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Labeling of Enveloped Virus via Metabolic Incorporation of Azido Sugars

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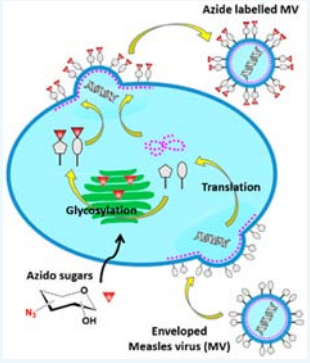
Supporting Information

ABSTRACT: Modification of an enveloped measles virus was achieved by metabolic incorporation of azido sugars in host cells through the protein glycosylation process. Based on this, the resulting measles virus particles could be modified with azido groups on the surface glycoproteins, which could be further labeled with fluorescence dyes using a strain-promoted azide−alkyne cycloaddition reaction. We envision this metabolic labeling approach to be applicable to a wide variety of enveloped viruses, allowing the facile conjugation and surface modification.



Enveloped viruses are surrounded by a phospholipid bilayer consisting of transmembrane glycoproteins distributed along the surface. These glycoproteins can regulate virus entry into host cells.[1] The selective surface modification of enveloped viruses is critical for many applications. For example, viral surface proteins or phospholipid layer modified with fluorophores or quantum dots (QDs) would facilitate tracking of the viral particles in host cells. This in turn could be used to report the underlying molecular mechanisms of virus entry.[2−4] In addition, the display of specific epitopes on the viral surface has been exploited to engineer potent immunization vehicles for the development of novel vaccines.[5] Furthermore, for cancer therapy purposes, surface engineering of the oncolytic viruses with targeting ligands could redirect the specific binding of viruses to cancer cells, which would mediate virus entry and promote the syncytial formation.[6,7]

The surface modification of enveloped virus can target either the phospholipid layer or surface proteins.[3,8,9] Because the surface proteins play a major role in the recognition event between viruses and host cell surface receptors,[1] the modification of surface proteins is now attracting much more attention in the application of enveloped viruses.[10,11] The conventional approaches for viral surface protein labeling rely upon genetic engineering and chemical conjugation.[12] The genetic modification of virus surface protein often suffers from attenuation of virus production, infectivity, cellular transduction, and size limitations on inserted peptides.[13] Chemical modification was also developed for surface protein modification. By taking advantage of surface exposed lysine on both vaccinia virus and influenza A virus, Hao et al. conjugated the clickable azido group onto the viral surface proteins using azide-PEG_4-NHS ester, which subsequently facilitated the chemical modification of QDs derived with dibenzocyclooctynes (DBCO) using a strain-promoted azide−alkyne cycloaddition (SPAAC) reaction.[14] Similarly, Cai et al. reported the DBCO labeling of surface proteins of baculoviruses through incubation with DBCO-PEG_4-NHS, followed by the subsequent modification of N_3-QDs.[15] As conventional bioconjugation strategy is dependent on the naturally occurring residues, it is often limited by availability of functional groups.

Recently, metabolic incorporation of azide bearing sugars into cell surface proteins has been applied for protein functional analysis and glycoprotein imaging.[16,17] During the metabolism process, azido sugars can be recognized and accepted by the cells, then transformed into substrates for the glycosylation of proteins. Carrico and co-workers have first demonstrated that nonenveloped adenovirus could be modified through metabolic incorporation of *O*-azidoacetylglucosamine (*O*-GlcNAz) into the viral fiber protein by treating the host cells with azido sugars during the virus production. This allowed subsequent chemical attachment of a variety of functionalities such as peptides, fluorophores, and small molecular targeting moieties without loss of either viral production or infectivity.[13,18] Considering that the surface proteins of enveloped viruses are glycosylated,

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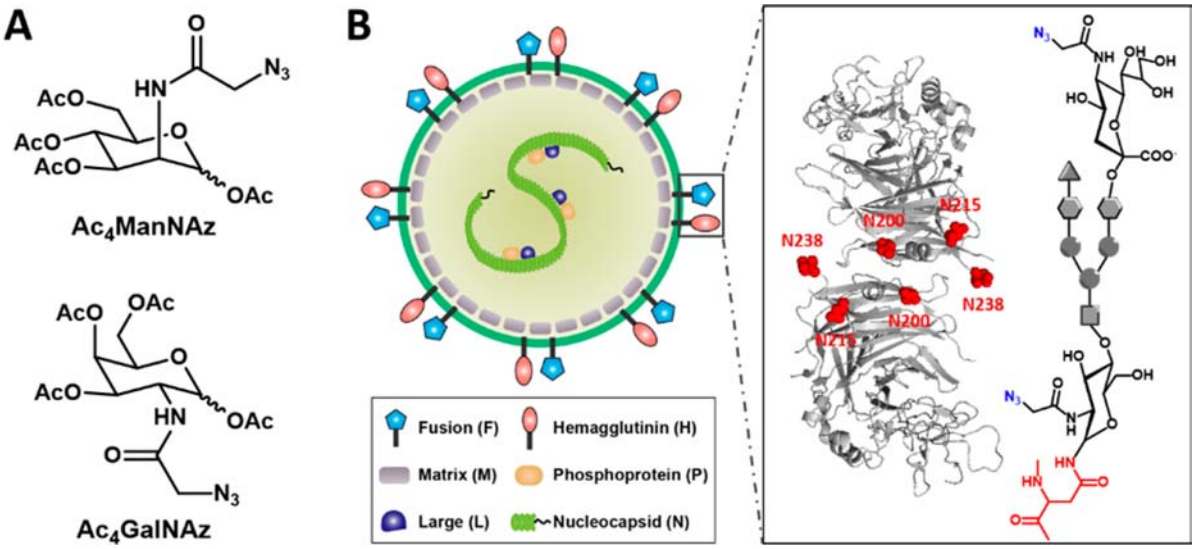
Scheme 1. (A) Chemical Formulas of Azido Sugars $Ac_4ManNAz$ and $Ac_4GalNAz$. (B) Schematic Illustration of the Structure of Measles Virus (MV) and the Metabolic Incorporation of Azido Groups on Virus Surface[a]



[a]Enlarged: structure of hemagglutinin (H) glycoprotein dimer and its *N*-linked glycan sites asparagine (Asn, N) residues (marked as red). Azido groups are incorporated on glycoproteins through *N*-linked glycosylation at Asn sites. Protein models were generated using PyMol (www.pymol.org) with coordinates obtained from the RCSB Protein Data Bank (www.pdb.org).

we hypothesize that they can be modified through similar metabolic incorporation of azido sugars in host cells.

Measles virus (MV), a member of the morbillivirus subgroup of paramyxoviruses, contains two glycosylated envelope proteins embedded on the phospholipid bilayer, hemagglutinin (H) and fusion protein (F) (Scheme 1B).[19] Generally, the H protein attaches the virus particle to receptors on the host cell surface, and then the F protein induces fusion of the virus envelope and the cell cytoplasmic membrane for the entry of MV into cells.[20,21] *N*-Azidoacetylmannosamine ($Ac_4ManNAz$) and *N*-azidoacetylgalactosamine ($Ac_4GalNAz$) are two of the most widely used azido sugars for glycan labeling (Scheme 1A).[17] In this paper, we investigated the modification of enveloped MV surface glycoproteins mediated by metabolic incorporation of azido sugars $Ac_4ManNAz$ and $Ac_4GalNAz$.

Azido sugars $Ac_4ManNAz$ and $Ac_4GalNAz$ were synthesized following the reported procedures.[22,23] Vero cells were used as the host cells for MV propagation, which were grown in complete media supplemented with azido sugars at specified concentrations. The azido sugars can compete with native monosaccharides in the culture media and subsequently be incorporated into the glycoproteins of the cells. $Ac_4ManNAz$ is taken up by the cell, deacetylated, and converted to N-azidoacetyl sialic acid (SiaNAz).[23] The activated sialic acid labeled with azide is subsequently incorporated at the terminus of *N*- and *O*-linked glycans.[16] Usually, $Ac_4GalNAz$ is utilized by cells as a substitute for GalNAc, which can append to serine or threonine residues to initiate mucin-type *O*-linked glycan biosynthesis.[24] Since it also has been reported that GalNAz could be partially interconverted to GlcNAz *in vivo* through conversion of the metabolic intermediate UDP-GalNAz to UDP-GlcNAz,[18,25] it may be possible that the core pentasaccharide $Man_3GlcNAc_2$ of *N*-linked glycans are also labeled with $Ac_4GalNAz$.[26] After 48 h incubation, the Vero cells were fixed, then stained with DBCO-Fluor 488 via a Cu-free SPAAC reaction (Figure S1). As shown in Figure 1A, the increase of fluorescence intensity correlated with an increasing

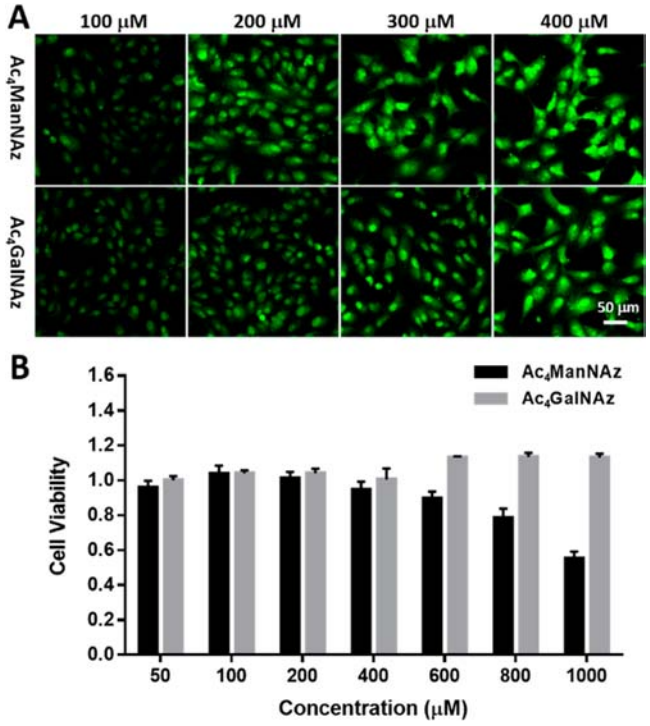


Figure 1. Fluorescence labeling and viability detection of azide-labeled Vero cells. (A) The metabolic incorporation of $Ac_4ManNAz$ and $Ac_4GalNAz$ into Vero cells. (B) CellTiter-Blue viability assay of Vero cells incubated with certain concentrations of $Ac_4ManNAz$ and $Ac_4GalNAz$ for 24 h.

molecular concentration of the azido sugars, suggesting the efficient incorporation of azido sugars into cells. The cell viability was above 90% when incubated with azido sugars at 400 μM for 24 h (Figure 1B). $Ac_4ManNAz$ showed some cytotoxicity at very high concentration, i.e., above 600 μM. A similar result was also reported by Almaraz and co-workers that

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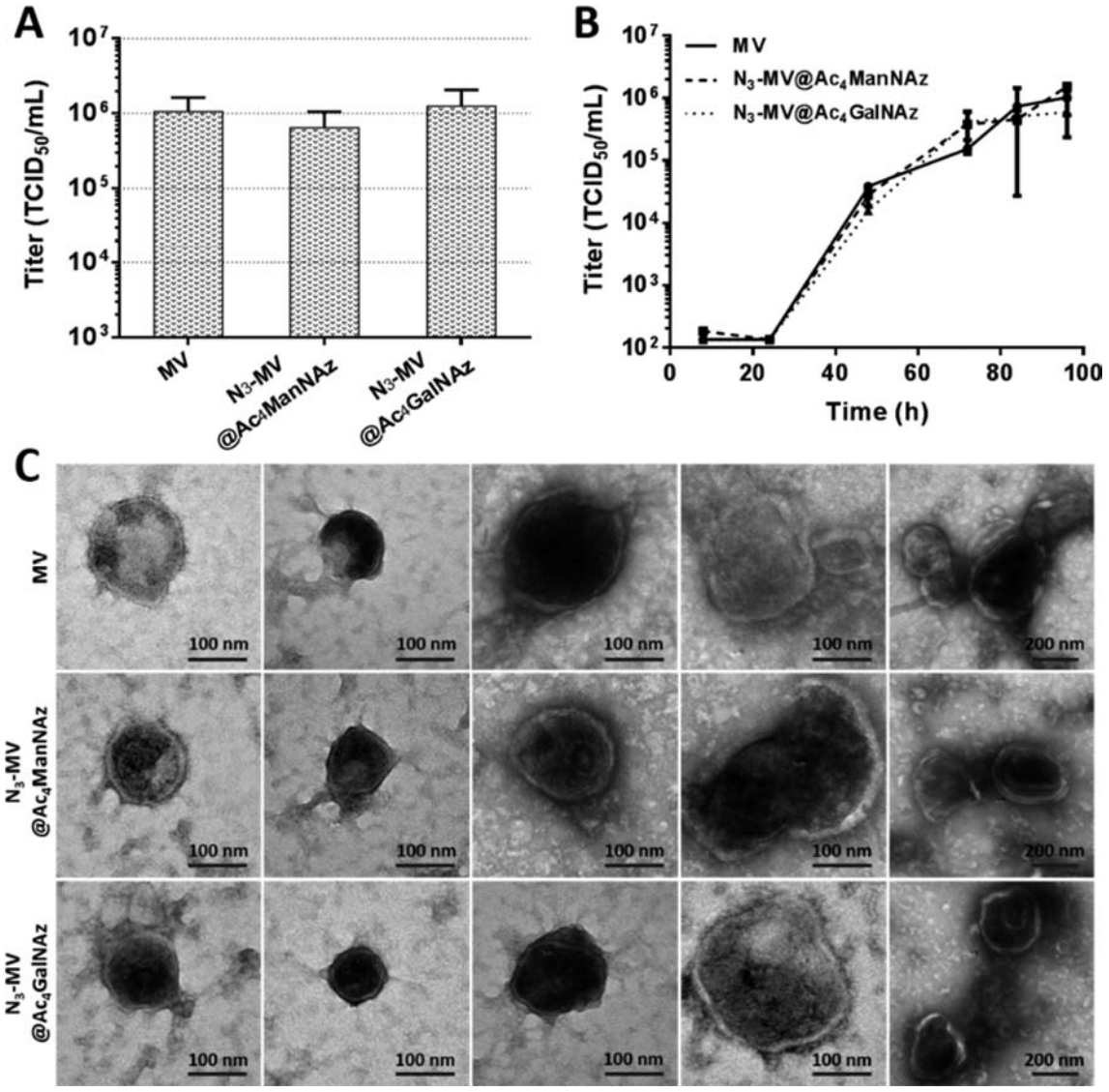

Figure 2. Detection of azide modified MV. (A) Production of control virus and azide labeled viruses by Ac_4ManNAz and Ac_4GalNAz. (B) Growth curves of control virus and azide labeled viruses by Ac_4ManNAz and Ac_4GalNAz. (C) Transmission electron microscopy (TEM) images of control virus and azide labeled viruses by Ac_4ManNAz and Ac_4GalNAz.

the cytotoxicity of Ac_4ManNAz to Jurkat cells could be observed at a concentration of ≥300 μM.[27]

Viruses were propagated on azide labeled Vero cells to afford the azide-labeling of MV particles. During the infection period, the culture media was supplemented with 400 μM of Ac_4ManNAz or Ac_4GalNAz. It is known that the F and H glycoproteins of MV are both *N*-glycosylated.[28] There are three potential *N*-linked glycosylation sites located on the F protein at asparagine (Asn) residues 29, 61, and 67,[29,30] while there are five located on the H protein at Asn168, 187, 200, 215, and 238.[31,32] Moreover, the carbohydrate content of the H protein was determined to be approximately 12% by weight, including mannose, galactose, fucose, *N*-acetylglucosamine (GlcNAc), and *N*-acetylneuramine acid (sialic acid).[33] By hijacking host cellular machineries, MV particles produced in the presence of azido sugar precursors Ac_4ManNAz and Ac_4GalNAz might be post-translationally modified with azido groups on the viral surface[13,18] (Scheme 1B).

Viruses produced from the infected Vero cells were purified on 20−60% sucrose gradient.[34] To evaluate the production of azide modified MV (N_3-MV), Vero cells were cultured until they reached 80−90% of confluence. Serially diluted virus samples (control MV or N_3-MV) of the same batch were added to the cells, which were fed with DMEM containing 2% FBS. Then, the cells were cultured for about 3 days. $TCID_{50}$ in Vero cells were calculated based on Reed-Muench formula,[35] showing that the titers of control MV and N_3-MV were comparable at around 10^6 $TCID_{50}$ mL^{-1}. This suggested metabolic labeling did not affect the propagation of MV in Vero cells (Figure 2A). The infectivity of N_3-MV was evaluated by one-step growth kinetics.[36] Vero cells were infected with virus samples at a multiplicity of infection (MOI) of 0.2 for the time indicated. As shown in Figure 2B, no obvious difference was observed in N_3-MV produced through Ac_4ManNAz and Ac_4GalNAz labeling, compared with that of normal MV. $TCID_{50}$ of control MV and N_3-MV labeled by Ac_4ManNAz and Ac_4GalNAz could reach 10^6 $TCID_{50}$ mL^{-1} at 96 h after infection. In addition, the azide modified viruses still maintained the intact structure like that of the control virus. The size of MV was heterogeneous, consistent with the

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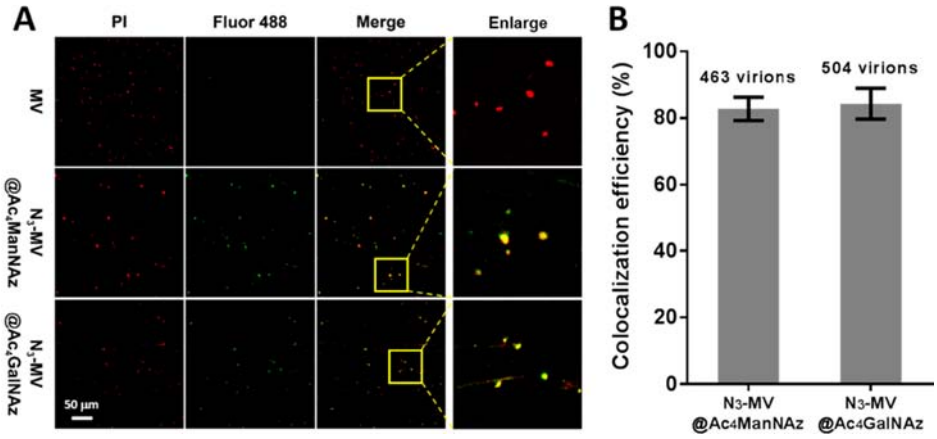

Figure 3. (A) Fluorescence imaging of control virus and azide labeled viruses stained with both DBCO-Fluor 488 (green) and propidium iodide (red), showing the accessible surface azido groups of MV particles derived from azido sugar treated Vero cells. (B) Colocalization efficiency of azide labeled viruses.

reported size range between 50 and 1000 nm.[37] No obvious size difference between the native and modified viruses was observed (Figure 2C). All of the above results suggested that the metabolic incorporation of azido sugars did not disturb the viral production, infectivity, or virus morphology.

To confirm that the azide-modified MV could be ready for further chemical modification with DBCO derived fluorophores, the viruses were overlaid on coverslips for 60 min at 37 °C. Then, the coverslips were fixed, permeabilized, and stained with DBCO-Fluor 488 for azido groups (green) and propidium iodide (PI) for nucleic acid (red). The fluorescence imaging results showed that most of fluorescence signal of PI colocalized with that of Fluor 488 for all N_3-MV, appearing as yellow in a merged image (Figure 3A). The colocalization efficiency of N_3-MV labeled by Ac_4ManNAz and Ac_4GalNAz were 82.8 ± 3.5% and 84.4 ± 4.6%, respectively (Figure 3B). However, no Fluor 488 signal was observed for the control sample (Figure 3A). The concentration of Fluor 488 was further analyzed based on the fluorescence measurement, which indicated that about 0.40 nmol and 0.28 nmol of dyes were attached on 1 mg of N_3-MV by Ac_4ManNAz and Ac_4GalNAz, respectively (Figure S2). Therefore, virus produced from azide labeled Vero cells has been further verified with azide modification on the viral surface. It was also concluded that the azido groups on the virus's surface could be consequently modified through a SPAAC reaction. Moreover, the fluorescence imaging of cells coincubated with QDs labeled viruses showed remarkable fluorescence signals on the cytomembrane, indicating the virus particles were still infective after further chemical modification through SPAAC reaction (Figure S4).

In summary, we have demonstrated that enveloped MV could be metabolically labeled with azido sugars assisted by host cells. Modification of the viral surface protein did not affect either the production or the infectivity of N_3-MV through azido sugars incorporation. Moreover, subsequent chemical modification of the azide modified virus allows the facile conjugation of a variety of functionalities, such as fluorescence dye, targeting reagent, peptide, and polymer. The remarkable ease and specificity of the metabolic labeling approach make it accessible to other enveloped viruses that contain the peripheral glycoproteins (e.g., respiratory syncytial virus, mumps virus, and henipaviruses). In particular, as the paramyxoviruses can be readily targeted to enter cells through designated receptors,[38] they were considered an effective platform to develop the next generation of cancer therapeutics by targeting viral entry to cancer cells.[39] Thus, this metabolic labeling approach not only is useful in the conjugation of enveloped viruses, but also has potential in the attachment of specific receptors to human virus systems for potential cancer virotherapy.[7]

■ ASSOCIATED CONTENT

Supporting Information

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General considerations, supporting methods, and supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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