for biological or clinical samples.[20] As another possibility, we believe that the approach presented in this study should contribute to a better understanding of ferroptosis in the future, which was recently proposed as a lipid-related cell death mechanism.[18a-c]

Experimental Section

Synthetic schemes, procedures, and compound characterizations are included in the supporting information.

General Information

BlotGlyco®H beads and aoWR were purchased from Sumitomo Bakelite (Tokyo, Japan). Ammonium carbamate (99%, ABC) 3-methyl-1-*p*-tolyltriazene (MTT), and other reagents and solvents were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) and Kanto Chemical Co., Inc., unless otherwise stated. MultiScreen Solvinert filter plates were purchased from Millipore. Mass spectra were recorded on MALDI-TOF/MS (UltraFlex III or UltrafleXtreme, Bruker Daltonics, Germany) and LTQ Orbitrap XL (Thermo Fisher scientific) machines equipped with a Shimadzu Prominence HPLC system. Calibration of the MALDI-TOF/MS setup was performed using Peptide calibration standard II (Bruker Daltonics, Germany). 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix in the MALDI analysis. The obtained MALDI data were analyzed using flexAnalysis (ver 3.4, Bruker Daltonics).

Protocol for lipid aldehyde analysis without MTT treatment

250 μL of BlotGlyco® H beads (10 mg mL^{-1}) were added to the wells of a MultiScreen Solvinert filterplate and the water was removed under reduced pressure. A lipid mixture (20 μL) was deposited in each well together with 180 μL of 2% AcOH/ACN. The plate was incubated at 80 °C for 45 min, followed by two successive washings using 200 μL of 2 M guanidine-HCl in 16.6 mM ammonium bicarbonate, water, and 1% triethylamine in MeOH. After acetyl capping using 100 μL of 10% acetic anhydride in MeOH at room temperature for 30 min, the remaining acetic anhydride was removed under reduced pressure. Each well was washed two times with 200 μL of 10 mM hydrochloric acid, MeOH, and water. Subsequently, 20 μL of 2.5 mM aoWR were added, accompanied by 180 μL of 2% AcOH/ACN, before the plate was incubated at 80 °C for 45 min. Finally, the labeled lipid aldehydes were eluted with 100 μL chloroform/methanol (1/1, v/v), collected, and analyzed by MALDI-TOF-MS.

Protocol for lipid aldehyde analysis including MTT treatment

250 μL of BlotGlyco® H beads (10 mg mL^{-1}) were added to the wells of a MultiScreen Solvinert filterplate and the water was removed under reduced pressure. A lipid mixture (20 μL) was deposited in each well together with 180 μL of 2% AcOH/ACN. The plate was incubated at 80 °C for 45 min, followed by two successive washings using 200 μL of 2 M guanidine-HCl in 16.6 mM ammonium bicarbonate, water, and 1% triethylamine in MeOH. After acetyl capping using 100 μL of 10% acetic anhydride in MeOH at room temperature for 30 min, the remaining acetic anhydride was removed under reduced pressure. Each well was washed two times with 200 μL of 10 mM hydrochloric acid, MeOH, and dioxane. On-bead methylation was performed by adding 100 μL of 100 mM MTT in dioxane, followed by incubating at 60 °C for 90 min. After consecutive washings with dioxane (200 μL), water, and MeOH/water, 20 μL of 2.5 mM aoWR were added, accompanied by 180 μL of 2% AcOH/ACN, before the plate was incubated at 80 °C for 45 min. Finally, the labeled lipid aldehydes were eluted with 100 μL chloroform/methanol (1/1, v/v), collected, and analyzed by MALDI-TOF-MS.

MALDI-TOF-MS and MS/MS conditions

Detection was performed in reflector-positive mode (600-2000 Da). Molecules with < 600 Da were deflected. MS/MS spectra were obtained in LIFT mode. For the analysis of the fatty acid composition, the conditions were changed to reflector-negative mode and LIFT-negative mode, respectively.

LC-ESI-Orbitrap Analysis

Labeled lipid core aldehydes (10 μL) were injected and separated on an HPLC with an Atlantis T3 column (3 μm x 2.1 mm x 150 mm; Waters) using an aqueous AcOH (0.1%)/acetonitrile gradient system (flow rate: 0.3 mL min^{-1}) as shown in Table 2. The temperature of the column oven was set to 40 °C. The following ESI conditions were applied: capillary voltage = 4000 V, desolvation temperature = 350 °C, sheath gas = 50 psi, and auxiliary gas = 10 psi. Precursor and product ions were detected in positive ion mode; for precursor ions: FTMS (resolution: 60,000); for product ions: ITMS (CID normalized collision energy = 35 V). The thus obtained data were analyzed using the Qual browser (Thermo Fisher Scientific). The extracted ion chromatogram (EIC) was obtained on the basis of the theoretical molecular weight (10 ppm error range). The calibration was performed using triple quadrupole calibration solution (Pierce™).

Table 2. Gradient system for the LC/MS analysis.

Time (min)	H_2O with 0.1% AcOH (%)	CH_3CN (%)
2	100	0
5	100	0
5	98	2
20	1	99
25	1	99
30	98	2
35	98	2

Preparation of oxidized liposomes

200 μL of PC solution (DLPC or PLPC; 5 mg/mL $CHCl_3$) were dried using an argon gas stream and kept under reduced pressure overnight. 500 μL PBS were added and mixed vigorously using a vortex and sonication. To the resulting white suspension, 5 μL of 500 μM $CuSO_4$ and 2.5 μM 30% H_2O_2 were added, before the resulting mixture was shaken for 2 days at 37 °C. Then, the oxidants were quenched by addition of 50 μL of sat. EDTA; the resulting solution was used in a glycoblotting experiment.

Preparation of oxidized LDLs

LDLs were isolated from normal adult serum in the presence of EDTA to prevent oxidation of the LDLs using ultracentrifugation.[21] This study was approved by the ethics review board of the Faculty of Health Sciences at Hokkaido University (approval no. 15-89-3). The concentration of the LDLs was determined as the protein concentration using a modified Lowry method. The LDLs were stored at 4 °C prior to use. The LDL solution was filtered through a 100 kDa cut-off filter (Amicon® ultra; Merck Millipore), followed by addition of 400 μL Dulbecco's phosphate buffered saline (DPBS) to the residues to remove the EDTA, sugars, and inorganic salts. This filtration was repeated six times. To 50 μL of the thus prepared LDL solution, 5 μL of 500 μM $CuSO_4$ was added, before the resulting mixture was shaken overnight at 37 °C. Then, the oxidants were quenched by addition of 5 μL of sat. EDTA; the resulting solution was used in a glycoblotting experiment.

Calibration curve

The calibration curve of PC-CHO **1** was calculated from the integrated peak areas of 1009.6 $[M]^+$ in 53.5, 26.8, 13.4, 3.3 μM samples. Each assay was conducted in four replications. The obtained data were analyzed using the Prism 6 software (GraphPad software Inc.)

In-situ NMR measurements

Benzyloxyamine hydrochloride **3** (5.4 mg, 33.6 μmol) was added to a solution of PC-CHO **1** (13 mg, 22.4 μmol) in 2% CD_3CO_2D/CD_3CN (850 μL) in an NMR tube, and the sample was immediately inserted in the NMR instrument. Every five minutes, a ^1H NMR measurement was conducted and the spectra were compared.

Acknowledgements

This study was financially supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT) in Sapporo health innovation "Smart-H", JSPS KAKENHI grant number JP25220206 and JP17K1576607. We would like to thank Dr. Maho Amano and Dr. Solomon T. Gizaw for helpful advice on glycoblotting experiments, as well as Mr. Tomohiro Hirose at Instrumental analysis division, global facility center, creative research institution, Hokkaido University for technical assistance with NMR measurements.

Conflict of interest

The authors declare the absence of any conflict of interests.

Keywords: glycoblotting • lipid aldehyde• oxidized lipidomics • liposome • lipoprotein

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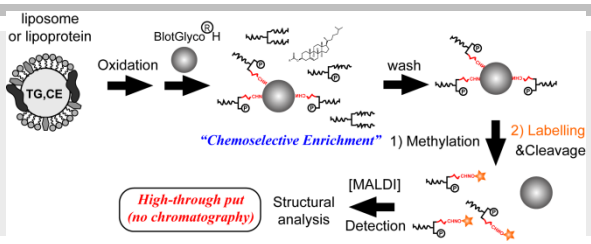
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COMMUNICATION



The oxidation of lipids triggered by reactive oxygen species produces a diverse variety of oxidized lipids. Herein, we show that the glycoblotting platform can be used to analyze such oxidized lipids. Using this platform, lipid aldehydes can be selectively captured, enriched, and detected in liposome or lipoprotein experiments. We furthermore show that 3-methyl-1-*p*-tolyltriazene (MTT) methylates phosphoric and carboxylic acid, and that the MTT-mediated methylation thus provides useful information for structural analyses of such oxidized lipids.

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An efficient glycoblotting-based analysis of oxidized lipids in liposomes and a lipoprotein