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AWARD REVIEW

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Functional design of glycan-conjugated molecules using a chemoenzymatic approach

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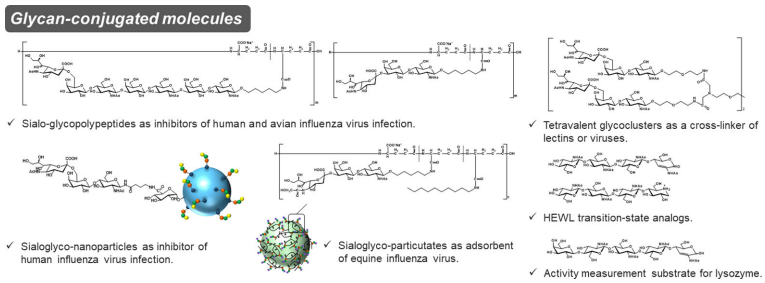
ABSTRACT

Carbohydrates play important and diverse roles in the fundamental processes of life. We have established a method for accurately and a large-scale synthesis of functional carbohydrates with diverse properties using a unique enzymatic method. Furthermore, various artificial glycan-conjugated molecules have been developed by adding these synthetic carbohydrates to macromolecules and to middle- and low-molecular-weight molecules with different properties. These glycan-conjugated molecules have biological activities comparable to or higher than those of natural compounds and present unique functions. In this review, several synthetic glycan-conjugated molecules are taken as examples to show design, synthesis, and function.

Graphical Abstract



Glycan-conjugated molecules synthesized using a chemoenzymatic approach.

Keywords: carbohydrate chemistry, chemoenzymatic, functional design, glycan-conjugated molecule, molecular recognition

Carbohydrates are the source of most organic compounds that exist on Earth and play a variety of roles in all living things (Varki 1993). These roles are diverse, including energy and metabolic intermediates, structural skeletons of nucleic acids, structures within cells, cell–cell recognition, quality control of glycoproteins, and participation in bacterial and viral infection processes (Varki 1993, 2007; Lis and Sharon 1998; Bishop, Schuksz and Esko 2007). The abundance of such properties is a result of the complexity of carbohydrate structures and their structural diversity enhanced by their ability to bind to molecules (proteins,

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lipids, etc) with a variety of properties. Currently, in the field of synthetic organic chemistry, attempts are being actively made to accurately reconstruct and utilize these diverse and complex natural carbohydrate molecules using the method for chemical or enzymatic synthesis (Yu and Chen 2016; Ando *et al.* 2017; Shoda 2017; Li and Wang 2018; Krasnova and Wong 2019). Furthermore, by simplifying or modeling natural, structurally complicated carbohydrate molecules, there are moves to create new carbohydrate materials that not only reproduce precise functions but also surpass natural products (Matsuura 2013; Cecioni, Imberty and Vidal 2015; Miura, Hoshino and Seto 2016).

Against this background, we have established a unique and practical enzyme-based method synthesizing functional sugar chains which utilizes a variety of recombinant glycosyltransferases expressed by the silkworm *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid system as biocatalysts (Ogata *et al.* 2009c, 2017a; Ogata, Usui and Park 2018). Furthermore, we have developed a highly versatile construction method for artificial glycan-conjugated molecules (glyco-polypeptides, glyco-nanoparticles, glycoclusters, etc) by introducing the synthesized sugar chains into molecules with different properties, and have demonstrated their usefulness as functional materials (Ogata *et al.* 2007, 2010d, 2016b; Masaka *et al.* 2010). We have also synthesized useful glycan-conjugated molecules by modifying/reconstructing natural chitin oligosaccharides by a chemoenzymatic method, and have shown that these are extremely effective as substrates for elucidating the hydrolysis reaction mechanism of hen-egg white lysozyme (HEWL) (Ogata *et al.* 2013, Ogata 2020). This review introduces the synthesis and utilization of glycan-conjugated molecules recently developed by our research group.

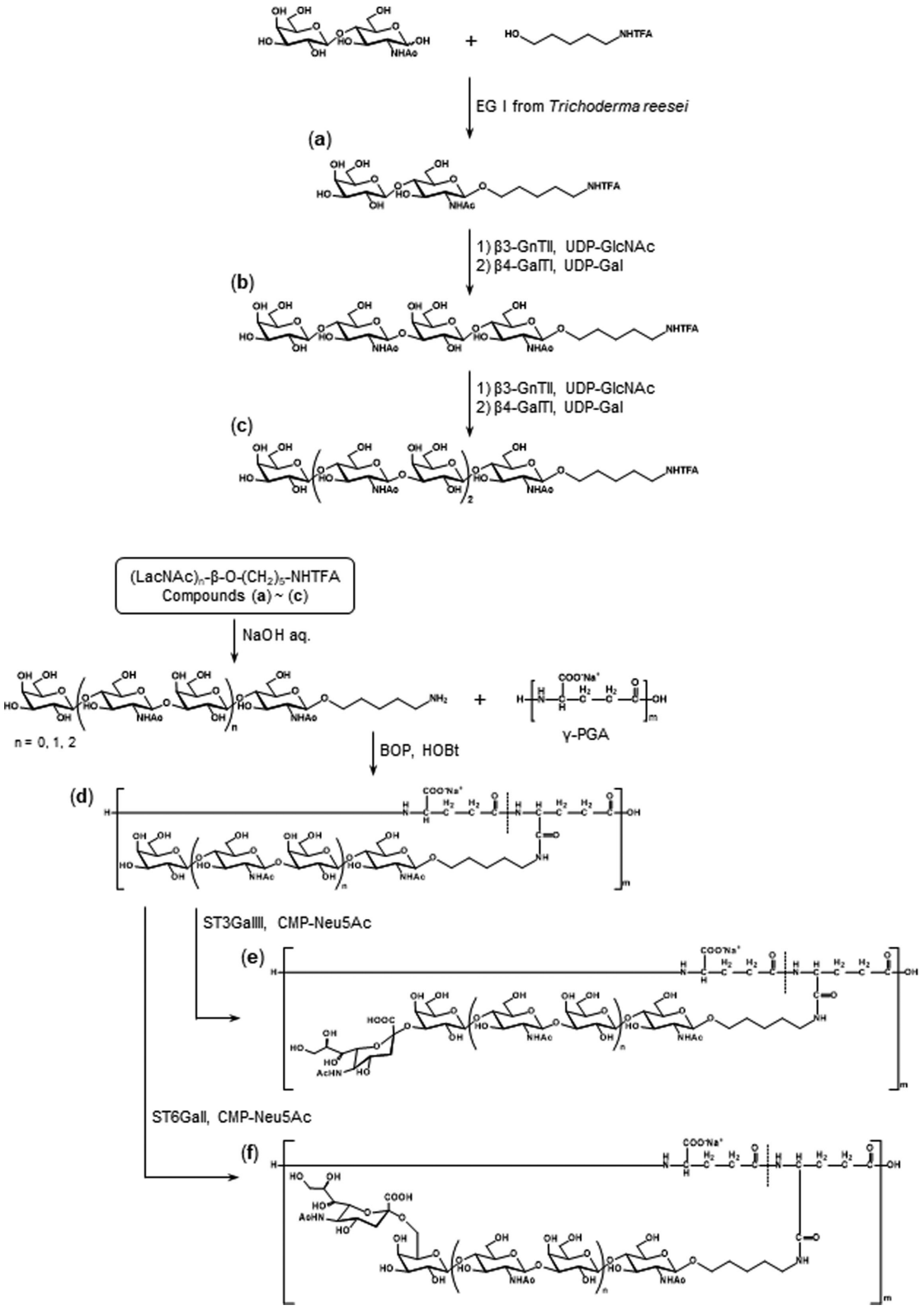
Synthesis of sialo-glycopolymers as inhibitors of influenza virus infection

Influenza virus (IFV) infection, which is a threat to humankind, begins when hemagglutinin (HA) on the surface of the IFV binds to sialo-glycans, such as glycoproteins, gangliosides, GPI anchors, and mucins, on the host cell membranes (Suzuki *et al.* 1986). Host mucins have highly glycosylated sugar chain structures and also function as barrier molecules against virus infection (Strous and Dekker 1992; McAuley *et al.* 2017). To date, many researchers have developed IFV infection inhibitors that mimic natural mucins by introducing multivalent sialoglycans into various polymer skeletons (synthetic polymers, biopolymers, dendrimers, etc) (Roy *et al.* 1992; Choi, Mammen and Whitesides 1997; Tsuchida *et al.* 1998; Gambaryan *et al.* 2005; Umemura *et al.* 2008; Suzuki *et al.* 2012; Tanaka *et al.* 2014).

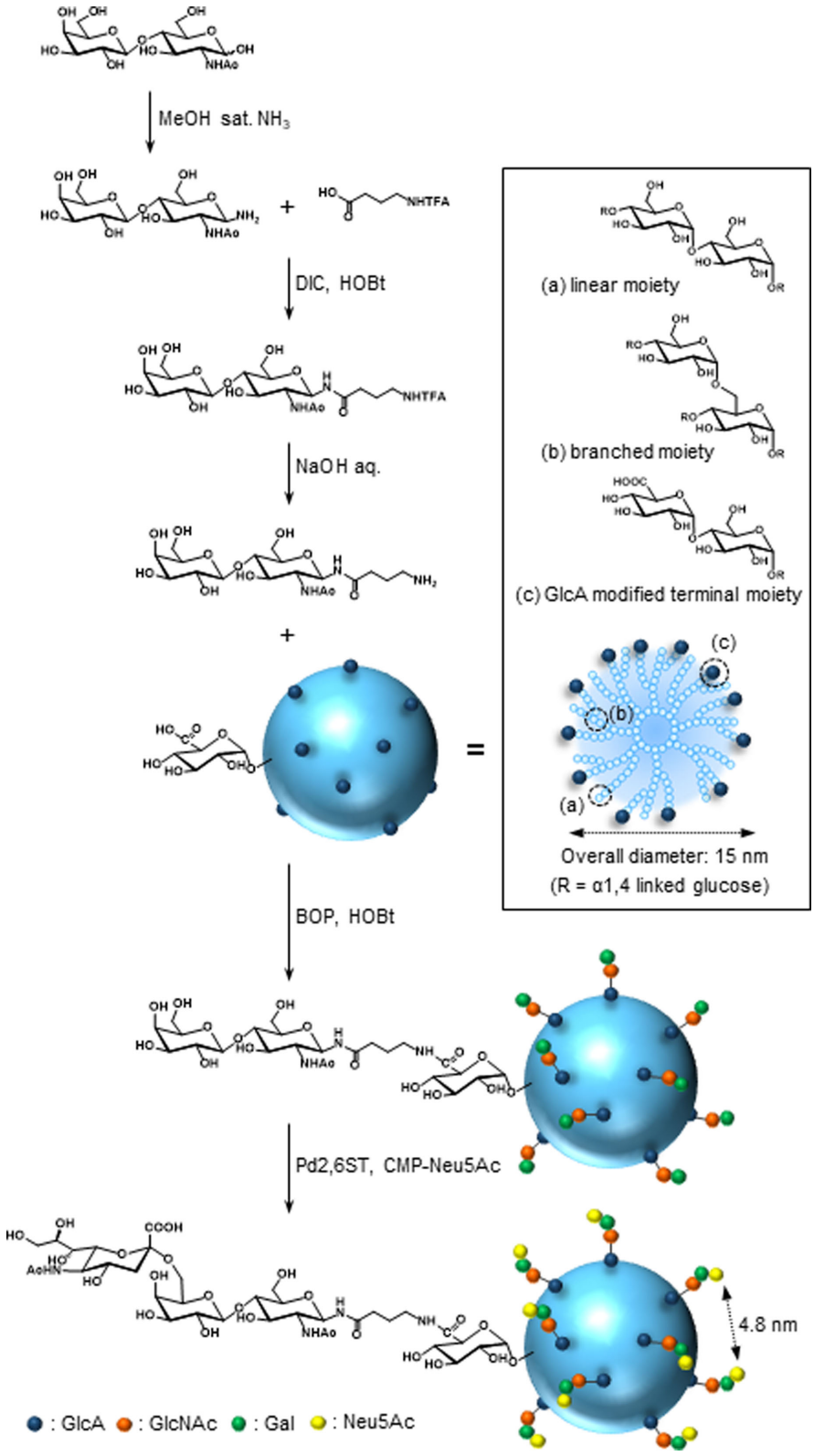
Our group focused on γ-polyglutamic acid (γ-PGA), which is a water-soluble natural polymer produced by *Bacillus subtilis* var. *natto*, and developed a glyco-polypeptide using this compound as a skeleton (Ogata *et al.* 2007, 2010c). Many of the conventional polymer skeletons used as IFV infection inhibitors have problems including cytotoxicity, but γ-PGA is a highly safe polymer skeleton which is used as a drug carrier (Wang *et al.* 2008). The synthesis of the artificial sialoglyco-polypeptides with γ-PGA as their skeleton is shown below. Initially, N-acetyllactosamine (LacNAc) and 5-(trifluoroacetamido)-1-pentanol were glycosylated in 1 step by utilizing the enzymatic condensation reaction of *endo*-β-1,4-glucanase I (EGI) contained in *Trichoderma reesei*–derived cellulase (Scheme 1a) (Ogata *et al.* 2007, 2010b). Subsequently, the synthesis of tetrasaccharide- and hexasaccharide-glycosides [$(LacNAc)_2$-glycoside and $(LacNAc)_3$-glycoside] was achieved by performing a sequential glycosylation reaction using 2 glycosyltransferases (β-1,3-*N*-acetylglucosaminyltransferase [β3GnTII] and β-1,4-galactosyltransferase [β4GalTI]) with the synthesized LacNAc-glycoside (Scheme 1b and c) (Ogata *et al.* 2009a, 2009b). After deprotecting the aglycone terminal of the different synthesized glycosides, the asialoglyco-polypeptides were easily prepared by connecting these to the γ-PGA side chains (Scheme 1d). In this method, the ratio of glycosides to γ-PGA can be easily adjusted by changing the reaction composition (Ogata *et al.* 2007). Finally, various sialoglyco-polypeptides with different sialic acid binding modes about linkages (Neu5Acα2,3Gal or Neu5Acα2,6Gal) and sugar chain lengths were constructed by incubating the synthesized asialoglyco-polypeptides with one of 2 recombinant sialyltransferases (α-2,3-sialyltransferase [ST3GalIII] and α-2,6-sialyltransferase [ST6GalI]) expressed by the silkworm-BmNPV bacmid system (Scheme 1e and f) (Ogata *et al.* 2009c, 2014, 2017a; Kato *et al.* 2012).

Subsequently, we succeeded in finding a sialoglycopolypeptide {poly[Neu5Acα2,6$(LacNAc)_3$-β-O$(CH_2)_5$NH-/γ-PGA]} capable of inhibiting human IFV (A/WSN/33 [H1N1] and A/Aichi/2/68 [H3N2]) cell infection at extremely low concentrations (picomolar level) in our synthesized compound library (Ogata *et al.* 2009b). The studies also showed that the sugar binding specificity of human IFV-HA depends on the linkage of the terminal sialic acid, and that the length of the sugar chain (human IFV: long sugar chain; avian IFV: short sugar chain) is also closely linked to inhibitory activity of IFV infection (Hidari *et al.* 2008; Ogata *et al.* 2009b). These results were in agreement with the results of sugar binding specificity analysis obtained by binding of IFV-HAs to glycan microarrays as reported by Chandrasekaran *et al.* 2008. Furthermore, we have shown that high inhibitory activity can be maintained even if part of the internal sugar chain length involved in the dramatic increase in human IFV infection inhibition is replaced with a linear alkyl chain (Ogata *et al.* 2009b). These results show that by using a chemoenzymatic method we have succeeded in developing a potent IFV infection inhibitor by synthesizing and simplifying the complex carbohydrates on the surface of host cells to which human IFV binds during infection.

In recent years, we have also reported on the chemoenzymatic synthesis of multivalent sialyllactosamine-carrying glyco-nanoparticles with a α-glucuronic acid-linked cyclic dextrin (GlcA-HBCD) backbone as a novel human IFV infection inhibitor (Scheme 2) (Ogata *et al.* 2016b). In this study, we adopted *N*-glycosylation, which is expected to have a high yield, for the purpose of efficient synthesis of LacNAc-glycoside. This *N*-glycosylation was carried out by a two-step procedure of conversion to an amino function of the anomer hydroxy group of LacNAc followed by coupling with a linker having a carboxy group. The linker moiety of the glycoside was introduced with the aim of reducing steric hindrance in binding to IFV-HA. The reaction proceeded stereoselectively within a few hours, yielding only β-glycosides in high yields (total yields 40%) without the need for protection and deprotection steps. The novel sialoglyco-nanoparticles prepared by enzymatic α2,6-sialylation after introduction of the resulting di-glycoside into GlcA-HBCD are noncytotoxic and have highly controlled particle size. In this study, it was proposed that the human IFV infection inhibitory ability of sialoglyco-nanoparticles is due to the proper arrangement between the sialo-glycans on the particle surface and the sialic acid binding sites of IFV-HA.



Scheme 1. Chemoenzymatic synthesis of sialoglyco-polypeptides as inhibitors of human and avian influenza virus infection.



Scheme 2. Chemoenzymatic synthesis of multivalent Neu5Acα2,6LacNAc-carrying glyco-nanoparticles with high affinity for the human influenza virus hemagglutinin.

Design, synthesis, and function of glycoclusters

In general, it is known that the one-to-one binding affinity between carbohydrates and proteins is extremely weak (millimolar level) (Mann *et al.* 1998; Dam *et al.* 2002). However, the "glycoside cluster effect" that dramatically increases these weak binding affinities by forming glycocluster structures on the cell surface is universally observed in the living body (Lee *et al.* 1983; Kiessling, Gestwicki and Strong 2006). This effect is used not only in the above-mentioned human IFV infection inhibitors but also for the development of various glyco-materials having molecular recognition ability (Mammen, Choi and Whitesides 1998; Dam and Brewer 2008; Fasting *et al.* 2012). Our research group has also reported on the design, synthesis, and function of various glycoclusters (Masaka *et al.* 2010; Ogata *et al.* 2010d, 2012b, 2016a, 2019, 2020; Endo *et al.* 2011; Kato *et al.* 2015). Here, we introduce the synthesis and utilization of 2 multivalent glycan-conjugated molecules developed by adding glycans to macromolecules and middle-molecular-weight molecules with applications other than as human IFV infection inhibitors.

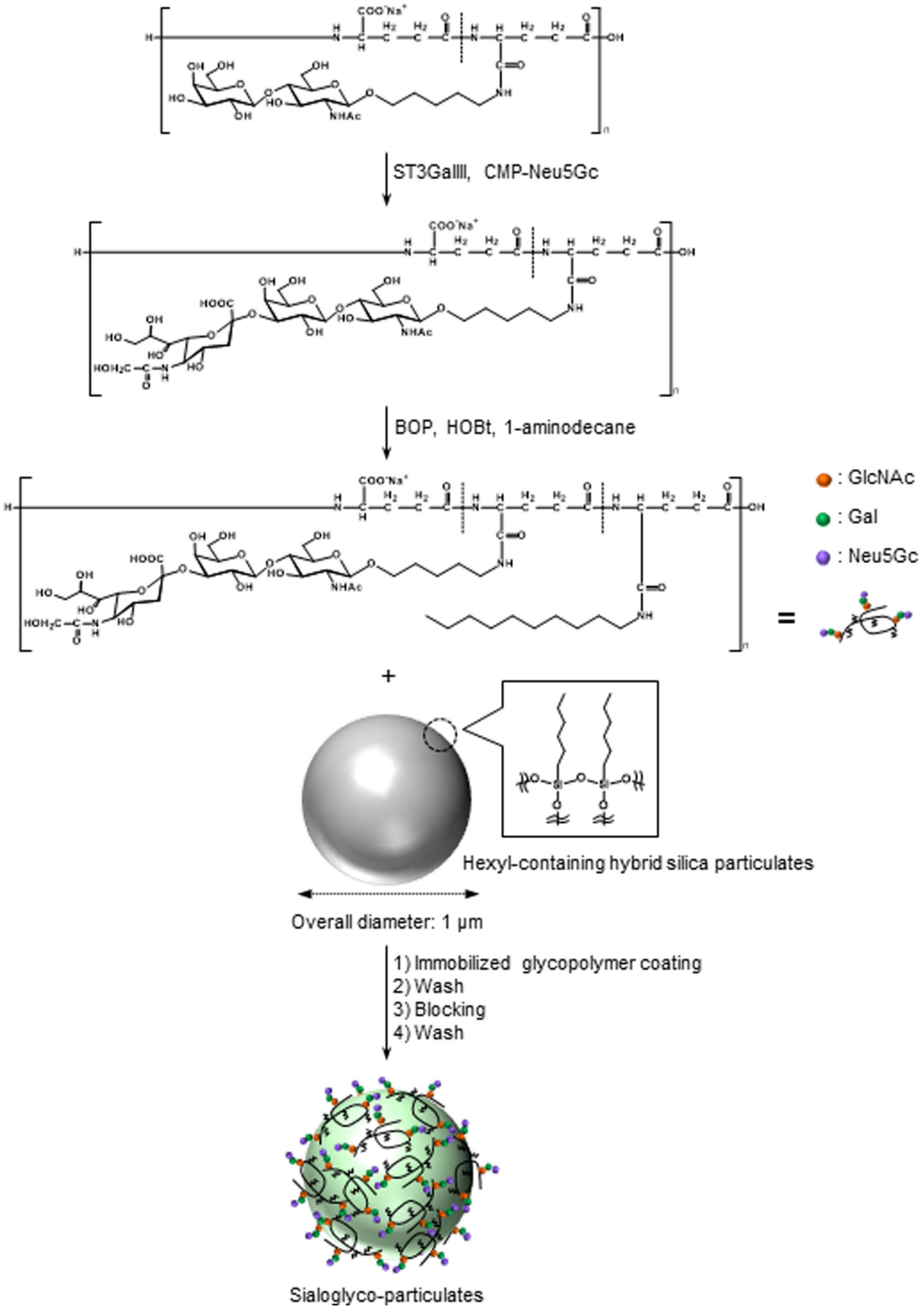
The first is the synthesis of sialoglyco-particulates and their use in the improvement of technology for equine influenza diagnosis. Equine influenza is a general term for acute respiratory diseases caused by equine influenza virus (EIV) infection (Wilson 1993; Cullinane and Newton 2013). It is characterized by its extremely strong infectivity and is the most alarming infectious disease in the horse-related industry. EIV is classified as a strain of the influenza A virus (Webster *et al.* 1992). Influenza A is classified into 18 subtypes of HA and 11 subtypes of neuraminidase (NA) according to the antigenic properties of HA and NA, which are spike-like proteins on the surface of the envelope. EIV subtypes are classified as H3N8 and H7N7, but there are no records of isolation of H7N7 virus since 1978 (Webster 1993). Therefore, in general, H3N8 is now the EIV subtype that causes epidemics among horse groups around the world. In Japan, a sudden outbreak of equine influenza occurred among racehorses in 2007, which had a great impact on related industries (Yamanaka *et al.* 2008). Therefore, a rapid, accurate and highly sensitive testing system for infected horses is important for the control of this infectious disease. For a definitive diagnosis of equine influenza, a method of detecting EIV in a small amount of sample collected from horse nasal discharge at the viral antigen or gene level is common. Typical methods are a lateral flow test for detecting an antigen which is both convenient and quick and a real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) method for detecting genetic material which has very high detection sensitivity. However, even with rRT-PCR, it is difficult to detect ultratrace EIV in a sample in the early stage of infection.

As mentioned for the human IFV infection inhibitors, influenza virus HA establishes infection by binding to the sialo-glycan receptor on the surface of the host cell. This infection process is also essential in horses, and it is known that HA of EIV exhibits binding affinity for a sialic acid called *N*-glycolylneuraminic acid (Neu5Gc), which does not exist in humans (Yamanaka *et al.* 2010). Therefore, we synthesized a sialoglyco-polypeptide in which a trisaccharide (Neu5Gcα2,3LacNAc) containing Neu5Gc, which is essential for adsorption of EIV to horse cells, was multivalently linked using a chemoenzymatic method (Scheme 3) (Ogata *et al.* 2017a). Furthermore, after hydrophobizing the synthesized sialoglyco-polypeptide, sialoglyco-particulates were synthesized by immobilizing the sialo-glycopeptides on the surface of hexyl-containing hybrid silica particulates with a diameter of 1 μm (Scheme 3) (Ogata *et al.* 2019). Subsequently, it was evaluated whether virus detection sensitivity could be significantly improved by selectively concentrating EIV in the specimen using the sialoglyco-particulates as a pretest treatment. Specifically, using nasal swabs obtained over time from 4 horses infected with EIV (A/equine/Malaysia/M201/2015), the amount of EIV genetic material was assayed before and after adsorption by sialoglyco-particulates using rRT-PCR. The results demonstrated that in all nasal swabs collected after EIV infection, there was a clear increase in the amount of EIV-specific gene after sialoglyco-particulates adsorption treatment (Ogata *et al.* 2019). In other words, by combining virus concentration technology using sialoglyco-particulates and rRT-PCR methodology we were successful in detecting small amounts of EIV, which were previously difficult to detect. We hope that this technology will be useful as an early detection method for highly contagious EIV and have a major impact on the horse industry.

Our second example is the synthesis of middle-molecular-weight glycoclusters with a well-defined structure in which the number of glycans is controlled. We have previously reported that the activity of glycoclusters can be enhanced even at low valences by proper arrangement of glycans (Masaka *et al.* 2010; Ogata *et al.* 2012b, 2016a, 2020). As a model compound, we produced chemoenzymatically synthesized tetravalent sialoglycoclusters in which the glycosides were bound to 4 carboxy groups of an ethylene glycol tetraacetic acid (EGTA) backbone, which is a metal chelating agent (Scheme 4a) (Ogata *et al.* 2012b, 2020). In the binding of the tetravalent sialo-glycocluster to *Sambucus sieboldiana* agglutinin (SSA) which has a polyvalent sugar binding site, a glycoside cluster effect and a multiply cross-linked complex were observed (Scheme 4b) (Ogata *et al.* 2012b). This interaction was a structure-specific cross-linking reaction similar to the antigen-antibody reaction. In recent years, we have evaluated whether the glycoside cluster effect and lectin cross-linking ability of tetravalent sialo-glycoclusters is observed with the pathogenic virus, human polyomavirus. As a result, it was demonstrated for the first time that tetravalent sialo-glycoclusters cause the formation of cross-linked complexes upon polyvalent binding to the sugar-binding protein (VP1) which is on the surface of polyomavirus (Scheme 4b) (Ogata *et al.* 2020). This series of results provided a chemically reliable example of a new detection, capture, and purification technique targeting morphological changes caused by sugar-binding properties between middle-molecular-weight glycoclusters and lectins or viruses.

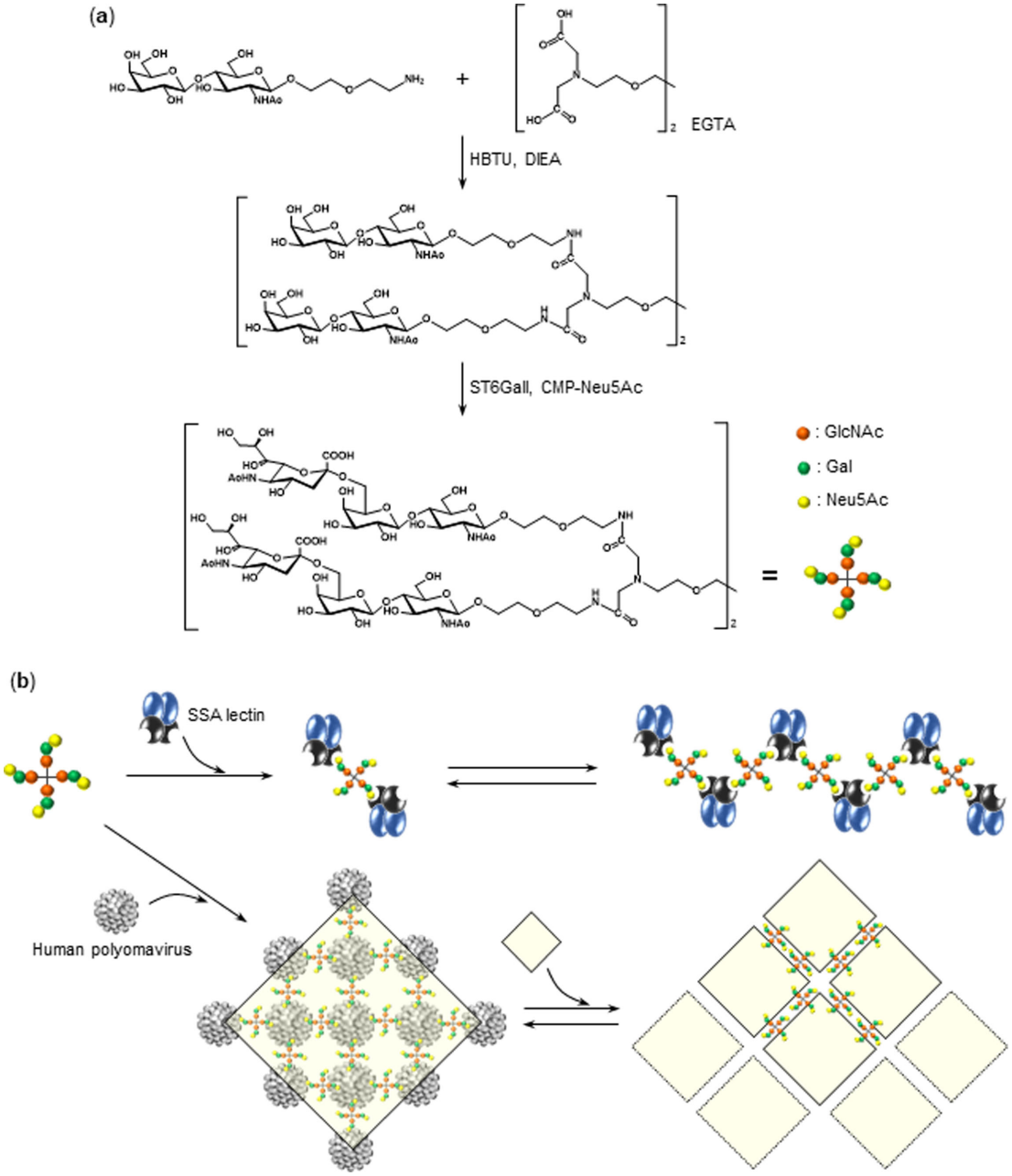
Molecular design of transition-state analogs and activity measurement substrates for lysozyme

Although the three-dimensional structure of HEWL has been determined by X-ray crystallography and as such was the first enzyme whose structure was known, its hydrolysis mechanism is still the subject of debate (Blake *et al.* 1962; Secemski, Lehrer and Lienhard 1972; Vocadlo *et al.* 2001). One of the most powerful methods for elucidating the reaction mechanism of such an enzyme is the use of a competitive inhibitor designed based on the three-dimensional structure of the enzyme and the reaction mechanism (reaction intermediate) (Lillelund *et al.* 2002; Ito *et al.* 2013). Therefore, as starting material, we designed and



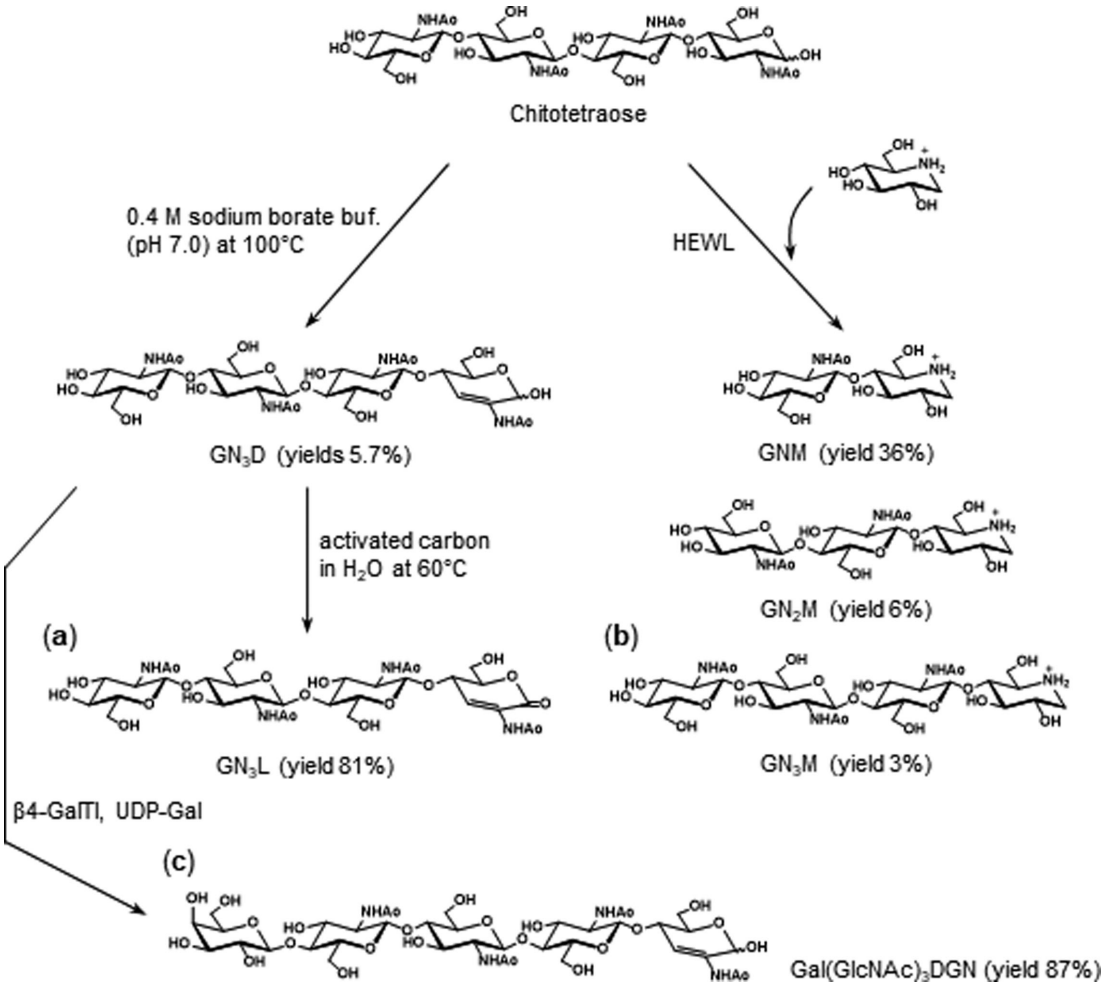
Scheme 3. Chemoenzymatic preparation of poly[Neu5Gcα2,3LacNAc-β-O$(CH_2)_5$NH-/$CH_3(CH_2)_9$NH-/γ-PGA]-immobilized sialoglyco-particulates as adsorbents of equine influenza viruses.

synthesized 2 types of HEWL transition-state analogs using chitin oligosaccharides, which are substrates of HEWL. The first type is a chitotetraose lactone "4-*O*-β-tri-*N*-acetylchitotriosyl-2-acetamido-2-deoxy-2,3-anhydro-glucono-δ-lactone (GN_3L)," which is modeled on the enzyme substrate complex formation mechanism proposed by Phillips (Scheme 5a) (Phillips 1966). GN_3L was synthesized by dehydration of the bond between C2 and C3 of the GlcNAc moiety at the reducing end of chitotetraose, followed by oxidation (Ogata *et al.* 2010a, 2012a). The terminal α,β-unsaturated δ lactone structure of GN_3L forms a stable half-chair conformation with a C1 displaying sp^2 hybridization. The second analog type is 4-*O*-β-tri-*N*-acetylchitotriosylmoranoline (GN_3M) with 1-deoxynojirimycin (moranoline) as the terminal structure, which is modeled on the



Scheme 4. (a) Chemoenzymatic synthesis of tetravalent Neu5Acα2,6LacNAc glycoside. (b) Schematics of agglutination of tetravalent sialo-glycoclusters/SSA lectin and tetravalent sialo-glycoclusters/human polyomavirus.

mechanism of Koshland (Scheme 5b) (Koshland 1953). GN_3M was enzymatically synthesized using the glycosyl-transfer reaction of lysozyme using chitotetraose as a donor and moranoline as an acceptor (Ogata *et al.* 2013). In contrast to GN_3L, the terminal moranoline group of GN_3M has a $^4C_1$ chair conformation with a C1 displaying sp^3 hybridization. Subsequently, the reaction mechanism of HEWL was re-examined using GN_3L and GN_3M. As a result, it was demonstrated that GN_3M not only acts as a potent competitive inhibitor, but also an X-ray cocrystal structure analysis with HEWL and GN_3M (PDB code: 4HP0) showed that the moranoline moiety located in the active center was a $^4C_1$ chair (Ogata *et al.* 2013). From these results, it was clarified that the moranoline moiety of GN_3M has strong affinity for the −1 subsite of HEWL. In fact, hydrogen bond formation was confirmed between the ring nitrogen atom of moranoline and multiple amino acid residues, including Asp52 in the active center (Ogata *et al.* 2013). So far, 2 main theories have been proposed for the formation of a complex between HEWL and a substrate, namely the oxocarbenium ion intermediate "Phillips mechanism" and the covalent bond intermediate "Koshland mechanism" (Koshland 1953; Phillips 1966). The result described here is an important finding as it used a Koshland-type transition-state analog that made it possible to analyze the X-ray cocrystal structure of the active center of natural HEWL for the first time. In recent years, these synthetic substrates have also been used as substrates for studying chitinolytic



Scheme 5. Chemoenzymatic syntheses of GN_3L and GN_3M as HEWL transition-state analogs and $Gal(GlcNAc)_3DGN$ as activity measurement substrate for lysozyme.

enzymes other than HEWL, and a variety of results have been obtained (Shinya *et al.* 2014; Leysen *et al.* 2015).

Taking inspiration from the HEWL transition-state analogs, we designed 2 substrates namely "4^4-O-β-D-galactosyl-β-tri-*N*-acetylchitotriosyl 2-acetamide-2,3-dideoxy-gluc-2-enopyranose [$Gal(GlcNAc)_3DGN$; Scheme 5c] and Galβ1,4GlcNAcβ1,4GlcNAc-β-*p*NP [Galβ1,4$(GlcNAc)_2$-β-*p*NP]" for assaying lysozyme activity by docking simulation. The characteristic of the chemical structure common to both substrates is that they are chitin oligosaccharide derivatives having a galactose residue at the nonreducing end. We have experimentally demonstrated that $Gal(GlcNAc)_3DGN$ undergoes limited hydrolysis by binding to −3 to +2 of the sugar-binding subsites in the cleft of HEWL while Galβ1,4$(GlcNAc)_2$-β-*p*NP binds from −3 to +1 (Ogata *et al.* 2017b; Matsui, Kono and Ogata 2018). These results indicate that the nonreducing end galactose residue of the chitin oligosaccharide derivatives preferentially binds to −3 of the sugar-binding subsites of HEWL. In other words, we newly demonstrated that neither substrates are subject to random hydrolysis by HEWL, even though they are oligosaccharide molecules having chitin oligosaccharides in their skeletons. As a result, by utilizing the hydrolysis properties of lysozyme with respect to synthetic substrates, it has become possible to dramatically simplify reaction kinetics analysis and activity evaluation, which have previously been difficult.

Conclusion

Our research group has consistently worked on the chemoenzymatic synthesis of glycan-conjugated molecules with the aim of developing functional materials for solving scientific problems. As a result, we have succeeded in creating several types of glycan-conjugated molecules introduced in this review. However, there are still many points that can be improved regarding the molecular design of these glycan-conjugated molecules. In the future, I would like to further develop research to advance the technology of carbohydrate synthesis and interdisciplinary research.

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Data availability

This article is an award review and does not contain any actual data, so it does not fall under this item.

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Disclosure statement

No potential conflict of interest was reported by the author.

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