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Identifying human milk glycans that inhibit norovirus binding using surface plasmon resonance

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Human milk glycans inhibit binding between norovirus and its host glycan receptor; such competitive inhibition by human milk glycans is associated with a reduced risk of infection. The relationship between the presence of specific structural motifs in the human milk glycan and its ability to inhibit binding by specific norovirus strains requires facile, accurate and miniaturized-binding assays. Toward this end, a high-throughput biosensor platform was developed based on surface plasmon resonance imaging (SPRi) of glycan microarrays. The SPRi was validated, and its utility was tested, by measuring binding specificities between defined human milk glycan epitopes and the capsids of two common norovirus strains, VA387 and Norwalk. Human milk oligosaccharide (HMOS)-based neoglycoconjugates, including chemically derived neoglycoproteins and oligosaccharide-glycine derivatives, were used to represent polyvalent glycoconjugates and monovalent oligosaccharides, respectively, in human milk. SPRi binding results established that the glycan motifs that bind norovirus capsids depend upon strain; VA387 capsid interacts with two neoglycoproteins, whereas Norwalk capsid binds to a different set of HMOS motifs in the form of both polyvalent neoglycoproteins and monovalent oligosaccharides. SPRi competitive binding assays further demonstrated that specific norovirus-binding glycans are able to inhibit norovirus capsid binding to their host receptors. A polyvalent neoglycoconjugate with clustered carbohydrate moieties is required for the inhibition of VA387 capsid binding to host receptor glycans, whereas both monovalent oligosaccharides and polyvalent neoglycoconjugates are able to inhibit Norwalk capsid binding to its host receptor. Binding of HMOS and HMOS-based neoglycoconjugates to norovirus capsids depends upon the specific strain characteristics, implying that HMOS and their polyvalent derivatives are potential anti-adhesive agents for norovirus prophylaxis.

Keywords: anti-adhesives / host–pathogen interactions / human milk glycans / norovirus / surface plasmon resonance

Introduction

Norovirus is a leading cause of acute gastroenteritis (Huang et al. 2003), affecting over 20 million people of all ages annually in the USA alone (Zhi et al. 2006). Due to the lack of specific treatment or vaccine prophylaxis, norovirus infection can lead to severe illness or death among infants, the elderly and immunocompromised patients (Tan and Jiang 2007, 2008; 2010). Recent studies have focused on the specificity of the initial binding that is the essential first step in norovirus infection (Tan et al. 2003; Zhang et al. 2006; Tan and Jiang 2008; Rydell et al. 2009). Norovirus capsid proteins adhere to human host glycosylated receptors via the histo-blood group antigens on the mucosa of the host gastrointestinal tract (Tan and Jiang 2007). The mucosal surface contains more than 100 oligosaccharide moieties bearing both Lewis (Le) and H(O)AB carbohydrate antigens (Robbe et al. 2004). The structures of these blood group antigens vary among individuals of different blood types and secretor status, and individual norovirus strains demonstrate a wide spectrum of binding specificities to their host receptors (Huang et al. 2005; Shirato et al. 2008; Rydell et al. 2009). The Norwalk strain recognizes A and O secretors, but not B secretors and non-secretors, whereas strain VA387 binds to all A, B and O secretors (Huang et al. 2005). Based on these previous findings, current efforts are focused on developing strategies to disrupt norovirus–blood group antigen interactions to prevent norovirus infection (Tan et al. 2008).

As histo-blood group antigens contain diverse carbohydrate structures, glycoconjugates bearing these antigens or their unbound oligosaccharide moieties have been synthesized and tested for their inhibitory activity against norovirus–host receptor interactions (Feng and Jiang 2007; Guiard et al. 2011; Rademacher et al. 2011). Chemical synthesis of diverse carbohydrate structures is arduous; therefore, synthesis of extensive libraries followed by their use for anti-norovirus high-throughput

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screening is a less appealing strategy than systematic screening of molecules from a natural mixture known to inhibit. Human milk glycans, specifically those fucosylated glycans that are structurally analogous to histo-blood group antigens, are potent natural anti-norovirus agents (Morrow et al. 2005) and are abundant in human milk. Glycosylated components in human milk can bind to norovirus capsids, blocking the adhesion of the norovirus capsids to the blood group antigens of saliva (Jiang et al. 2004; Ruvoen-Clouet et al. 2006; Huang et al. 2009). By fractionating human milk and saliva by molecular weight, Huang et al. further demonstrated that a glycosylated component of high molecular weight ($1.3–2.0 \times 10^6$ Da) can inhibit norovirus binding to histo-blood group antigens; deglycosylation of this fraction abrogated its ability to inhibit (Huang et al. 2009). These results suggest that both glycan composition and presentation on a high molecular weight carrier are prerequisites for norovirus-receptor binding inhibition, implicating the polyvalent nature of this interaction. However, the diversity of the human milk glycome complicates the isolation and identification of discrete glycan moieties that inhibit norovirus binding.

A high-throughput biosensing platform was developed in this study. A surface plasmon resonance imaging (SPRi) glycan microarray was validated and used to characterize the binding specificities of two norovirus strains to a defined set of human milk carbohydrates. SPRi is a label-free bioanalytical technique for interrogating biomolecular interactions at a metal/dielectric interface (e.g., gold/water; Kanda et al. 2004). SPRi can be used to study and quantify receptor–ligand binding, as well as inhibitory activity of candidate anti-adhesives (Mullett et al. 2000). A glycan microarray containing the milk glycans of interest was printed on a gold-coated SPRi sensor chip utilizing the divinylsulfone (DVS)-based immobilization strategy previously developed by our group (Cheng et al. 2011; Shang et al. 2012; Yu et al. 2012). The viral capsids of two common strains of norovirus (VA387 and Norwalk) were screened for their binding to the SPRi glycan microarray. Using direct binding assays, the human milk glycan binding specificity was determined for these two norovirus strains, and competitive binding assays characterized the glycans capable of inhibiting norovirus binding to its host receptor.

Results

Bioactivity of the immobilized glycans

Human milk contains diverse glycans with multiple carbohydrate structures, including free human milk oligosaccharide (HMOS) and glycoconjugates—glycopeptides, glycoproteins, glycolipids and glycosaminoglycans (Morrow et al. 2005). To identify discrete carbohydrate moieties capable of inhibiting norovirus, the main HMOS, containing select carbohydrate antigen analogs (H, Le^a, Le^x, Le^b, Le^y and Le^c), were tested (Table I). These carbohydrates were either conjugated to proteins [bovine serum albumin (BSA) or human serum albumin (HSA)] or modified with a glycine residue (-Gly derivatives), representing polyvalent or monovalent presentations of these HMOSs. Note that the BSA/HSA conjugates were synthesized via reductive amination, during which the reducing pyranose of the terminal monosaccharide residue is opened (Gray 1974). The Gly derivatives were modified at the reducing end to retain the pyranose ring form of the reducing sugar (Bejugam and Flitsch 2004; Likhosherstov et al. 2012). Therefore, differences in binding by these presentations could represent valency per molecule, or the retention of the natural form of the monosaccharide residue at the reducing end of the HMOS. DVS-based biofunctionalization was used to capture these neoglycoconjugates onto 11-mercaptoundecanol-modified gold chips (Cheng et al. 2011). To conserve precious glycans and enable simultaneous analyses, a microarray was printed onto an SPRi chip using a microarrayer (Supplementary data, Figure S1).

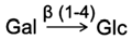
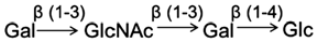
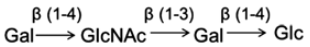
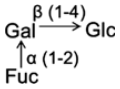
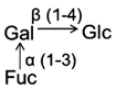
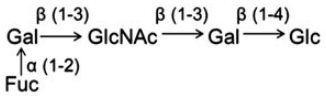
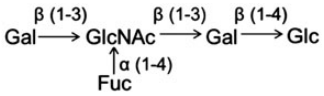
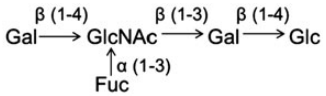
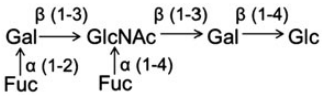
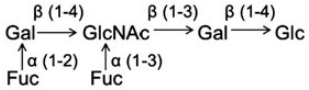
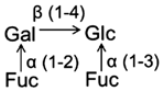
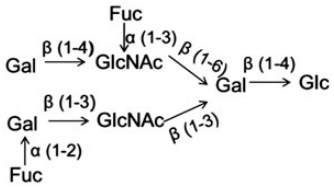
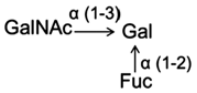
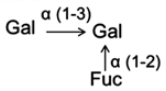
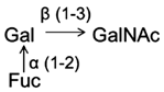
Two lectins, carbohydrate-binding proteins, were employed to evaluate the bioactivity of the immobilized glycans: *Lotus tetragonolobus* lectin (LTL) and *Ricinus communis* agglutinin I (RCA_{120}). LTL recognizes both α1,2- and α1,3-fucose (Fuc) that is linked to Galβ1-4Glc [H type 6, e.g. 2′-fucosyllactose (2′-FL)] or Galβ1-4GlcNAc [H type 2, e.g. lacto-*N*-fucopentaose III (LNFP III) and lacto-*N*-neodifucohexaose I (LNnDFH I); Pereira and Kabat 1974], whereas RCA_{120} binds to terminal β-galactose (Gal; Newton et al. 1992). When LTL passed over the glycoarray surface, the correct fucosylglycans bound (Figure 1A), validating that these glycans retained their binding activity on the SPRi surface. Conversely, three fucosyloligosaccharides, LNFP I, LNFP II and lacto-N-difucohexaose I (LNDFH I), as BSA or Gly derivatives, which do not normally bind LTL, did not exhibit strong specific LTL binding (Pereira and Kabat 1974); but LNFP II bound avidly to monoclonal antibody specific for Le^a, and LNDFH I bound to anti-Le^b antibody (Supplementary data, Figure S2). This indicates that fucosylated HMOS retained their binding specificity when immobilized to the SPRi surface. Due to the structural similarities between LNFP I and LNDFH I, the glycans containing LNFP I also demonstrated binding to the antibody specific for Le^b (LNDFH I) (Supplementary data, Figure S2B), consistent with previous reports that anti-Le^b antibody binds specifically to both LNDFH I and LNFP I (Huang et al. 2005). Thus, LNFP I-Gly and LNFP I-BSA also retained their bioactivities on the sensor surface.

For HMOS containing the core carbohydrate structures without Fuc decoration, RCA_{120} was used to assess their ability to bind when immobilized on the SPRi surface. Neoglycoconjugates containing terminal βGal [lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT) and lactose (Lac)] showed strong binding to RCA_{120} (Figure 1B), verifying the availability of these glycans on the surface. Weak binding by the fucosylated glycans to RCA_{120} (the curves under the sensorgram of LNT-Gly in Figure 1B and Supplementary data, Figure S3) can be attributed to low affinity interactions between RCA_{120} and the core Gal in some fucosyloligosaccharides (Wu et al. 2005; Itakura et al. 2007). These lectin- and antibody-binding results provided confidence in the capability of the glycan microarray chip to screen for the carbohydrate binding specificity of norovirus capsids.

Norovirus–glycan interactions

The norovirus-like particles used in this study are recombinant complexes of 24 protruding domains of norovirus capsid proteins, with a total molecular weight of ~850 kDa (Tan and Jiang 2008). The norovirus capsids are organized in octahedral symmetry and exhibit native virus receptor-binding specificity (Tan et al. 2008). Two strains of norovirus capsids, VA387 and Norwalk, were tested in this study. VA387 capsid binds to neoglycoproteins LNFP III-HSA and 2′-FL-BSA (Figure 2A), but

Table I. Structures of carbohydrate epitopes employed in this study

Name	Structure	Name	Structure
Lac (-BSA, -Gly)		LNT (-BSA,-Gly)	
H2 (-BSA)		LNnT (-Gly)	
2′-FL (-BSA, -Gly)		3′-FL (-Gly)	
LNFP I (-BSA, -Gly)		LNFP II (-Gly)	
LNFP III (-HSA, -Gly)		LNDFH I (-BSA, -Gly)	
LNnDFH I (-BSA)		LDFT (-Gly)	
DFLNHa (-Gly)		Blood group A antigen	
Blood group B antigen		Blood group H type 3 antigen	

Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; -BSA, carbohydrate-bovine serum albumin conjugates; -HSA, carbohydrate-human serum albumin conjugates; -Gly, carbohydrate-glycine derivatives.

not to the other neoglycoconjugates, including LNFP I-based structures. In addition, LNFP III-Gly and 2′-FL-Gly, which are the monovalent oligosaccharide counterparts to LNFP III-HSA and 2′-FL-BSA, did not bind to VA387 capsids. In contrast, Norwalk capsid binds to a different set of glycans (Figure 2B), including LNFP I and LNDFH I as both the neoglycoprotein and Gly conjugates, and several other carbohydrate-Gly derivatives [2′-FL-Gly, lactodifucotetraose (LDFT)-Gly and difucosyllacto-N-hexaose(a) (DFLNHa)-Gly]. These results demonstrate significant differences in glycan-binding specificities between VA387 and Norwalk and suggest the identity of potential norovirus-binding inhibitors. The dissociation shown in Figure 2 is very low, as illustrated by the minimal decrease in binding response after passing buffer over the norovirus particle-bound surface. This is consistent with previous reports of low affinity of monovalent carbohydrate–protein binding, but in polyvalent form, binding avidity dramatically increases (Sacchettini et al. 2001). The slow dissociation of norovirus–glycan binding could reflect high avidity or apparent affinity between glycans and norovirus particles.

Inhibitory activity of HMOS against norovirus–host receptor interactions

The glycans that bound most strongly to VA387 and Norwalk capsids were evaluated for their ability to inhibit norovirus capsid binding to host receptor glycans. Polymeric glycoconjugates containing blood group antigens were employed to provide a surface on which glycan epitopes are expected to be clustered. To facilitate glycan immobilization and simultaneous analysis, half of the SPRi chip was functionalized with the polymer-blood group antigen conjugates through biotin–streptavidin capture, whereas the other half of the sensor chip was functionalized with four neoglycoproteins (including LNFP III-HSA, 2′-FL-BSA, LNFP I-BSA and LNDFH I-BSA) that specifically bind to either VA387 or Norwalk.

As 2′-FL and LNFP I are the two most abundant fucosyloligosaccharides in human milk (Morrow et al. 2005) and they showed specific binding to VA387 and/or Norwalk capsids, we selectively investigated the activity of these two HMOSs (in the form of free oligosaccharides and/or neoglycoproteins) to

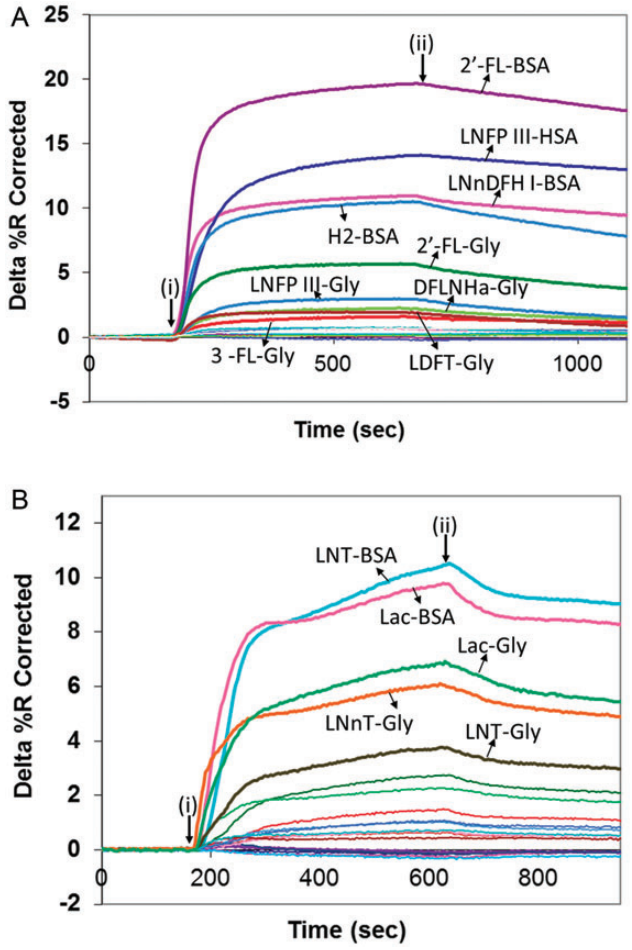


Fig. 1. SPR sensorgrams of LTL (**A**) and RCA_{120} (**B**) binding to the immobilized neoglycoconjugates. (i) Lectin solution introduced and (ii) buffer rinse.

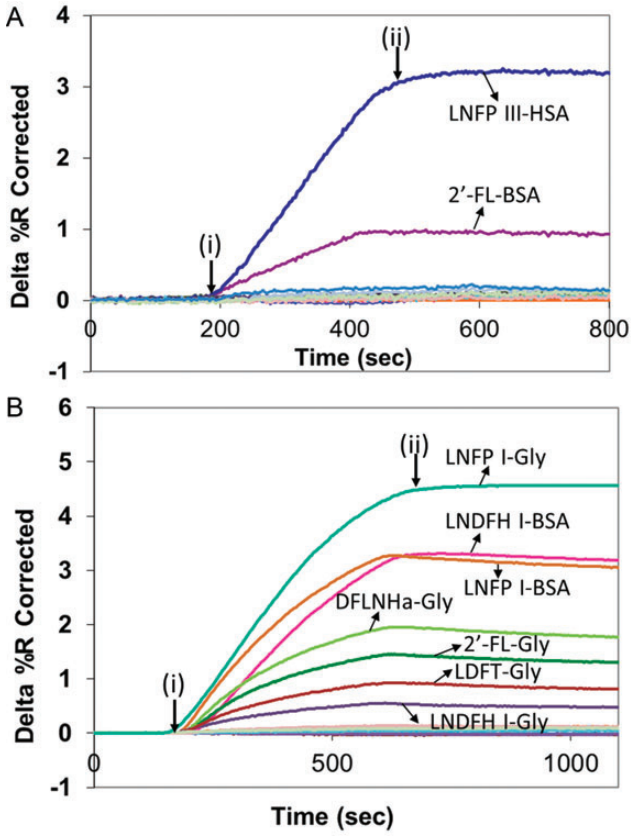


Fig. 2. SPR sensorgrams of norovirus VA387 (**A**) and Norwalk (**B**) capsid binding to the immobilized neoglycoconjugates. (i) Norovirus capsid introduced and (ii) buffer rinse.

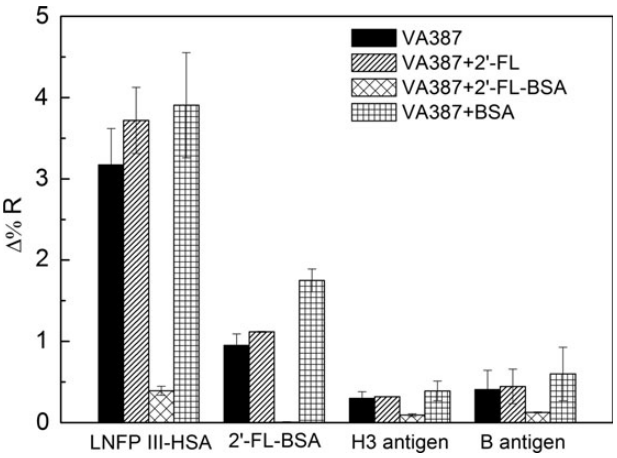


Fig. 3. SPR responses of immobilized glycans to VA387 capsid (10 μg/mL, ~12 nM), VA387 capsid (10 μg/mL) with 2′-FL (10 mM), VA387 (10 μg/mL) with 2′-FL-BSA (1 μM) and VA387 (10 μg/mL) with BSA (1 μM). Δ%R is the maximal SPR following 5 min of binding by solutions containing VA387 capsid alone or VA387 capsid mixed with glycan.

inhibit norovirus binding to blood group antigens. Using SPR competitive binding assays, we compared binding to the glycan-functionalized gold surface by norovirus capsids alone to the binding by norovirus capsids in the presence of milk glycans or neoglycoproteins. Binding of VA387 capsid to the immobilized LNFP III-HSA and 2′-FL-BSA and its host receptors [H type 3 (H3) and B antigens] was significantly reduced by 2′-FL-BSA, but not affected by free 2′-FL oligosaccharide or free BSA (Figure 3). In contrast, Norwalk capsid binding to LNFP I-BSA, LNDFH I-BSA neoglycoproteins, H3 and A antigen host receptors was almost entirely inhibited by both free 2′-FL and LNFP I-BSA, but not by BSA (Figure 4). Thus, the glycans that had bound to VA387 or Norwalk capsids most strongly were able to inhibit binding of VA387 and Norwalk norovirus capsids to their host receptors, consistent with competitive inhibition.

Discussion

Human milk glycan concentrations are inversely associated with infant risk of diarrheal diseases caused by a variety of pathogens including *Campylobacter*, stable toxin of *Escherichia coli* and norovirus (Morrow et al. 2005). These glycans competitively inhibit pathogens from binding to their host cell receptors (Morrow et al. 2005). For noroviruses, the

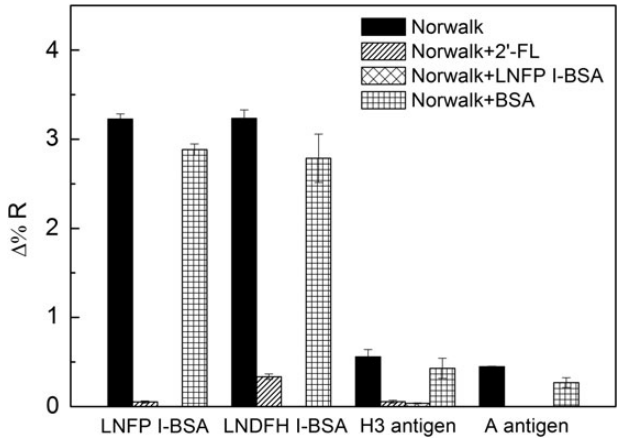


Fig. 4. SPR responses of the immobilized glycans to Norwalk capsid (10 μg/mL, ~12 nM), Norwalk capsid (10 μg/mL) with 2′-FL (10 mM), Norwalk capsid (10 μg/mL) with LNFP I-BSA (1 μM) and Norwalk capsid (10 μg/mL) with BSA (1 μM). Δ%R is the maximal SPR response following 5 min of binding by the solutions containing Norwalk capsid alone or Norwalk capsid mixed with glycan.

structures of these anti-infective glycans have not been fully elucidated. In this study, HMOS binding to two common norovirus strains (VA387 and Norwalk) was measured. This group covers the main carbohydrate antigens of human milk, including core structures (LNT, LNnT and Lac), monofucosylated HMOS [LNFP I, LNFP II, LNFP III, 2′-FL and 3-fucosyllactose (3-FL)], difucosylated HMOS (LNDFH I, LNnDFH I, LDFT) and a more complex biantennary difucosylated structure (DFLNHa; Newburg et al. 2005). Some of the HMOSs were tested as both polyvalent neoglycoproteins and monovalent oligosaccharides. These two glycan valencies coexist in human milk, and their relative binding characteristics are critical to defining the role of glycan presentation in norovirus binding and in inhibition of norovirus binding.

The results in this study suggest that the glycans that bind to VA387 capsid must be fucosylated, as the non-fucosylated glycans (e.g., LNT-BSA, Lac-BSA) did not bind to VA387 capsid. Among the fucosylated glycans, VA387 capsid recognizes glycans with the type 2/6 core structure of Galβ1-4GlcNAc (e.g., LNFP III-HSA) and Galβ1-4Glc (e.g., 2′-FL-BSA), but not with the type 1 core structure of Galβ1-3GlcNAc (e.g., LNFP I-BSA or LNDFH I-BSA). Furthermore, 2′-FL and LNFP III must be in the form of neoglycoproteins for VA387 capsid binding, as VA387 capsid did not bind to the 2′-FL-Gly and LNFP III-Gly conjugates. This finding is consistent with previous reports from Huang et al. (2009), in which only the high molecular weight glycoconjugates in human milk can inhibit VA387–receptor binding.

The strong binding of VA387 capsid to neoglycoproteins rather than oligosaccharides could be attributed to the higher carbohydrate surface density (Huang et al. 2003; Marionneau et al. 2005). The lectin- and antibody-binding data provide a relative binding response for different glycans, but they cannot be used to determine glycan surface densities due to the complex factors involved in a binding response, including the saccharide structure, the carrier and the surface density. Therefore, biophysical and chemical analysis was used to estimate glycan densities. Oligosaccharide monovalent derivative was estimated to be 0.15 molecule/nm^2 by X-ray photoelectron spectroscopy from a previous study (Cheng et al. 2011). Neoglycoconjugate densities are more difficult to quantify, as they depend on multiple factors, including the number of carbohydrate moieties per macromolecule, conformation of the protein on the surface and the contact area of proteins with the surface. Using mass spectrometry, the number of carbohydrate moieties per BSA molecule was measured to be ~37/protein. Previous studies reported that the globular BSA molecules have a dimension of 14 × 4 × 4 nm and are adsorbed onto hydrophilic surfaces sidelong (Su et al. 1998). Assuming that the BSA conjugates are attached to the DVS-activated SPR surface with a projection area of 14 × 4 nm^2, and at least half of the carbohydrate moieties are exposed, the surface density of carbohydrates for neoglycoproteins is at least 0.33 molecule/nm^2. This is significantly higher than the density of oligosaccharide–Gly derivatives and likely underestimates the surface density. Furthermore, the neoglycoconjugates confer three-dimensional flexibility to the carbohydrates, potentially allowing "induced fit" binding pockets to be formed around ligands; whereas the oligosaccharide–Gly derivatives are fixed on a planar surface modified with well-packed self-assembled monolayers with more rigid structures, limiting their ability for multivalent interactions with spherical norovirus particles (Mendoza et al. 2007). Therefore, we postulate that neoglycoproteins interact with VA387 capsid in a multivalent or polyvalent manner, resulting in higher binding strength than their monovalent oligosaccharide counterparts.

The binding specificity of VA387 capsid differs between this study and previous reports with regard to binding strength and other characteristics. Huang et al. (2005) demonstrated little interaction between VA387 capsid and Le^x antigen (LNFP III) using enzyme immunoassay (Tan and Jiang 2007), whereas Han et al. (2013) detected weak binding of VA387 capsid protein dimer to Le^x oligosaccharide by electrospray ionization mass spectrometry. SPRi indicates strong binding of VA387 capsid to Le^x neoglycoproteins. These differences in binding could reflect different binding surface density or the preservation of the terminal glucose ring structure.

In contrast to VA387 capsid, Norwalk capsid bound strongly to the H type 1 fucosyloligosaccharides, LNFP I and LNDFH I (Le^b), both in the form of monovalent glycyl-oligosaccharides and polyvalent neoglycoproteins, consistent with previous reports of binding specificity (Marionneau et al. 2002; Rydell et al. 2009). Norwalk capsid also recognized the fucosyloligosacchardes, 2′-FL and LDFT, demonstrating an affinity to Fucα1-2Gal in both H type 1 and H type 6 moieties. Thus, Norwalk possesses a broader glycan-binding specificity than VA387, and it does not require polyvalent glycan presentation for carbohydrate binding. Interestingly, the SPR response of the neoglycoproteins to Norwalk capsid was not necessarily higher than the response of glycyloligosaccharides. For instance, although LNDFH I-BSA showed a higher binding to Norwalk capsid than its oligosaccharide counterpart, LNFP I-BSA had a lower response than LNFP I-Gly (Figure 2B). This suggests that the surface density of glycans may not be the primary factor that determines the binding strength of Norwalk capsid

to neoglycoconjugates. Other factors, such as the affinity of Norwalk capsid to monovalent oligosaccharides, could contribute to the overall binding strength. In addition, Norwalk capsid binds to 2′-FL-Gly but not 2′-FL-BSA, which is the opposite of VA387 capsid. This could be attributed to the synthetic differences between the carbohydrate moieties on 2′-FL-BSA and 2′-FL-Gly. As was discussed in *Results*, the pyranose ring form of the glucose on 2′-FL-BSA is opened during reductive amination, while the glucose ring structure on 2′-FL-Gly retains its ring structure. Therefore, 2′-FL-BSA contains only an intact disaccharide (Fucα1-2Gal), whereas 2′-FL-Gly has the full trisaccharide 2′-FL moiety. Thus, Norwalk capsid may have strongest affinity for the trisaccharides Fucα1-2Galβ1-4Glc and Fucα1-2Galβ1-3GlcNAc.

To evaluate the inhibitory effect of the norovirus-binding glycans on norovirus–host receptor interactions, we employed polyvalent forms of blood group antigens to better approximate the presentation of carbohydrate receptors on the cell surface. The neoglycoprotein 2′-FL-BSA conjugate inhibited binding of VA387 capsid to its putative host receptors, blood group B and H3 antigens and to the two known VA387 ligands, 2′-FL-BSA and LNFP III-HSA. Monovalent 2′-FL, even with a 100–1000-fold molar excess (the molar ratio of 2′-FL to VA387 is $\sim 8.3 \times 10^5$), cannot inhibit receptor binding. Blood group A antigen has been reported as a VA387 receptor (Huang et al. 2005; Cao et al. 2007), but did not exhibit a specific response to the VA387 capsid in our experiments. This may be due to the low surface density of A antigen immobilized on the SPR chip. The combination of inhibition results and direct binding data confirm that a glycoconjugate presenting multiple carbohydrate moieties is required for VA387 binding and inhibition. For the Norwalk strain, the glycan inhibition profile is quite different from that of VA387. Both free 2′-FL and LNFP I-BSA blocked the binding of Norwalk to its host receptors (blood group A and H3 antigens) and two Norwalk-binding glycans (LNFP I-BSA and LNDFH I-BSA), suggesting that polyvalency is not critical for its binding and inhibition. Differences in binding inhibitor activity between the two strains probably reflect the differences in the capsid protein structures of these two norovirus strains (Bu et al. 2008). These results lay the groundwork for defining inhibitory potency of various norovirus-binding glycans to select effective inhibitors of norovirus binding to host receptors. Quantitative in vitro and in vivo studies could further define the anti-adhesive capacity of the norovirus-binding glycans. The quantity of glycans needed to reduce the incidence of norovirus infection in vivo is 300–400 mg/L (Morrow et al. 2005; Newburg et al. 2005). High quantities of human milk glycans for large-scale norovirus infection studies are not currently available, as human milk is their only source. However, genetically modified microbes are now being developed for scalable production of HMOS, which will obviate the need for larger quantities of high purity material needed for kinetic studies, and provide a starting point for synthesis of an assortment of neoglycoproteins.

In conclusion, the SPRi glycoarray platform developed in this study can identify HMOS structures that bind to norovirus VA387 capsid and/or Norwalk capsid. This platform provided an efficient and multiplexing biophysical method for characterizing the binding and inhibitory effects of HMOS on norovirus binding to host receptor glycan moieties. The glycan structures required for inhibiting VA387 capsid or Norwalk capsid binding to host receptors are unique to each strain. For the VA387 capsid, a neoglycoprotein with multiple pendant carbohydrate moieties is necessary to achieve strong binding and inhibition, whereas for Norwalk capsid, both free oligosaccharides and neoglycoproteins demonstrate binding and inhibition. These results suggest that carbohydrate moieties and their presentation on a macromolecular carrier must be considered when designing glycan-based agents to inhibit norovirus–host receptor interactions. The anti-adhesive glycans identified in this study and the technology developed to measure their binding could significantly advance the search for norovirus-binding glycans that may protect against norovirus infection.

Materials and methods

Reagents and materials

All chemical reagents were from Sigma-Aldrich (St Louis, MO) and were used without further purification. LTL and RCA_{120} were from Vector Laboratories (Burlingame, CA). All neoglycoproteins were prepared based on the procedure described in the literature (Gray 1974): LNFP III-HSA, H disaccharide (H2)-BSA, LNT-BSA, Lac-BSA, LNFP I-BSA, LNnDFH I-BSA, LNDFH I-BSA and 2′-FL-BSA. Gly-oligosaccharide conjugates (2′-FL-Gly), 3-FL-Gly, LNT-Gly, LNnT-Gly, Lac-Gly, LNFP I-Gly, LNFP III-Gly, LNDFH I-Gly, LNFP II-Gly, LDFT-Gly and DFLNHa-Gly were synthesized according to the procedure reported in the paper (Likhosherstov et al. 2012). Free 2′-FL was synthesized by Glycosyn, Inc. (Medford, MA). Virus-like particles of norovirus strains (VA387 and Norwalk) were prepared based on the procedure described previously (Tan et al. 2008). Biotin-polyacryamide (PAA)-blood group antigen [A(tri), B (tri) and H type 3(H3-tri)] conjugates were from Glycotech (Rockville, MD). Monocolonal antibodies specific for Lewis antigens (Le^a and Le^b) were from Gamma Biologicals, Inc. (Houston, TX).

Functionalization of SPR sensor with human milk glycans

DVS chemistry. Fresh SPR gold chips were immersed in 11-mercaptoundecanol (0.1 mM in ethanol) overnight at room temperature to form a hydroxyl-terminated self-assembled monolayer on the surface. The modified gold chips were rinsed with ethanol and dried under a stream of nitrogen. The chips were then immersed in 10% DVS (v/v, 0.5 M carbonate buffer, pH 11) solution for 1 h at ambient temperature. The DVS-activated chips were thoroughly rinsed with ~10 mL water and dried by a stream of nitrogen. HMOS neoglycoproteins (1 mg/ml) and Gly conjugates (~5 mM) were dissolved in carbonate buffer (10 mM, pH 10) and printed on the DVS-activated SPR chip using a manual microarrayer (V&P Scientific, Inc., CA). The printed chip was incubated in a 75% relative humidity chamber overnight. After incubation, the chip was immersed in 1 mg/mL BSA solution (10 mM carbonate buffer, pH 8.5) for 1 h to passivate the surface, then rinsed with water and dried under a stream of nitrogen. A

schematic microarray printing map and real binding images were shown in Supplementary data, Figure S1.

Biotin-streptavidin binding method. To immobilize biotin-PAA-blood group antigens on the SPR surface, half of an SPR gold chip was immersed in streptavidin [0.5 mg/mL, phosphate-buffered saline (PBS), 10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4] solution and incubated for 2 h. Subsequently, the chip was rinsed with water and dried with a stream of nitrogen. A(tri)-, B(tri)- and H3(tri)-saccharide-PAA biotin (1 mg/mL, PBS) were printed on the streptavidin-functionalized chip surface. The other half of the unmodified gold chip was printed with neoglycoproteins (1 mg/mL, PBS). After overnight incubation, the chip was washed with BSA (1 mg/mL, PBS) and incubated in the BSA solution for 1 h. Finally, the chip was rinsed with water and dried with nitrogen.

Validating the bioactivity of immobilized glycans by SPR

Glycan–protein binding studies were performed on a SPRimagerII (GWC Technologies, Madison, WI, USA). The SPRimagerII was operated at room temperature using a standard flow cell and a peristaltic pump (BioRad-EconoPump, Hercules, CA, USA) at 100 µL/min. Fuc-binding lectin LTL was dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM $CaCl_2$, pH 7.5) at 2 µM, and Gal-binding lectin RCA_{120} was in PBS buffer at 500 nM. Monocolonal antibodies specific for Le antigens (Le^a and Le^b) in PBS buffer were used at a dilution of 1:100. In a typical binding experiment, a buffer solution is flowed over the glycan-functionalized surface for 10 min to stabilize the baseline signal, followed by passing a protein solution over the surface for 5–8 min. Buffer is flowed over the surface again for dissociation for another 10 min. 8 M urea was used to regenerate protein-bound surfaces. All binding curves were normalized by subtracting the background signal, represented by the area modified with BSA.

SPR detection of interactions between human milk glycan and norovirus capsid particles

After verifying the bioactivity of the immobilized glycans, norovirus capsid particles from strains VA387 and Norwalk were passed over the sensor surface to test their binding to the immobilized glycans. The norovirus capsid particles were dissolved in PBS pH 7.4 at 10 µg/mL, unless otherwise specified. The SPR procedure for norovirus capsid binding is the same as that for protein binding. All binding curves were normalized by subtracting the background signal, represented by the area modified with BSA.

Inhibition of norovirus capsid binding to host receptors by human milk glycans

2′-FL-BSA (1 µM), BSA (1 µM) and free 2′-FL oligosaccharide (10 mM) were mixed with VA387 virus capsid particles (10 µg/mL). LNFP I-BSA (1 µM), BSA (1 µM) and free 2′-FL oligosaccharide (10 mM) were mixed with Norwalk virus capsid particles (10 µg/mL). The norovirus/glycan or norovirus/BSA mixtures were preincubated for 30 min at room temperature before being passed over the SPR chip functionalized with norovirus–host receptors and norovirus-binding neoglycoproteins. Binding by the mixed solutions to the immobilized glycans was compared with binding assays without soluble inhibitor.

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

Prof. David S. Newburg is a shareholder in Glycosyn, LLC, which synthesizes individual HMOS. This potential conflict is managed by Boston College.

Abbreviations

BSA, bovine serum albumin; DFLNHa, difucosyllacto-*N*-hexaose(a); DVS, divinylsulfone; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; Fuc, fucose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gly, glycine; H2, H disaccharide; H3, H type 3; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMOS, human milk oligosaccharide; HSA, human serum albumin; Lac, lactose; LDFT, lactodifucotetraose; Le, Lewis antigen; LNDFH I, lacto-*N*-difucohexaose I; LNFP III, lacto-*N*-fucopentaose III; LNnDFH I, lacto-*N*-neodifucohexaose I; LNnT, lacto-N-neotetraose; LNT, lacto-*N*-tetraose; LTL, *Lotus tetragonolobus* lectin; PBS, phosphate-buffered saline; PAA, polyacryamide; RCA_{120}, *Ricinus communis* agglutinin I; SPRi, surface plasmon resonance imaging.

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