



Characterization of N-glycosylation profiles from mammalian and insect cell derived chikungunya VLP



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ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes severe arthralgia. The envelope of CHIKV is composed of 240 copies of two glycoproteins: E1 and E2. In this work, we have characterized the N-glycosylation patterns of CHIKV virus-like particles (VLPs), containing both E1 and E2 proteins, derived from mammalian and insect cells using hydrophilic interaction liquid chromatography (HILIC) with fluorescence (FL) and mass spectrometry (MS) detection. While HEK293 derived CHIKV VLPs contain oligomannose, hybrid and complex glycans, VLPs derived from SfBasic predominantly contain oligomannose glycans. This strong host dependence of N-glycosylation pattern resembles other alphaviruses such as SINV. The VLPs from HEK293 and SfBasic, with significantly different N-glycosylation profiles, are valuable reagents enabling future in-depth correlation studies between immunogenicity and glycosylation. In addition, the characterization tools presented here allow one to monitor glycosylation during vaccine process development and ensure process consistency.

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1. Introduction

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus. It is a re-emerging virulent human pathogen that has caused millions of cases of disease around the Indian Ocean and Southeast Asia [1–3]. The adaptation of CHIKV from its primary vector *Aedes aegypti* to *Aedes albopictus* (an Asian tiger mosquito that is widespread in western countries) is responsible for the re-emergence of this pathogen [4,5]. Although rarely fatal, chikungunya infection causes severe morbidity including joint pain that can persist for months to years. The disease has become a global health concern. Both academia and industry have been actively pursuing vaccine approaches, including DNA [6], live and inactivated [7,8], VLP [9–11], and subunit vaccines [12]. It has been demonstrated in a phase 1 dose-escalation trial that CHIKV VLPs expressed in human embryonic kidney cell 293 (HEK293) are immunogenic, safe and well tolerated [13]. VLPs have multiprotein structures that mimic native viruses. Because VLPs are non-replicating particles that are capable of eliciting high immune response, they are attractive anti-

gen vaccine candidates compared to traditional live or attenuated viruses.

In recent years there has been increased attention paid to N-glycosylation of vaccine antigens and its impact on immunogenicity. Different N-glycosylation patterns on hemagglutinin (HA) profoundly impact immunogenicity including T cell activation, *in vitro* cytokine production and *in vivo* IL-2 production in a transgenic mouse model [14]. It is also found that recombinantly expressed HA elicits different antibody responses. While high IgG titers are induced by HA proteins carrying oligomannose glycans, higher neutralizing titers are induced by HA with complex glycans [15]. N-glycosylation is not only dependent on the host cells and virus strains [16,17], but also impacted by process changes. Adapting host cells from adherent cells to serum-free suspension significantly changes HA N-glycosylation [18]. N-glycosylation has also influenced HIV vaccine development. Researchers have found a dramatic difference in N-glycan profiles of viral envelope glycoprotein gp120 between primary isolates and recombinantly generated gp120, revealing an attractive epitope for vaccine development [19,20].

The CHIKV envelope is composed of 240 copies of glycoproteins E1 and E2 arranged in a T4 icosahedral symmetry. E1 has one N-linked glycosylation site on N141, and E2 has two glycosylation

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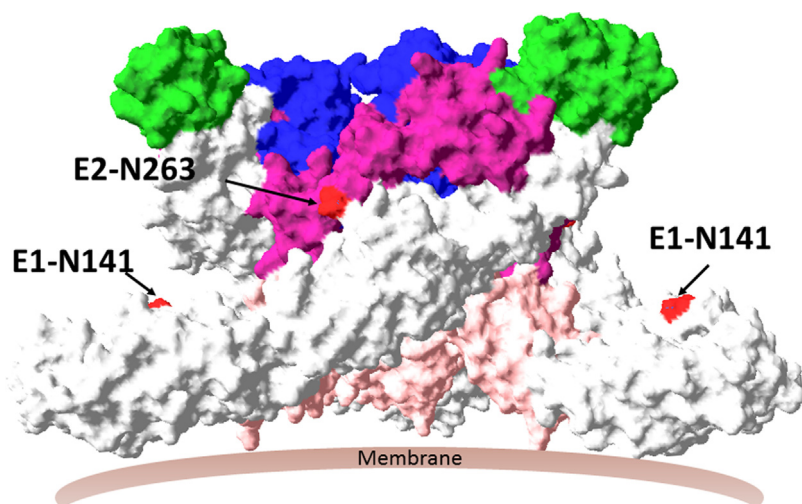


Fig. 1. Localization of the *N*-glycosylation sites on CHIKV VLP. This is a horizontal view from the VLP surface of one CHIKV VLP spike formed by three E1/E2 heterodimers. The molecular surface is drawn using PDB ID 2XFB and SwissPDB software. The E1 surface is colored grey. The E2 surface is colored blue for domain A, green for domain B, light brown for domain C, and dark purple for β -ribbon connector. Two glycosylation sites E1-N141 and E2-N263 are exposed on the surface and are highlighted red. The third glycosylation site E2-N345 is close to the transmembrane region and is not visible from the crystal structure.

sites on N263 and N345. The glycosylation of E1-N141 and E2-N263 are confirmed by the crystal structure of p62-E1 heterodimer [21]. Both glycosylation sites are exposed on the VLP surface (Fig. 1). E2-N345 is located close to the transmembrane region and is not visible in the crystal structure.

Unlike influenza or HIV, little is known about the *N*-glycan structures of CHIKV. The only alphavirus whose glycosylation has been well studied is Sindbis virus (SINV). The SINV envelope is composed of glycoproteins E1 and E2 with two glycosylation sites on each glycoprotein. SINV has shown strong host-dependent glycosylation variation. SINV derived from baby hamster kidney cells (BHK), chick embryo fibroblast (CEF), and Chinese hamster ovary cells (CHO) has oligomannose, hybrid and complex glycans [22,23], while mosquito cell grown SINV has predominantly oligomannose [24]. The differences in SINV *N*-glycosylation pattern are due to the host cell glycosylation pathway and machinery and the steric accessibility of the glycans to cellular processing enzymes.

In this study, we characterize the major glycan structures of CHIKV VLPs derived from both mammalian and insect cells, by utilizing 2-aminobenzamide (2AB) labeling of *N*-glycans followed by HILIC-FL. For confirmation of glycan identifications, we utilize LC-MS, 2AB glycan standard correlation, and exoglycosidase treatments. To our knowledge, this is the first in-depth characterization of *N*-glycan profiles of CHIKV VLPs from HEK293 and SfBasic expression systems. This work confirms a strong host cell dependent *N*-glycan profile for this VLP. This knowledge is valuable during vaccine process development. First, selection of a VLP expression platform will be based on not only the titer and yield but also other parameters including glycosylation of VLPs. Second, the VLPs from HEK293 and SfBasic can be used in detailed glycosylation-immunogenicity correlation studies to evaluate if the differences in glycan profile yield differences in immunogenicity. Lastly, monitoring glycosylation will be critical part of process development to ensure product consistency.

2. Materials and methods

2.1. Materials

PNGase F, acetonitrile, ethanol, formic acid, ammonium hydroxide, and sodium dodecyl sulfate (SDS) were purchased from Sigma

(St. Louis, MO, USA). 2-Aminobenzamide (2AB) labeling kit, α (1-2,3,6) mannosidase, α (1-2,3,4,6) fucosidase, β (1-3,4) galactosidase, and α (2-3,6,8,9) sialidase A (each including its own 5 $\times$ Reaction Buffer) were purchased from Prozyme (Hayward, CA, USA). β -mercaptoethanol was from Acros (Fair Lawn, NJ, USA). NP-40 and Slide-A-Lyzer 10 K cassette were obtained from Pierce (Rockford, IL, USA). Wizard SV 96 Lysate Clearing Plate was from Promega (Madison, WI, USA). 2AB labeled oligomannose glycans (M5, M6, M7, M8, and M9) were purchased from QA Bio LLC (San Mateo, CA, USA). The expression and purification of HEK293 and SfBasic derived CHIKV VLPs were described by Wagner et al. [25].

2.2. Glycan release and labeling

An aliquot of 1 mL of approximately 300 μ g/mL CHIKV VLP, containing both E1 and E2 proteins, was dialyzed into PBS using a Slide-A-Lyzer 10 K cassette. The dialyzed sample was completely dried in a SpeedVac (Savant) for $\sim$ 5 h. The dried sample was incubated with 90 μ L PBS, 2 μ L 5% SDS and 1 μ L 10% β -mercaptoethanol at 37 $^{\circ}$ C for 10 min. To release the *N*-glycans from the reduced and denatured sample, 5 μ L NP-40 followed by 5 Units PNGase F were added to the sample. The mixture was incubated for 20 h at 37 $\pm$ 5 $^{\circ}$ C. A 300 μ L aliquot of ethanol was added and the mixture was centrifuged at 10,000 rpm (10,620g) at 2–8 $^{\circ}$ C for 20 min to precipitate the proteins. The supernatant containing the released glycans was dried in a SpeedVac overnight ($\sim$ 20 h).

The dried glycans were labeled with 2AB at 65 $\pm$ 5 $^{\circ}$ C in the dark for 3 h using the 2-AB Labeling Kit. To remove excess dye, the mixture was loaded onto a Wizard SV 96 Lysate Clearing Plate that was pre-washed with acetonitrile. The labeled glycans were allowed to flow through the wells by gravity. Each well on the plate was washed with 1 mL 96% acetonitrile three times and glycans were eluted with 300 μ L Milli Q water. The eluted glycans were dried using SpeedVac and resuspended in 150 μ L of milliQ water.

2.3. HILIC profiling

The 2AB derivatized glycans were separated on a Waters Acquity UPLC BEH glycan column (1.7 μ m, 2.1 $\times$ 100 mm) designed for HILIC separation of 2AB labeled *N*-glycans. The column was maintained at 60 $^{\circ}$ C on a Waters Acquity UPLC system using Empower 2 soft-

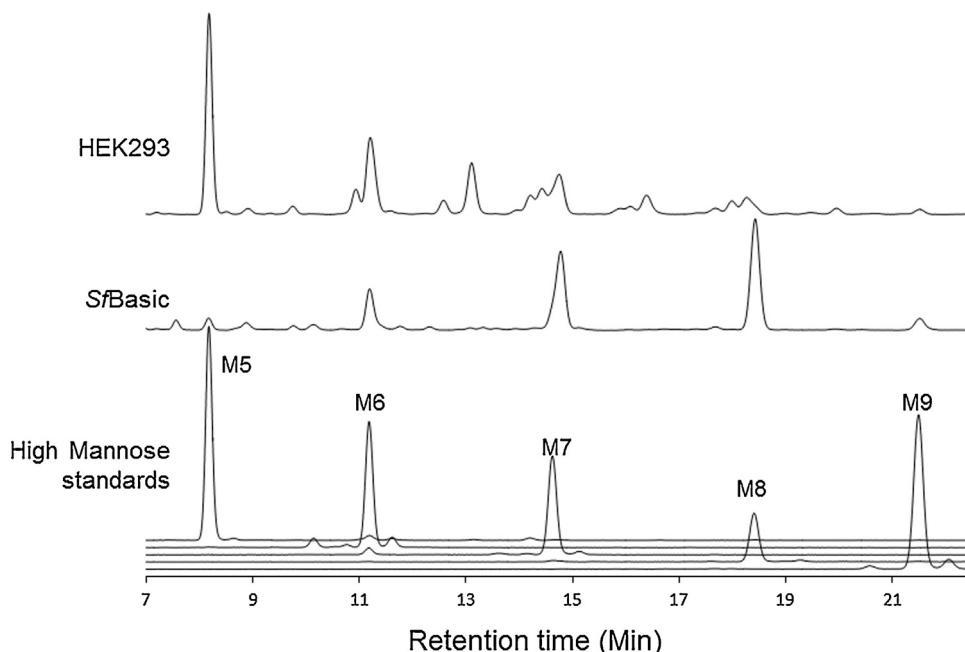


Fig. 2. HILIC profiles of 2AB labeled *N*-glycans from CHIKV VLPs derived from HEK293 and SfBasic and 2AB labeled oligomannose standards (M5, M6, M7, M8 and M9) by fluorescence detection (330 nm excitation, 420 nm emission).

ware. Mobile Phase A contained 100 mM ammonium formate pH 4.5 and Mobile Phase B contained 100% acetonitrile. An aliquot of 5 μ L of derivatized glycan was injected onto the HILIC column and eluted using a gradient from 72% to 62% B over a period of 30 min at a flow rate of 0.4 mL/min. The eluted glycans were monitored by fluorescence (330 nm excitation/420 nm emission).

2.4. LC–MS identification

In order to identify the chromatographic peaks, the eluted 2AB derivatized glycans were analyzed by LC–MS on a Waters SYNAPT G2 Time of Flight (ToF) mass spectrometer equipped with an elec-

tro spray ionization source. The mass spectrometer was operated in a sensitivity mode with capillary voltage of 2 kV, cone voltage of 15 V, and extraction cone voltage of 4 V. Nitrogen was used as a desolvation gas at a flow rate of 1000 L/hr. The desolvation temperature was 350 °C, and the source temperature was 120 °C. The mass spectra were acquired in an *m/z* range of 300–2400 and analyzed using Waters MassLynx 4.1 software.

2.5. Exoglycosidase cleavage

The 2AB derivatized glycans were digested with α (1-2,3,6) mannosidase, α (1-2,3,4,6) fucosidase, β (1-3,4) galactosidase, and

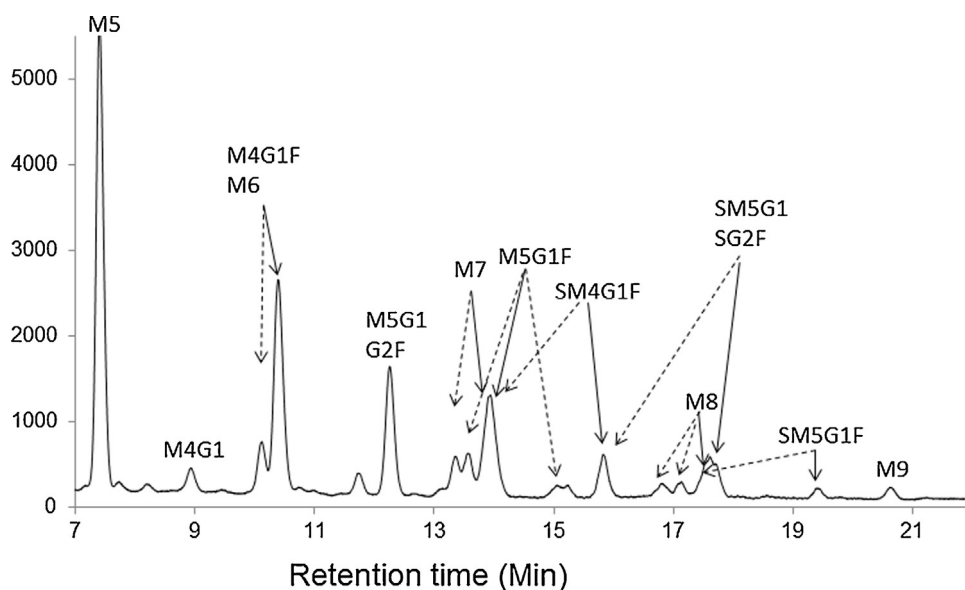


Fig. 3. In-line LC–MS analysis results showing preliminary assignments (based on comparison of observed and theoretical mass) for 2AB labeled *N*-glycans from HEK293 derived CHIKV VLPs. Peaks were monitored at 330 nm (chromatogram as shown) as well as by MS. In cases where multiple peaks show the same mass, indicating isobaric structures, solid line arrows point to the major species, while dashed lines point to the minor forms.

Table 1

Proposed structures of 2AB derivatized glycans detected in HEK293 derived CHIKV VLPs are shown, based on comparison of calculated and observed mass.

Glycans	Proposed Glycan Structures	Calculated MW	Observed MW
M5		1354.50	1354.51
M6		1516.56	1516.56
M7		1678.61	1678.62
M8		1840.66	1840.66
M9		2002.71	2002.69
M4G1		1557.59	1557.57
M4G1F		1703.65	1703.64
SM4G1F		1994.74	1994.75
M5G1		1719.64	1719.63
M5G1F		1865.70	1865.69
SM5G1		2010.74	2010.72
SM5G1F		2156.79	2156.78
G2F		1906.73	1906.73
SG2F		2197.83	2197.82

: N-acetylglucosamine,
 : Mannose,
 : Galactose,
 : Fucose,
 : Sialic acid

α (2-3,6,8,9) sialidase A, respectively. A control was carried out using water instead of enzymes. In each experiment, 4 μ L of enzyme (or water for control reaction) and 4 μ L of 5 $\times$ Reaction Buffer were added to 16 μ L 2AB labeled glycan. Mixtures were incubated at 37 $^{\circ}$ C for 20 h at which time the samples were transferred to an LC vial for injection.

3. Results

3.1. N-linked glycan profiling by HILIC

To profile the *N*-glycosylation of CHIKV VLPs produced from HEK293 and *Sf*Basic, glycans were released by PNGase F and derivatized with a fluorescence tag, 2AB. The labeled glycans were separated on a HILIC column and detected by fluorescence. The main glycan peaks derived from *Sf*Basic overlaid nicely with oligomannose standards and were identified as M5, M6, M7, M8 and M9 (Fig. 2). Conversely, the glycan profile of HEK293 derived VLPs was complex and not as easily identifiable. Therefore, glycan identification was performed by LC-MS in order to supplement the HILIC profiling with fluorescence detection. Glycan identification was further confirmed in many cases by exoglycosidase treatments of the glycans from both expression systems.

3.2. Glycan identification by LC-MS

In order to further understand the identity of glycans from HEK293 derived CHIKV VLPs, the 2AB labeled glycans were analyzed on a Waters SYNAPT G2 mass spectrometer after HILIC separation. Glycan structures were proposed based on the exact match between the observed and the expected molecular mass of glycans with the caveat that isobaric (including isomeric) structures may exist (Table 1). Fig. 3 shows the glycan assignments of each chromatographic peak. It is not surprising that we observe co-eluting glycans in some cases. It is, however, striking that we observed multiple peaks with the same mass for some assigned glycans shown in Fig. 3. This is represented using solid arrows pointing to where the major glycan elutes and dashed arrows pointing to the

minor species. Complete identification of these peaks, which could be isomeric or other isobaric structures of those proposed here, will rely on future studies. These studies may consist of a combination of carbohydrate derivatization and additional MS analyses [26]. From the existing analysis, we conclude that HEK293 derived CHIKV VLPs contain a broad range of glycans including oligomannose, hybrid and complex glycans and tentative assignments of these glycans have been made based on the mass.

3.3. Confirmation of N-glycan structures by exoglycosidase treatment

To further confirm the *N*-glycan structures identified by HILIC and LC-MS, the 2AB labeled glycans were cleaved with exoglycosidases including α -mannosidase, α -fucosidase, β -galactosidase and sialidase A, and analyzed by HILIC UPLC. As shown in Fig. 4A for the HEK293 derived VLPs, all oligomannose and hybrid glycans were cleaved by α -mannosidase. M5G1F, SM4G1F and SM5G1F were cleaved by α -fucosidase. While there was some drop in M4G1F upon α -fucosidase, it was not as significant likely due to co-elution of this glycan with M6. M5G1 and M5G1F were cleaved by β -galactosidase. We did not observe the drop of M4G1F after β -galactosidase cleavage likely due to the co-elution of some cleavage products. SM4G1F, SM5G1 and SM5G1F were all cleaved by sialidase A. The exoglycosidase cleavage experiments confirm the monosaccharide structure at the non-reducing end of the proposed glycans. Unfortunately this experiment was not able to conclusively confirm the identities of M4G1, G2F and SG2F, most likely due to their low abundance and co-elution with other glycans. Fig. 4B shows that all the oligomannose glycans from SfBasic derived VLP were cleaved by α -mannosidase. This result is consistent with the oligomannose assignments of the major glycans from SfBasic derived VLP.

4. Discussion

Utilizing 2-AB labeling of *N*-glycans followed by HILIC UPLC, we have observed a strong host dependent *N*-glycosylation profile for CHIKV VLPs. CHIKV VLPs derived from HEK293 consist of oligomannose, hybrid and complex glycans while VLPs from SfBasic contain predominantly oligomannose. This observation is consistent with the glycosylation pattern of SINV derived from mammalian and insect cells. The glycan characterization also supports an earlier intact protein mass analysis in which a combination of hybrid and oligomannose glycans were identified with E2 from HEK293 derived VLPs [27]. We have not yet addressed the potential isobaric (including isomeric) structures of the glycans proposed, nor have we characterized the site specific glycosylation or occupancy at each site. In the future, the site specific *N*-glycosylation, along with site occupancy, could be studied using tryptic digestion and LC-MS with and without PNGase cleavage. The VLPs derived from HEK293 and SfBasic are modified with significantly different *N*-glycans and are therefore valuable reagents for a future in-depth glycosylation/immunogenicity correlation study. A preliminary study in guinea pig shows that SfBasic derived VLPs elicited neutralization titers not significantly different from that of HEK293 derived VLPs [25]. Since that study was carried out with adjuvants, it will be interesting to further evaluate the impact of adjuvant and glycosylation on immunogenicity separately. Furthermore, multiple neutralizing antibodies targeting different epitopes on E2 have been identified recently [28] [29] [30]. It will be informative to compare the antigenicity of VLP glycovariants against those known neutralizing antibodies. Since CHIKV VLP glycosylation is highly dependent on the expression host, the knowledge we gained here is relevant to selecting the manufacturing platform for CHIKV VLPs.

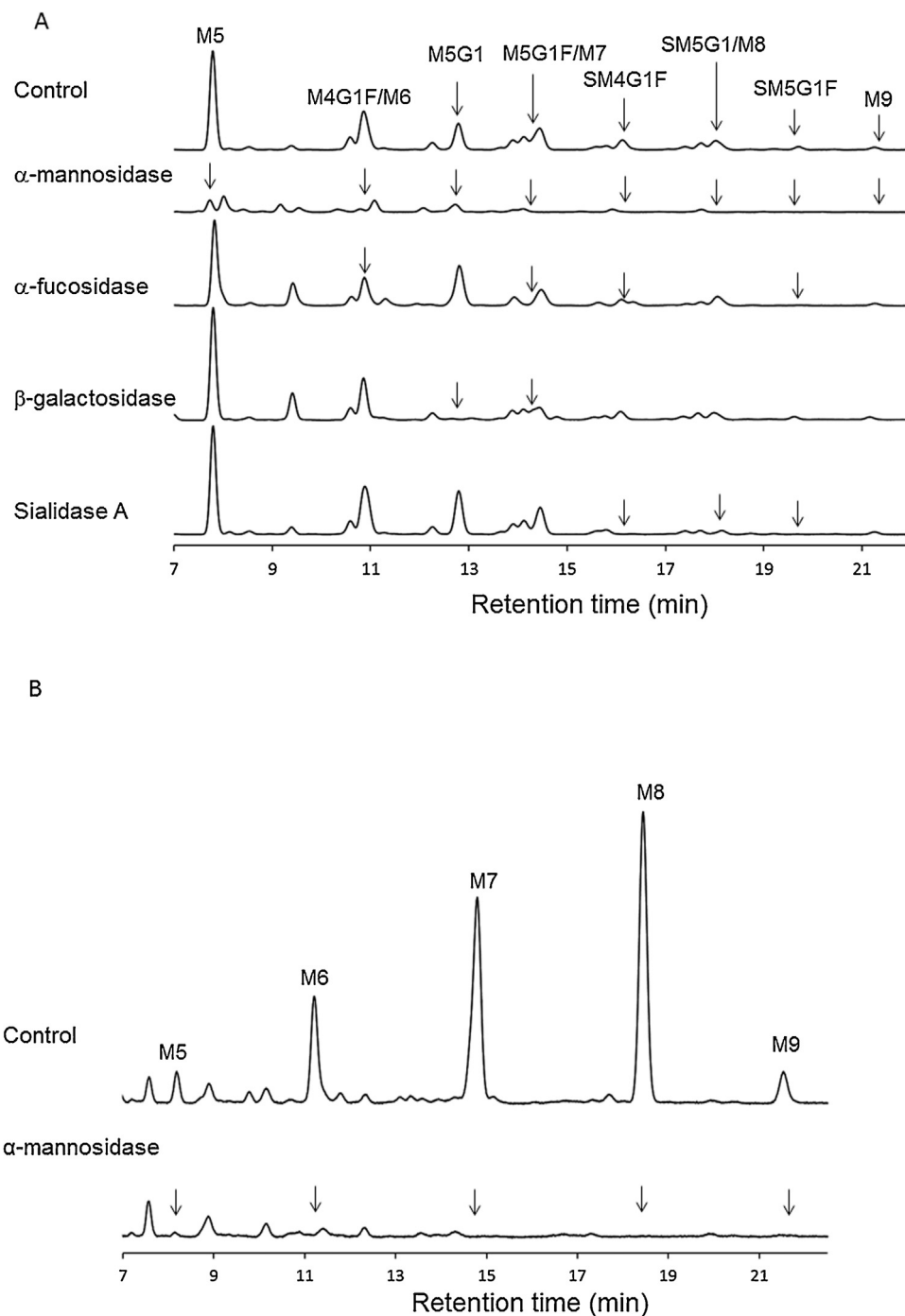


Fig. 4. HILIC profiles of 2AB labeled glycans before and after exoglycosidase cleavage. (A) N-glycans from HEK293 derived CHIKV VLPs compared to those cleaved by α -mannosidase, α -fucosidase, β -galactosidase and sialidase A. (B) N-glycans from SfBasic derived CHIKV VLPs compared to those cleaved by α -mannosidase.

The characterization tools presented here also allow for the monitoring of N-glycosylation during vaccine process development.

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