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Research Article

Effect of the elapsed time between sampling and formalin fixation on the *N*-glycosylation profile of mouse tissue specimens

Formalin-fixed, paraffin-embedded (FFPE) samples are generally used for histology-study, however, they also possess important molecular diagnostics information. While it has been reported that the *N*-glycan moieties of glycoproteins is not affected by the FFPE process, no information is available about the effect of the elapsed time between sampling and fixation on the resulting *N*-glycosylation profile. In this study, lung, brain, heart, spleen, liver, kidney, and intestine mouse tissue specimens were used for *N*-glycan profiling analysis and the elapsed sampling time effect was investigated with the lung tissue. *N*-glycan extraction from the tissue samples was performed by glycoprotein retrieval from the FFPE specimens using radioimmunoprecipitation assay (RIPA) buffer followed PNGase F digestion. The released oligosaccharides were fluorophore labeled and analyzed by capillary electrophoresis-laser induced fluorescent detection (CE-LIF). *N*-glycosylation profiles of freshly collected lung-tissue samples (zero time point), as well as 1 and 2 h after sampling were compared by carbohydrate profiling and exoglycosidase treatment based deep glycomics analysis. It was found that up to two hours of room temperature storage of tissue specimens apparently did not cause changes in the *N*-glycosylation profiles of complex carbohydrates, but resulted in considerable decrease in the amount of linear glucose oligomers and high mannose type glycans present in the samples.

Keywords:

capillary electrophoresis / FFPE sampling / *N*-glycans

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1 Introduction

Formalin-fixed, paraffin-embedded (FFPE) samples are routinely prepared in hospitals and pathology laboratories for histology, and kept for decades to archive tissue specimens [1], representing an unexploited clinical information source about biological processes and diseases. Albeit these specimens were traditionally used for histology-study based diagnostics, the advent of highly sensitive bioanalytical methods enabled the utilization of FFPE samples for molecular level investigation including genomics, proteomics,

metabolomics, and glycomics [2]. However, for this latter one, no information is available about the effect of elapsed time between sampling and fixation as glycomolecular level changes can occur during that time. It is generally assumed that tissue specimens are immediately fixed after sampling to prevent any post-sampling biochemical processes, which might change their global molecular profile. In the instance of *N*-glycosylation type post-translational protein modifications, this elapsed time might affect some labile sugar residues such as sialylation and fucosylation (for the latter one, antennary and/or core), which play important structural and modulatory roles in many normal and pathological processes [3–5].

While postmortem degradation was extensively examined in proteomics and genomics studies, changes in *N*-glycan profiles have been investigated only in living organisms. It was found that protein kinase and phosphatase inhibitors preserved actin and myosin heavy chains from

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Abbreviations: APTS, 8-aminopyrene-1,3,6-trisulfonic acid; CE-LIF, CE-laser induced fluorescent detection; ER, endoplasmic reticulum; FFPE, formalin-fixed, paraffin-embedded; RIPA, radioimmunoprecipitation assay

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degradation [6]. So far, alterations of biologically important oligosaccharides were mainly associated with processes in the Golgi apparatus and in the endoplasmic reticulum (ER) [7]. It was found that in case of immunoglobulin light chains, *N*-glycan degradation increased in the presence of glucosidase inhibitors, however, addition of proteasome and mannosidase inhibitor stopped the degradation [8]. ER associated degradation in *Arabidopsis* revealed that during a chilling stress, smaller *N*-glycan intermediates were synthesized (Man_{5,7}GlcNAc₂ instead of Man_{8,9}GlcNAc₂) [9]. Robb et al. analyzed *N*-glycosylation degradation in *Streptococcus pneumoniae* using ribonuclease B as model glycoprotein and showed that various mannosidases were essential for trimming high mannose type *N*-glycans [10]. Cytosolic oligosaccharide chains degrade in the proteasome by autophagy, a lysosomal degradation process of cytoplasmic materials. Proteasomal degradation is controlled by *N*-glycans attached to substrates of ER associated degradation [11]. Deglycosylation of unfolded proteins catalyzed by enzymes such as cytosolic endo- β -*N*-acetylglucosaminidase [12] and cytoplasmic PNGase cuts the glycans from the misfolded proteins [13].

In complex type *N*-glycans, sialic acid residues are considered as the most sensitive elements for environmental degradation effects. Kiyohara et al. found that the degradation of sialylated human milk oligosaccharides is regulated by the expression of the exo- α -sialidase gene. Interestingly, it kept 80% of its activity even after 30 min incubation at 80°C, but the linkage preference of the enzyme changed from $\alpha(2,6)$ and $\alpha(2,8)$ to $\alpha(2,3)$ [14]. It was reported that lyase epimerase and kinase enzymes controlled the catabolism of sialyl-mucin glycoproteins [15]. Sam et al. investigated the glycosylation of Chinese hamster ovary cells where the decrease in temperature reduced the level of sialylation. However, the increase of the amount of dissolved oxygen enhanced the activity of sialyltransferases, thus the sialic acid content of these glycoproteins were increased [16].

Our earlier results revealed that the *N*-linked carbohydrates of glycoproteins with neutral, sialylated and high mannose structures were not affected by the formalin fixation and paraffin embedding process [17, 18]. Thus, it was considered that the fixation process archived the *N*-glycan structures and compositions of the sample at the very moment it was prepared. However, the elapsed time between sampling and fixation was not evaluated in respect to its effect on glycomolecular level changes. To the best of our knowledge, this is the first report examining possible time dependent degradation of asparagine-linked glycans in tissue specimens prior to formalin fixation.

2 Materials and methods

2.1 Chemicals

Formaldehyde for the buffered formalin (3.7% formaldehyde in 10 mM phosphate buffer, pH 7.4), the paraffin for

embedding, RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), dithiothreitol (DTT), iodoacetamide, xylene, ethanol, acetonitrile, sodium cyanoborohydride (1M in THF), and glucosidase enzyme (EC 3.2.1.20) were from Merck and (Kenilworth, NJ, USA). All other enzymes including PNGase F (EC 3.5.1.52), *Arthrobacter ureafaciens* sialidase (ABS) (EC 3.2.1.18), and $\alpha(1-2,3,6)$ mannosidase (EC 3.2.1.24) were from Prozyme (Hayward, CA, USA). The 8-aminopyrene-1,3,6-trisulfonate (APTS), the magnetic cleaning beads and the bracketing standards of APTS-labeled maltose and maltopentadecaose were from the Fast Glycan Sample Preparation and Analysis kit of SCIEX (Brea, CA, USA).

2.2 Sample preparation

The SCID male mice were from the National Institute of Oncology (Budapest, Hungary) and treated in accordance with the Guidelines for Animal Experiments by the Institutional Ethics Committee (permission # 22.1/722/3/2010). For the *N*-glycan time-degradation study, lung-tissue samples from three SCID male mice were used. The freshly removed specimens were volume aliquoted into three equal portions (parallel samples), followed by sample preparation for *N*-glycan profile analysis. The first aliquots were immediately processed. The second and third aliquots were stored at room temperature and processed 1 and 2 h after the tissue removal, respectively. In clinical practice, tissue samples are usually fixed within 2 hours after removal, so the experiments were planned accordingly.

The FFPE samples were washed twice by xylene and ethanol for 20 min, respectively, followed by centrifugation at 5000 \times g for 10 min. For glycoprotein release (also referred to as antigen retrieval), 40 mg of specimens were first homogenized in a Tapered Tissue Grinder (VWR, Budapest, Hungary), followed by the addition of 100 μ L of RIPA buffer and 10 μ L of 50 mM DTT to the homogenized samples and kept at 100°C for 20 min, then at 65°C for 120 min. After the protein extraction step, 10 μ L of 50 mM iodoacetamide was added to the reaction mixture and incubated at 37°C for 30 min in dark to alkylate the SH groups. This was followed by buffer exchange using 10 kDa spinfilters (VWR, Budapest, Hungary) and the *N*-glycan release took place on the filter using 49 μ L of 20 mM NaHCO₃ buffer (pH 7.0) and 1 μ L PNGase F (2.5 mU). The reaction mixture was incubated at 50°C for 60 min. To collect the released sugars, the filters were centrifuged at 5000 \times g for 10 min. The spinned content were dried in a centrifugal vacuum evaporator (Thermo Fisher Scientific, Waltham, MA, USA) followed by fluorophore labeling with the addition of 6 μ L of 20 mM APTS and 2 μ L sodium-cyanoborohydride (in 1M THF) and incubated overnight at 37°C. The fluorescently labeled samples were purified by magnetic bead-based technology [19] and analyzed by CE with laser induced fluorescence detection (CE-LIF). All experiments were done in triplicates and the RSDs were calculated.

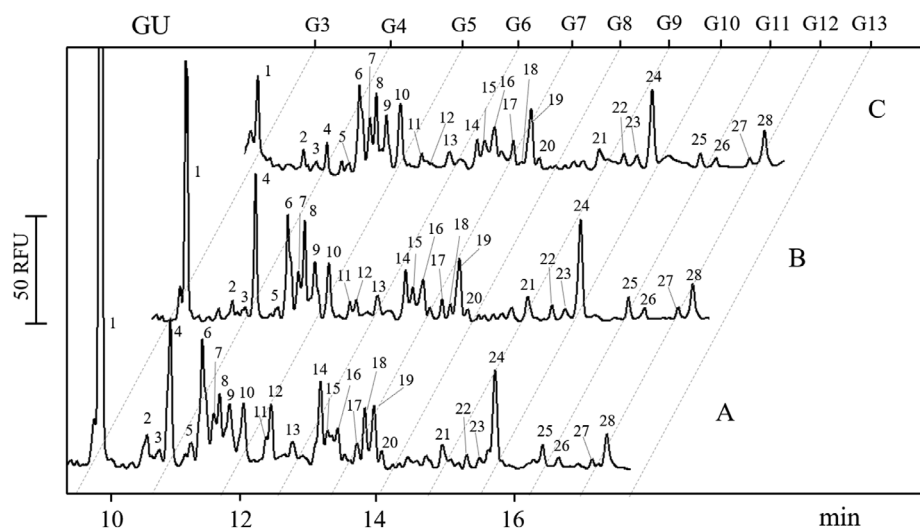


Figure 1. CE separation traces of APTS labeled *N*-glycan pools of mouse lung-tissue specimens processed at different times after sampling. (A) immediately prepared, (B) kept for 1 h at room temperature before processing, (C) kept for 2 h at room temperature before processing. Separation conditions: bare fused silica capillary (50 μ m i.d., 60 cm total length, 50 cm effective length) filled with NCHO separation buffer, injection: 5 psi for 5 s, Separation voltage: 30 kV at 25°C.

2.3 CE

All CE separations were performed using a PA800 Plus (SCIEX) Pharmaceutical Analysis system with laser induced fluorescence detection (488 nm excitation wavelength and a 520 nm emission filter) providing a low fmol detection limit. The bare fused silica capillary (effective length: 50 cm, total length: 60 cm, internal diameter: 50 μ m) was filled with N-CHO separation gel buffer (SCIEX) and the separations were performed in reversed polarity mode (cathode at the injection side) by the application of 30 kV electric potential. Samples were injected by 1 psi pressure for 5 s. APTS-labeled bracketing standards (maltose and maltopentadecaose) were co-injected (10 nL) with each sample to assure migration time reproducibility of <0.1% RSD. GU value calculation and structural *N*-glycan assignment were accomplished by using the GUcal software and database (www.GUcal.hu).

2.4 Exoglycosidase digestion based structural elucidation

Deep structural determination of the oligosaccharides of interest found in the released *N*-glycan pools from the mouse lung samples were accomplished by exoglycosidase-based studies [20]. *Arthrobacter ureafaciens* sialidase (ABS) was used to remove the α (2-3,6,8,9) bound sialic acids, Jack bean (*Canavalia ensiformis*) mannosidase (JBM) to cut α (1-2,3,6) mannoses (each 0.5 U, Prozyme,) and α -glucosidase (100 U from Merck and Co. Kenilworth, NJ, USA) to hydrolyze terminal, non-reducing α 1-4-linked D-glucose residues. The aliquoted APTS-labeled samples were subject to exoglycosidase digestions separately with the application of one enzyme at a time. The exoglycosidase reaction mixtures were incubated overnight at 37°C in 50 mM ammonium acetate buffer (pH 5.5) and analyzed by CE-LIF as described above.

3 Results

For mouse lung tissue, the effect of the elapsed time between the actual sampling and the formalin fixation, paraffin embedding process has been investigated to understand its influence on *N*-glycosylation degradation in zero time point as well as after 1 and 2 h of room temperature storage. All other mouse tissue sample specimens were profiled for their *N*-glycosylation since different tissue sample types have unique *N*-glycan profiles, specific to that particular organ [17] containing diverse proportions of high and low sialylated, high mannose as well as neutral carbohydrate structures in different size ranges, and interestingly a few low degree of polymerization glucose oligomers.

Figure 1 compares the CE-LIF separation patterns of the APTS labeled *N*-glycan pool of mouse lung-tissue samples processed at different time points after sampling (0, 1 and 2 h). All measurements were done in triplicates and the resulting peak area reproducibility was 8.17% RSD. Trace A depicts the resulting profile of the immediately prepared samples after tissue specimen removal. Traces B and C show the glycoanalysis results of the corresponding aliquots of the samples kept at room temperature for one and two hours prior to processing, respectively. The numbers above the peaks correspond to the structures listed in Table 1. As one can observe, peaks 1, 4, 12, 14, and 18 were significantly decreased after 1 and 2 h of room temperature storage prior to processing, but with different kinetics. For example, Peaks 1 and 14 decreased gradually between 0 and 2 h, Peak 4 practically did not change up to 1 hour of room temperature storage but significantly decreased between 1 and 2 h. The size of peak 18 dropped between 0 and 1 h of storage. Quantitative data for each of the 28 peaks at the three different times are provided in Supporting Information Table 1.

GU values were calculated for all the peaks (zero hour time point) (Table 1) and their corresponding structures were suggested using the GlycoStore database (www.glycostore.org). To verify the suggested GU value based structures and

Table 1. Glycan structures found in FFPE mouse lung, brain, heart, spleen, liver, kidney, and intestine tissue specimens. GU: glucose unit value

Peak	Structure*	GU	Lung	Brain	Heart	Spleen	Liver	Kidney	Intestine
1	Maltotriose	3	+	+	+	+	+	+	+
2	A4G(4)S(6,6,6)4	3.650	+	–	–	–	–	–	–
3	A4G(4)S(3,3,3)4	3.850	+	–	–	–	–	–	–
4	Maltotetraose	4	+	+	+	+	+	+	+
5	A3G(4)3S(6,6,6)3	4.250	+	–	–	+	+	+	+
6	A2G(4)2S(6,6)2	4.549	+	+	+	+	+	+	+
7	A2G(4)2S(3,6)2	4.721	+	+	+	–	+	+	+
8	F(6)A2G(4)2S(6,6)2	4.819	+	+	+	+	+	+	+
9	F(6)A2G(4)2S(3,6)2	4.996	+	+	+	+	–	–	–
10	A1[3]G(4)2S(3)1	5.240	+	–	+	+	+	+	–
11	A2[6]BG(4)1S(6)1	5.490	+	–	+	+	–	+	–
12	M4	5.580	+	+	+	+	+	+	+
13	F(6)A2[3]BG(4)1S(6)1	6.200	+	–	+	+	–	+	–
14	M5	6.607	+	–	+	+	+	+	+
15	F(6)A2[6]G(4)2S(6)1	6.713	+	+	+	+	+	+	+
16	F(6)A2G1Ga1S1	6.936	+	–	+	+	+	+	+
17	F(6)A2	7.326	+	+	+	+	+	+	+
18	M6	7.595	+	+	+	+	+	+	+
19	F(6)A2[3]G(4)2S(3)1	7.691	+	–	–	–	–	–	–
20	A2[6]BG(4)1	8.020	+	+	–	–	–	–	–
21	A2G(4)2	9.039	+	+	+	–	–	+	+
22	A2BG(4)2	9.530	+	+	+	+	+	+	+
23	F(6)A2BG(4)1	9.950	+	+	–	–	–	+	+
24	F(6)A2G(4)2	10.106	+	+	+	+	+	+	+
25	F(6)A2BG(4)2	10.860	+	+	–	–	–	–	+
26	A(6)3G3	11.250	+	–	–	–	–	–	+
27	A3G(4)3	11.970	+	+	+	–	–	+	+
28	FA2G2Ga2	12.366	+	+	–	–	–	+	–
29	A1[3]G(4)2	9.039	+	–	–	–	–	–	–
30	F(6)A2G2Ga1	11.096	+	–	–	–	–	–	–

*Abbreviated structure names followed the nomenclature suggested in the GlycoStore site (www.glycostore.org). Ga: α (1-3) galactose residue; (+) the structure was present in the tissue; (–) the structure was not present in the tissue

identify the oligosaccharides susceptible for delayed specimen processing, exoglycosidase digestion based structural analysis was also accomplished for the lung specimen. Figure 2 shows the results of the specific enzymatic releases of monosaccharide units in case of intact mouse lung-tissue sample after glucosidase (trace A), mannosidase (trace B), and sialidase (trace C) treatments. Structures were determined for all peaks >0.1% (peak area). Glucosidase cut off all α -D-glucose units, apparently eliminating peaks one and four from trace A. The mannosidase treatment released all α (1-2,3,6) mannose residues eliminating peaks 12, 14, and 18 from trace B. Finally, the sialidase removed all α (2-3,6,8,9) sialic acid residues, shifting most of the peaks from the early migration time regime to the later migrating neutral species region. The Lung column in Table 1 lists all structures found during this exercise

All other mouse tissue samples of brain, heart, spleen, liver, kidney, and intestine were profiled by CE-LIF for N-glycosylation characterization only (no storage time effect) and the results are summarized in Table 1 in the corresponding columns.

4 Discussion

Elongated room temperature storage (up to 2 h) of mouse lung-tissue specimens resulted in the decrease of peak heights for some glucose oligomers and high mannose type N-glycan features. GU values from public databases and exoglycosidase digestion-based studies were implemented using glucosidase, mannosidase and sialidase enzymes to identify all carbohydrate structures in the lung samples. The glucosidase treatment resulted in disappearance of some of the low DP glucose oligomers of maltotriose and maltotetraose present in the samples. On the other hand, the formalin fixation, paraffin embedding process apparently prevented their decomposition, so these structures were readily identifiable in the samples immediately fixed after removal. Mannosidase treatment eliminated all high mannose type structures but also affected the sizes of the maltotriose and maltotetraose peaks, assumably due to its apparent nonspecific glucosidase activity. As a first approximation, we suggest that the cause of the time based decrease in the size of these low DP glucose oligomers and high mannose N-linked structures was the

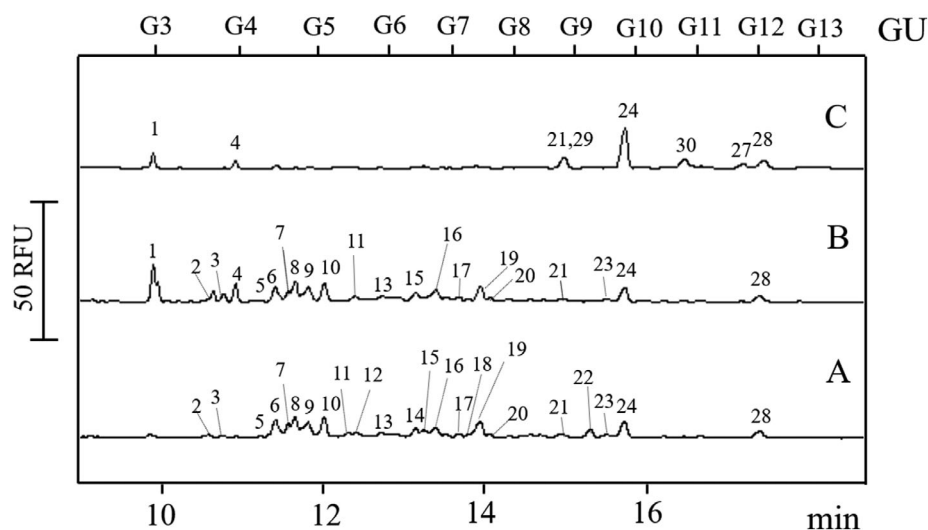


Figure 2. Exoglycosidase digestion based structural analysis of the *N*-glycans and maltooligosaccharides found in mouse lung-tissue specimens. (A) $\alpha(1-4)$ glucosidase, (B) $\alpha(1-2,3,6)$ mannosidase, and (C) $\alpha(2-3,6,8,9)$ sialidase treated samples. Separation conditions were the same as in Fig. 1.

result of non-ATP mediated enzymatic reactions, which might still keep on working under postmortem conditions (personal communications with Prof Tamas Freund). Sialidase treatment removed all $\alpha(2-3,6,8,9)$ bound sialic acids, resulting in shifts of all sialylated structures (see list in Table 1).

Tissue specimens are supposedly formalin fixed immediately after sampling at clinical and hospital facilities, but its exact time is usually unknown, and may significantly vary. In this paper, we investigated the effect of elapsed time between sampling and formalin fixation, paraffin embedding on the *N*-linked glycan profiles of mouse tissue specimens. In all tissue types studied, two major peaks significantly decreased with delayed processing, identified as maltotriose and maltotetraose, based on their GU value and glucosidase treatment results. It is important to note that no significant changes were observed in the profile of complex *N*-linked glycans including the sialylated structures up to two hours of storage of the removed specimens at room temperature.

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The authors have declared no conflict of interest.

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