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# Initial Step of Virus Entry: Virion Binding to Cell-Surface Glycans

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cell-surface glycans, attachment factors, atomic force microscopy, virus entry, virus-glycan complexes, single-virion level

## Abstract

Virus infection is an intricate process that requires the concerted action of both viral and host cell components. Entry of viruses into cells is initiated by interactions between viral proteins and cell-surface receptors. Various cell-surface glycans function as initial, usually low-affinity attachment factors, providing a first anchor of the virus to the cell surface, and further facilitate high-affinity binding to virus-specific cell-surface receptors, while other glycans function as specific entry receptors themselves. It is now possible to rapidly identify specific glycan receptors using different techniques, define atomic-level structures of virus-glycan complexes, and study these interactions at the single-virion level. This review provides a detailed overview of the role of glycans in viral infection and highlights experimental approaches to study virus-glycan binding along with specific examples. In particular, we highlight the development of the atomic force microscope to investigate interactions with glycans at the single-virion level directly on living mammalian cells, which offers new perspectives to better understand virus-glycan interactions in physiologically relevant conditions.

## INTRODUCTION

**Glycan:** carbohydrate structure made of mono- or polysaccharides that can be free or covalently linked to a core molecule

**Attachment factor:** cell-surface structure that allows virus attachment and promotes binding to specific entry receptors

**Entry receptor:** cell membrane structure that triggers virus entry inside permissive cells upon virus binding

Viruses are obligate intracellular parasites. Therefore, viruses must bind to and enter permissive host cells in order to hijack their host cellular machinery. Viruses can be seen as passive and metabolically inactive entities with limited available strategies to cross the plasma membrane of their target cells. However, mammalian cells are dynamic and provide various structures and processes allowing the uptake of macromolecular assemblies. Throughout evolution, viruses developed various strategies to take advantage of numerous cellular functions (e.g., genome replication, transcription) and uptake mechanisms such as endocytosis (1). Cellular membranes consist of complex assemblies of three of the four building blocks of life: proteins, carbohydrates, and lipids. While carbohydrates (or glycans) have so far been the most understudied, the rise of glycomics has highlighted their complexity and their faceted roles in physiology. They play a crucial role in many fundamental cellular processes including signal transduction, extracellular matrix formation, protein folding, cellular identity, and host-pathogen interactions.

Cellular glycans play a crucial role in virus infection. In this review, we describe the role of glycans during the initial step of virus-cell binding. We discuss different techniques available to study virus-glycan binding as well as recent advances to understand the function of glycan engagement during virus infection of living cells.

## THE ROLE OF GLYCANS IN VIRAL INFECTION

To replicate and avoid immune recognition within the extracellular medium, viral particles must enter susceptible cells. Furthermore, conditions within the extracellular medium can be hostile for certain virus types. Therefore, to limit free diffusion in an unfavorable environment, viral particles should initiate entry into cells relatively rapidly (2). To do so, the most efficient strategy is to bind the first cellular structure encountered while approaching the plasma membrane: the glycocalyx. This dense, 50- to 200-nm thick layer of carbohydrates coats the surface of almost every mammalian cell and plays both structural and functional roles to ensure physical integrity, cellular signaling, and cell-to-cell communication (3). The glycocalyx is composed of various sugars linked to lipids or proteins, such as glycoproteins, glycolipids, and proteoglycans (4). Binding to sugar moieties is of critical importance to gain access to target cells covered by carbohydrates (e.g., within the mucus). Using cell-surface oligosaccharides as initial attachment factors enhances viral infectivity (5), mainly by concentrating viral particles on the cell surface and facilitating the subsequent binding of specific virus receptors for internalization (6). In addition to that, some cell-surface carbohydrates also can be used by viruses directly as entry receptors (7). The distinction between attachment factors and entry receptors is however not as clear in practice. As knowledge about virus entry increases, it becomes evident that attachment to cell surfaces is more complex than initially thought. Functions of attachment, internalization, and uncoating are mediated by several host factors, which can be cellular glycans or proteins depending on the cell type. Therefore, the definitions of attachment factors and entry receptors can somehow overlap, as both functions can be mediated by similar glycan or protein moieties.

Glycans are abundant on cell surfaces, where they are covalently associated with proteins and lipids, which confers additional structural and functional features to these molecules (6). Glycans found on the surface of animal cells display a wide diversity of constituents and structures (8). Glycoconjugates can therefore be classified based on their core structure, the type of macromolecule they are attached to, and the type of linkage to which they attach. Glycoconjugates consisting of cell-surface proteins with covalently attached oligosaccharides are called glycoproteins. The type of linkage by which the proteins are glycosylated allows discriminating *N*- and *O*-glycans,

depending on whether the glycans are linked to amino acids through nitrogen (9) or oxygen atoms (10). The second type of branched glycoconjugate is composed of oligosaccharides attached to lipid molecules. Two main classes of glycolipids can be distinguished: the glycosphingolipids (8, 11) and the glycophospholipid anchors or glycosylphosphatidylinositol (GPI) anchors (8). Finally, a third type of glycoconjugate molecule exists, consisting of linear chains of sugars attached to a core protein. These structures are called proteoglycans, in which the glycan moieties are long chains of repeating disaccharide units called glycosaminoglycans (GAGs) (11).

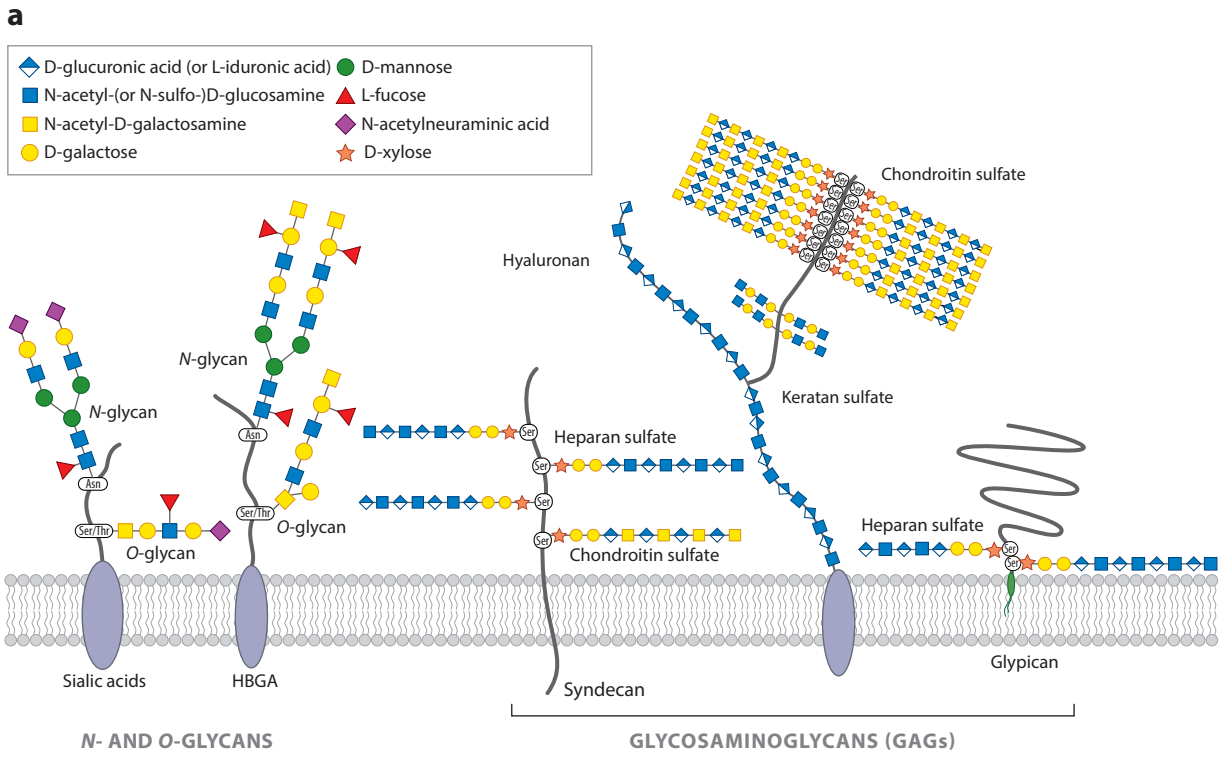
The cellular tropism of a virus is, among other aspects, influenced by its initial binding step, which frequently involves glycans present at the surface of susceptible cells (2). Therefore, the tropism of a virus for a tissue or a particular cell type is primarily affected by the type of glycans displayed on their surfaces and the capacity of viral proteins to recognize them. In addition to the presence of a specific glycan, the type of glycosidic linkage also can influence the host range specificity, as is the case for influenza viruses (12, 13).

## Glycan Types

Viruses mainly bind three different glycoepitopes: sialic acids on *N*- and *O*-glycans and glycolipids (e.g., orthomyxoviruses and reoviruses), GAGs within proteoglycans (e.g., herpesviruses and retroviruses), and neutral carbohydrate moieties such as histo-blood group antigens (HBGAs) on *N*- and *O*-glycans (e.g., caliciviruses) (3).

***N*- and *O*-glycans.** Viruses use two main protein glycan modification types during cellular attachment. The first type, *N*-glycan, is initiated in the endoplasmic reticulum on an asparagine residue and further processed in the Golgi apparatus. *N*-glycosylations are often highly branched. *O*-glycosylation occurs in the Golgi apparatus at serine or threonine residues by an *O*-linkage to the free hydroxyl group of the amino acid. *O*-glycans tend to be smaller and less branched than *N*-glycans. Sialic acids are often found at the terminal branches of *N*-glycans and *O*-glycans (14) (**Figure 1*a***). The most common form of sialic acid in humans is α5-N-acetylneuraminic acid (Neu5Ac), which consists of a nine-carbon backbone that can be extensively modified by acetylation, methylation, hydroxylation, and sulfation (3). Those modifications can be crucial in the context of virus tropism. For example, the preferred attachment factor for influenza A and B viruses is Neu5Ac not 9-*O*-acetyl-Neu5Ac residues, whereas the reverse is true for influenza C virus (15). In addition, various types of glycosidic linkages can be used to connect sialic acid to the subsequent carbohydrate unit of the glycan chain (16). Therefore, more than 50 variants of sialic acids exist and account for a high level of diversity. Sialic acids are usually attached by α2,3- or α2,6-glycosidic linkages to galactose or *N*-acetylgalactosamine at the tail end of glycan chains (16). This arrangement allows the protruding functional groups (hydroxyl, glycerol, carboxylate, and *N*-acetyl) to be accessible for interactions with viral particles (17). Several viruses exploit these structural properties of sialylated glycans as species-specific attachment factors. Influenza A virus is a well-studied example for which the presence of α2,3- or α2,6-linked sialylated glycans is suggested to function as a species barrier between avian and human hosts. While these specific glycans are certainly important, recent glycomics studies have yielded a more complex diversity of sialylated attachment factors and sialic acid derivatives (18–20). Additional examples of sialic acid–dependent viruses are parainfluenza viruses (12), reoviruses (21), polyomaviruses (22), and coronaviruses (23) (**Figure 1*b***).

HBGAs are neutral carbohydrate moieties present on *N*- and *O*-glycans from the surface of red blood cells as well as on most epithelial cells (24) (**Figure 1*a***). These terminal structures on




**b**

| | | Attachment factors | Receptors |
|---|---|---|---|
| **Glycosaminoglycans** | Heparan sulfate and chondroitin sulfate | Togaviridae *Sindbis virus*<br>Flaviviridae *Yellow fever virus* *Hepatitis C virus*<br>Herpesviridae *Herpes simplex virus 1*<br>Papillomaviridae *Human papillomavirus 1*<br>Retroviridae *Human immunodeficiency virus 1* | Adenoviridae *Adenovirus 5*<br>Picornaviridae *Enterovirus 71*<br>Paramyxoviridae *Human orthopneumovirus*<br>Coronaviridae *Human coronavirus NL63*<br>Polyomaviridae *Merkel cell polyomavirus* |
| ***N*- and *O*-glycans** | Sialic acids | Caliciviridae *Feline calicivirus*<br>Reoviridae *Mammalian orthoreovirus 3* *Porcine rotavirus CRW-8* | Orthomyxoviridae *Influenza A virus* |
| | | | Adenoviridae *Human adenovirus 52*<br>Picornaviridae *Enterovirus 68*<br>Paramyxoviridae *Human respirovirus 1*<br>Coronaviridae *Avian infectious bronchitis virus*<br>Polyomaviridae *Merkel cell polyomavirus* |
| | HBGA | Reoviridae *Human rotavirus HAL1166* | Caliciviridae *Norwalk virus* |

(*Caption appears on following page*)

**Figure 1** (*Figure appears on preceding page*)

Glycans used as attachment factors or entry receptors by different viral species. (*a*) The most-used glycoconjugates from the cell surface are sialic acid terminated *N*- and *O*-glycans, glycosaminoglycans (heparan sulfate, chondroitin sulfate, hyaluronan, and keratan sulfate), and histo-blood group antigens (HBGAs). (*b*) Viruses from different families use one or two types of glycans as attachment factors or receptors depending on the virus type. Examples of viruses are provided in italics. For some viruses, it is still not clear whether binding to sialylated glycans is sufficient to induce virus internalization (e.g., influenza A virus for which other specific receptors were identified, such as epidermal growth factor receptor).

glycan chains are used as attachment factors by human noroviruses (25) and some rotavirus strains, switching specificity from sialylated glycans (26) (**Figure 1*b***).

Glycolipids containing terminal sialic acids also can function as attachment factors or receptors for viruses binding to sialylated moieties, as exemplified by simian virus 40 binding to ganglioside GM1 (27).

**Glycosaminoglycans.** GAGs are linear chains of polysaccharides attached to a core protein embedded in the plasma membrane. These proteins, called proteoglycans, are highly glycosylated, as they can bear multiple extended chains of carbohydrate moieties. GAGs are abundant on the surface of most mammalian cells (28) and constitute the main form of glycans within the glycocalyx (**Figure 1*a***). The carbohydrates constituting the GAG chains are repeating units of disaccharides, one being either uronic acid (glucuronic acid or iduronic acid) or galactose, while the second sugar is *N*-acetylglucosamine or *N*-acetylgalactosamine (28). One or both of the sugars bear negative charges inherent to the carboxylate side groups of uronic acid or the sulfation of the sugar moieties (16). Therefore, GAG chains have a high overall negative charge density. Although their structure consists of repeating disaccharide units, GAG chains are highly heterogeneous. This is mainly due to the post-synthetic addition of sulfate groups to the constituents of the chains. The sulfation patterns on the GAG chains are of main importance and interest, as sulfation motifs and their location on the chains are tissue specific and related to the developmental state of the cell as well as disease-induced physiological conditions (29). The sulfation pattern of GAG chains influences their interactions with viruses (30). For example, the degree of GAG sulfation directly influences the binding affinity of herpes simplex virus 1 (HSV-1) (31).

Proteoglycans can be classified based on their cellular and subcellular localization as well as genetic homologies (32). Among those localized at the cell surface, the two main proteoglycans involved in virus attachment to cell surfaces are syndecans and glypicans. Syndecans are transmembrane proteins containing an intracellular domain, a transmembrane region, and an ectodomain bearing the GAGs (33). Some syndecans are responsible for the receptor-mediated endocytosis of ligands (e.g., low-density lipoprotein and transferrin, growth factors). Therefore, virus binding to these receptors serves as an entry ticket to the interior of the cell by endocytosis (34). For example, human rhinovirus 2 undergoes receptor-mediated endocytosis after interaction with the low-density lipoprotein (35). Glypicans are proteoglycans attached to the plasma membrane through a C-terminal lipid moiety GPI. Glypicans are exclusively extracellular proteins that take a globular shape (6). In contrast to syndecans, the anchorage region of the GAG chains on the proteoglycan is located near the juxtamembrane domain (32), bringing virus particles in close proximity to their cognate receptor. GAG chains can themselves be classified into four different categories, depending on the nature of the disaccharide repeating units. Hyaluronan has the highest number of repeating units and is the only GAG that is not sulfated, while keratan sulfate has the shortest GAG chain. The other two GAGs, heparan sulfate (HS) and chondroitin sulfate (CS), have a high net negative charge and are the main GAGs used by viruses as attachment factors (2). HS and CS are composed of 10 to 100 repeating units (36) but differ in the type of disaccharides, sulfation pattern, and location on the cell surface. HS chains are mainly attached to syndecans and are

**Binding affinity:** strength at which a ligand binds its receptor; expressed as the ligand concentration that occupies half the receptors at equilibrium

therefore situated relatively close to the cell surface. However, while CS chains also can be found attached to syndecans, the vast majority are attached to another family of proteoglycans, aggrecans, which are carried by hyaluronan within the extracellular matrix and are thus located further away from the cell membrane (28). Proteoglycans and syndecans in particular serve as receptors for several viruses, including human immunodeficiency virus type 1 (HIV-1) (37), HSV (38), and human papillomavirus (39) (**Figure 1*b***).

As viral membrane proteins are synthesized using the host cell machinery and traverse the secretory pathway, they are modified with host-dependent glycans. Depending on their location, these glycans can influence viral replication and pathogenicity. Besides affecting immune recognition (40), protein folding, and protein conformation (41, 42), glycosylation is an important modulator for virus-cell binding. Glycosylation of the influenza virus hemagglutinin affects folding and pH stability when near the stalk and cleavage site of the protein (41, 42). When near the binding site, glycosylation alters the affinity for sialylated attachment factors (43), as is also the case for avian coronaviruses (44).

## Virus-Cell Binding and Entry

Due to the ubiquity of glycans on cell surfaces, viruses have evolved to use glycans as attachment factors. Examples include hepatitis C virus (6), influenza virus (12), HIV (45), Ebola virus (46), human papillomavirus (47), and viruses from the herpesvirus family (48). In order to take advantage of glycans to gain access to cellular membranes, viral attachment proteins recognize specific glycan patterns on the surface of target cells. As the constituents of the glycocalyx bear a substantial amount of negative charge, electrostatic interactions are of primary importance for virus binding, as positively charged domains of viral proteins can readily attach to negatively charged glycans. However, a high degree of binding specificity is often reached, implemented by precise hydrogen bonding or hydrophobic interactions between viral proteins and glycoepitopes (49, 50).

Interactions between viral proteins and cellular glycans are usually of low affinity, with dissociation constants in the millimolar range (51–53). However, the surface of virus particles is covered with several hundred copies of the glycan-binding proteins (54), which allows viruses to form multiple simultaneous interactions with cell-surface sugars, i.e., multivalent interactions (55). Engaging simultaneously with multiple viral proteins allows compensation for the inherently weak affinity of the glycan-protein interactions and strengthens the binding of viral particles to cell surfaces. The strength of such a multivalent interaction depends on the number of connections (i.e., the number of simultaneously interacting pairs of viral protein-glycan moieties), which itself mainly depends on the density and spatial arrangement of the interacting molecules on both surfaces. Therefore, the number of available binding partners on viral particles directly controls and modulates the interactions taking place with cell surfaces. Multivalent binding is a property of a wide variety of viruses, indicating a principal mode of cell attachment (55, 56). The affinity of adenovirus serotype 37 (Ad37) binding to sialic acid is increased 250-fold when bivalent interactions are involved, relative to monovalent binding (51, 57). As another example, trimers of recombinant influenza A hemagglutinin bind to multiple glycans depending on the host origin (58). Thus, a dense glycan organization on the cell surface appears to be required for functional binding. Interestingly, super-resolution microscopy has visualized the presence of dense submicrometer glycan nanodomains, which provide a multivalent virus-binding platform. Following this hypothesis, single-virus tracking supports the idea of a compartmentalized organization of sialylated glycans (59).

Once attached to the cell surface, viral particles mostly show two types of behavior that can be depicted as land-and-stick and land-and-seek approaches (60). In the former, virus particles

interacting with attachment factors or receptors are internalized at the location of the initial interaction. This involves spatial confinement of the virions waiting on the cell surface for endocytosis or the recruitment of a coreceptor (60). In the latter, virions undergo a complex mobility process at the cell surface in order to find a suitable location to be internalized. In that case, after binding to their attachment factors or primary receptors, viral particles undergo random diffusion or directional displacement on the cell surface to reach a specific location on the plasma membrane enabling virus internalization (61, 62). This strategy is used by influenza A virus, mouse polyomavirus, and simian virus 40 (63–65). The structural basis for this explorative motion often is elusive due to the nanoscale size of the virus-cell interface. Here, super-resolution microscopy can be used to visualize the organization of viral proteins and virus-binding factors (discussed below). While virus displacement on the cell surface can be achieved by diffusion of a virus-bound protein within the plasma membrane, it also can be achieved by cell-surface glycans. For example, stable binding of simian virus 40 to cell surfaces is highly dependent on the motion of virions bound to the glycosphingolipid GM1 to gather a sufficient number of receptors (66). The motion of influenza A virus is highly dependent on the modulation of the number of sialylated receptors engaged by hemagglutinin proteins as well as the action of the cleaving enzyme neuraminidase (67, 68). The polarized intravirion organization of hemagglutinin and neuraminidase is thought to be important for viruses to avoid immobilization and provide the opportunity for directional virus movement (69).

While the glycoepitopes on sialylated glycoproteins are usually a terminal unit of a large glycoconjugate molecule, GAG molecules provide multiple glycoepitopes, as they can be internal sequences of the chain (6). This structure provides a powerful diffusion platform for viral particles, as the long chains of repeating glycans allow sequential binding and unbinding of ligands to neighbor binding sites or from one GAG chain to another (70, 71). Virus mobility on GAG chains was demonstrated for HSV-1, which displays different diffusion coefficients depending on the GAG sulfation pattern (31). In this context, the number of physical bonds between viral particles and receptors directly influences the virus diffusion potential. While multiple parallel interactions allow strong virus attachment, a low number of bonds could be preferred to allow diffusion to specific entry receptors.

**Random diffusion:** random movement of a particle driven by a concentration gradient that depends on particle random walk mechanisms

**Directional displacement:** constrained movement of a particle along specific cellular structures or pathways

**Multivalent inhibitor:** molecule interfering with virus binding to cell surfaces using structures displaying multiple copies of glycans

## Glycan-Based Antiviral Strategies

Because attachment of viral particles to glycans is usually the first step of infection for many virus families, interfering with this process could limit viral spread and block the development of virus-related diseases. An attractive strategy of impeding virus attachment to cell-surface glycans is to make use of those molecules to physically interfere with virus-cell interactions (72). Therefore, antiviral compounds that compete with cell-surface glycans for virus binding have potential as therapeutic agents (73). The overall strength of virus attachment to cell surfaces is usually enhanced by engaging multiple glycans simultaneously. Therefore, to mimic virus-cell interaction, compounds capable of forming multivalent interactions with virions are of primary interest to block viral attachment. Consequently, several multivalent inhibitors have been developed to interfere with attachment of different viral families. Various polyvalent assemblies targeting the influenza virus hemagglutinin protein inhibit attachment, with dramatic enhancement of efficiency relative to their monovalent counterparts. These structures include functionalized polymers (74), liposomes (75), and solid or soft nanostructures (76, 77) to obtain glycoconjugates carrying multiple sialic acid residues. In addition to that, GAG-mimicking macromolecules such as heparin (78), sulfated polysaccharides (79), or functionalized polymers, dendrimers, or nanoparticles decrease GAG-mediated virus attachment in vitro. HS-coated nanoparticle structures also display virucidal

activities in vivo (80). Moreover, giant multivalent glycofullerene structures inhibit infection by pseudotyped Ebola virus particles (81). Unfortunately, despite these promising results, antiviral effects are rarely observed in vivo (82, 83), which often correlates with low biocompatibility and high cytotoxicity. Some of these problems have been successfully addressed, revealing that an optimal scaffold size and geometry play important roles in modulating the efficacy and host compatibility of synthetic compounds in vivo (76, 84). Currently, a few glycan-based antiviral drugs involved in clinical trials include carrageenan-, dextran-, and alginate-based assemblies (85). Two sialic acid analogs are licensed and effectively used to fight influenza virus infection: zanamivir and oseltamivir. Their action mechanisms involve inhibition of the neuraminidase enzyme and therefore prevention of virus release from infected cells and cell-to-cell spread (85).

Despite the great potential of inhibiting viral attachment by targeting virus-glycan interactions and the numerous molecules that show positive effects, only a few such antiviral drugs are licensed and effectively used. Therefore, gaining new insights into the molecular details of virus attachment to cell-surface glycoconjugates is of prime interest to foster development of antiviral strategies based on glycan-binding inhibitors.

**Glycofullerene:** spherical macromolecular carbonaceous structure capped with glycan moieities

**Antiviral drug:** medication used to treat viral infections by blocking the viral cycle at a specific step (cell binding, genome replication)

**Cryo–electron microscopy:** electron microscopy technique applied to frozen samples allowing high-resolution imaging of biomolecules

**Atomic force microscopy (AFM):** microscopy technique using a fine tip attached to a cantilever to image various surfaces and force-probe their constituents

## EXPERIMENTAL APPROACHES TO STUDY VIRUS BINDING

There are many techniques available to study virus binding to cell-surface glycans as purified interactants. The current challenge is to address this fundamental question using physiologically relevant conditions. New developments include imaging and tracking techniques, such as cryo–electron microscopy (cryo-EM), fluorescence-based techniques, and biophysical approaches, including optical tweezers and atomic force microscopy (AFM). We provide an overview of these techniques and discuss their strengths and weaknesses.

### Virus-Binding Assays

Early virus-binding assays relied on the capacity of a virus to agglutinate (i.e., clump) cells such as heavily glycosylated erythrocytes [i.e., hemagglutination (86)]. While this type of assay is still used to quantify viruses and probe their specificity (e.g., for influenza virus), more recent approaches take advantage of synthetic glycans. In general, a virus-binding assay relies on monitoring viral adsorption to a known molecule of interest (i.e., specific receptor, antibody, or glycan). A detection method is used to quantify the presence and extent of virus binding to the molecule of choice. Many virus-binding assays follow this principle but differ in the presentation of the molecule and the detection method used to monitor virus binding (e.g., liquid- or solid-phase, radioactive, or fluorescence assays).

Solid-phase binding assays are widely used to study interactions in vitro and have the advantages of speed and simplicity. These assays are based on the binding of virions in solution to an immobilized receptor on a solid phase (e.g., protein or glycan). Binding is usually monitored using fluorescence-based detection methods (immunofluorescence analysis or ELISA) or radioactive methods (87). Although solid-phase binding assays with purified receptors lack the complexity of biological samples, this method is useful as a primary screen of a virus-receptor interaction. In the context of virus-glycan binding, glycan arrays can be used to better define differences in virus-glycan interactions by enabling fast, high-throughput screening of several glycans as potential virus-binding molecules (88). In such screens, recombinant viral proteins, either alone or immobilized on nanoparticles, as well as whole virions are analyzed for binding to a wide array (>600) of synthetic glycans that have been robotically printed on glass slides (89). Glycan arrays differ in their glycan composition (90) and mode of glycan immobilization (91), which have been previously reviewed (92). Glycan arrays have become the standard for many virus-glycan studies,

e.g., for adenovirus (57), influenza virus (93), polyomavirus (94), and reovirus (21). However, a requirement for the use of synthetic glycan libraries is structural knowledge of cellular glycans in a respective host tissue. Glycomics approaches now allow determination of glycan structure and composition of native tissues, revealing a much more complex picture than previously assumed (18, 19). Some studies have already begun to adapt these technologies to employ natural glycan libraries by harvesting glycans from cells or tissues and imprinting them in a glycan array format (95).

To better mimic physiologically relevant conditions, virus-binding assays also can be conducted using living cells. In these assays, viruses are incubated with a confluent layer of cells (preferably at 4°C to avoid endocytosis), and virus binding is monitored by fluorescently labeled viruses (96). Labeled viruses are usually obtained in vitro by incorporating radioactive nucleotides (97), radioactive lipids for enveloped viruses (98), or chemical modification of exposed surface proteins with fluorescent dyes (99). However, in the latter case, the labeling of the virus can alter its binding due to changes in the intrinsic nature of a particular protein responsible for virus-cell interaction.

Thanks to their simplicity, virus-binding assays are widely used as screening tools. However, they lack quantitative and dynamic information.

**X-ray crystallography:** technique to determine the three-dimensional structure of macromolecules down to atomic resolution

## Structural Insights into Virus-Glycan Interactions

Structural studies provide detailed knowledge of the structure of viral proteins involved in binding to host attachment factors as well as insights into their entry mechanisms such as snapshots of membrane fusion mechanisms. A wide variety of methods are used to obtain structural insights, ranging from electron microscopy (EM) or cryo-EM techniques to more classical approaches such as nuclear magnetic resonance and X-ray crystallography.

EM is an important tool in virology. The first images of viruses date from the late 1930s (100) thanks to the transmission electron microscope (TEM). The high resolving power of the TEM permits studies at the nanometer scale, providing direct images of viruses for diagnosis and research. Since then, many improvements in structural techniques yielded enhanced resolution and maintenance of the biological sample in near-native conditions. In this context, the development of cryo-EM has been particularly important, with resolution approaching that of X-ray crystallography without a requirement for crystallization, fixation, or large amounts of biological material. Single-particle cryo-EM reconstruction is often used for studies of purified viral proteins, macromolecular complexes, or whole virions with the aim to resolve the organization of multicomponent assemblies. Being mostly applied to resolve structures of a variety of viruses in the subnanometer range (reviewed in 101), this technique also has been used to study viral particles in complex with glycans. Some elegant examples include the study of Eastern equine encephalitis virus host cell interactions revealing a binding site for the cellular attachment factor heparin sulfate as well as describing the mechanism for the nucleocapsid core release (102), the discovery of a glycan shield of a coronavirus spike protein together with its epitope masking (103), and the description of the structural basis for human coronavirus attachment to sialic acid receptors (104).

To gain insights into complex specimens or viruses undergoing dynamic processes, we can use cryo–electron tomography (cryo-ET), another electron beam–based method. Cryo-ET extends imaging in two to three dimensions in which specimens are tilted usually by 1° or 2° from about −70° to +70° along an axis perpendicular to the electron beam. A series of two-dimensional (2D)-projection images are collected, aligned with each other, and then back-projected to form 3D images (105). This technique has been applied to study a variety of viruses (reviewed in 106) and has provided structures of glycoproteins in situ on the surface of virions, including HIV-1

Env (reviewed in 107) and influenza virus hemagglutinin (54). Because these approaches depend on detection of morphologically recognizable structures or on crystals of purified material, the application of these techniques is mainly restricted to the analysis of free extracellular virions or particles attached to or being released from a host cell.

**Force spectroscopy:** technique allowing the study of interactions and binding forces between individual molecules down to the single-molecule level

**Nuclear magnetic resonance spectroscopy:** analytical chemistry technique providing structural and dynamic information on biomolecular samples based on the detection of oscillating magnetic fields

**Mass spectrometry:** analytical technique for discriminating various substances according to their masses

At the atomic level, interactions between viral proteins and glycans have been captured in numerous X-ray crystal structures of such complexes. While not a new technique, X-ray crystallography has improved in nearly every step of the crystallographic and protein purification process, accelerating structural determination (108). As examples of the application of X-ray crystallography to studies of virus-glycan interactions, X-ray crystal structures reveal that the reovirus σ1 capsid protein binds differently to sialylated glycans depending on the serotype (type 1 or type 3) (21, 109) and the influenza virus hemagglutinin varies in its binding to differently linked sialic acids (110, 111). Other examples include identifying the structural basis of adenovirus (51, 57), coronavirus (104), and rotavirus (112) binding to glycans. An interesting application of crystallographic data is their use in simulated force spectroscopy experiments (113, 114). Knowing the exact molecular coordinates of a protein-ligand complex allows for the application of a specific pulling force and visualization of the complex coming unbound. By recording structural and quantitative data, this method enables studies of unbinding pathways bond by bond. As applied to an influenza hemagglutinin-glycan complex, simulated force spectroscopy revealed a variety of unbinding pathways and dynamic binding/unbinding behavior of the interaction (115). However, X-ray crystallography requires studies of isolated proteins and thus can provide only a single picture of many conformations accessible to the protein.

Nuclear magnetic resonance (NMR) spectroscopy provides an approach to map virus-glycan interactions in real time and in solution. This technique provides additional information about the dynamics of glycan motion in free and protein-bound states. In its basic form, saturation transfer difference NMR, experiments require no isotope labeling and only relatively small amounts of virus or viral receptor-binding protein. However, the virus (or protein)-glycan complex must have a clear and detectable chemical shift distinct from the unbound material, and often NMR techniques work best for relatively low-affinity interactions. In studies of virus-glycan interactions, NMR analyses of different glycan receptors bound to different viral attachment proteins can give information about glycan-protein contacts that is complementary to X-ray structures (21, 51, 111) or serve as a standalone technique to reveal the molecular basis of glycan recognition, as is the case for calicivirus-glycan interactions (116).

Finally, modern mass spectrometry (MS) techniques enable studies using native conditions, opening avenues to explore the dependence of glycan binding on virus assembly (117). Conformational changes also can be mapped with hydrogen/deuterium exchange MS, as exemplified for hepatitis B virus (118). These techniques lack insights into dynamics, yielding snapshots rather than capturing the whole dynamic process essential for a full understanding of binding and subsequent infection.

## Insight into the Thermodynamics and Kinetics of Virus Binding

In addition to structural studies of virus-glycan interactions, there is a crucial need to examine the thermodynamic and kinetic parameters regulating virus binding to receptors. Especially in the context of drug design and protein mutability, a better understanding of the interplay between thermodynamics and kinetics will enable improved decision-making for selecting drug candidates (119). Several techniques are discussed below that allow studies of the kinetics and thermodynamics of virus-glycan interactions.

Surface plasmon resonance (SPR) is a label-free biosensor technique to study ligand-receptor interactions in real time. SPR exploits the fact that the binding of biomolecules on the sensor

surface will result in a change in refractive index, which is measured as a change in resonance angle or resonance wavelength. The change in the refractive index on the surface is directly proportional to the number of bound molecules. In the context of virus-glycan interactions, this instrument allows determination of association and dissociation kinetics. SPR methodology has been used to explore engagement of many viruses with glycans, including influenza virus–glycan interactions (120) and inhibition of influenza virus infection by sialic acids (121). The main limitation of this method is the poor control of glycan density and orientation, which may affect binding (76). Moreover, if the affinity of the virus for the glycan is low, relatively large amounts of the binding partners are required. Finally, for multivalent interactions, SPR could underestimate the dissociation rate due to the formation of multiple parallel bonds.

**Fluorescence microscopy:** optical imaging technique relying on the spontaneous emission of light by specific fluorescent dyes upon excitation

In addition to SPR, isothermal titration calorimetry (ITC) is a rigorous means to define the equilibrium-binding constant of a glycan and a viral binding partner. ITC is based on the detection of the heat released or absorbed during a biomolecular binding interaction and extends to the nanomolar range. ITC has been used to determine the binding stoichiometry and equilibrium dissociation constant of HIV-1 and different glycoproteins (122) and to identify a trisaccharide containing α2,3-linked sialic acid as a receptor for mumps virus (123).

Biolayer interferometry (BLI) and microscale thermophoresis (MST) are additional affinity-based techniques increasingly used to analyze virus-glycan interactions. BLI is a label-free optical and analytical technique that can be used to study interactions between biomolecules on a 2D biosensor surface. This technique analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized molecules on the biosensor tip and an internal reference layer. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured. In contrast, MST measures the diffusion of an analyte in solution following laser-induced heating (124). Both techniques allow monitoring of the interactions of intact virions or isolated viral proteins with receptors in real time, allowing assessment of binding specificity, rates of association and dissociation, and concentration with high precision and accuracy. BLI has been used to analyze the kinetics of the interactions of influenza A virus hemagglutinin and neuraminidase during infection (125) and validate the glycan-mediated enhancement of reovirus-receptor binding (126).

## Optical Microscopy and Spectroscopy Toward Dynamic Studies

When defining glycan organization and dynamic processes on living cells, fluorescence microscopy is a method of choice. Different fluorescence-based techniques are available to determine how glycans are organized within the plasma membrane and how virions interact with cell-surface attachment factors or receptors leading to virus entry. Studies of post-entry steps using fluorescence microscopy have revealed how the cytoskeleton and its motors support virion trafficking and uncoating, leading to the delivery of its genetic cargo into the cytoplasm (127).

High-resolution optical techniques such as confocal laser scanning microscopy (CLSM) and spinning-disc confocal microscopy enable localization of virions during their initial interactions with cells. CLSM revealed that influenza A virus colocalizes with GM1-based lipid rafts during virus attachment to the cell surface (128). Furthermore, dynamic studies at high temporal resolution (~millisecond) allow single-virus tracking, as has been done to image early infection steps of influenza A virus (130), murine leukemia virus (131), and Sindbis virus (129), as well as many other viruses (reviewed in 77). For an in-depth review of the different microscopy methods, see Reference 132; on imaging, tracking, and computational analyses of different viruses and their attachment, entry, and replication, see References 133 and 134.

With its high sensitivity, total internal reflection microscopy (TIRF) yields high-contrast images at low background, reduced cellular photodamage, and rapid exposure times. Due to the

intrinsic properties of the evanescent field, the TIRF excitation of fluorophores decreases exponentially with distance from the coverslip, such that only fluorophores close to the coverslip (e.g., within ~100 nm) are selectively illuminated. Thus, TIRF is a useful technique to track movement of single virions on artificial surfaces and cell membranes. TIRF has been used to study influenza A virus interacting with specific glycans, which has improved our understanding of virus binding, infectivity, transmissibly, pathogenicity, and host specificity (67, 68).

**Optical/magnetic tweezers:** precision biophysical tool allowing the measurement of the mechanical properties of biomolecules thanks to an optical/magnetic trap

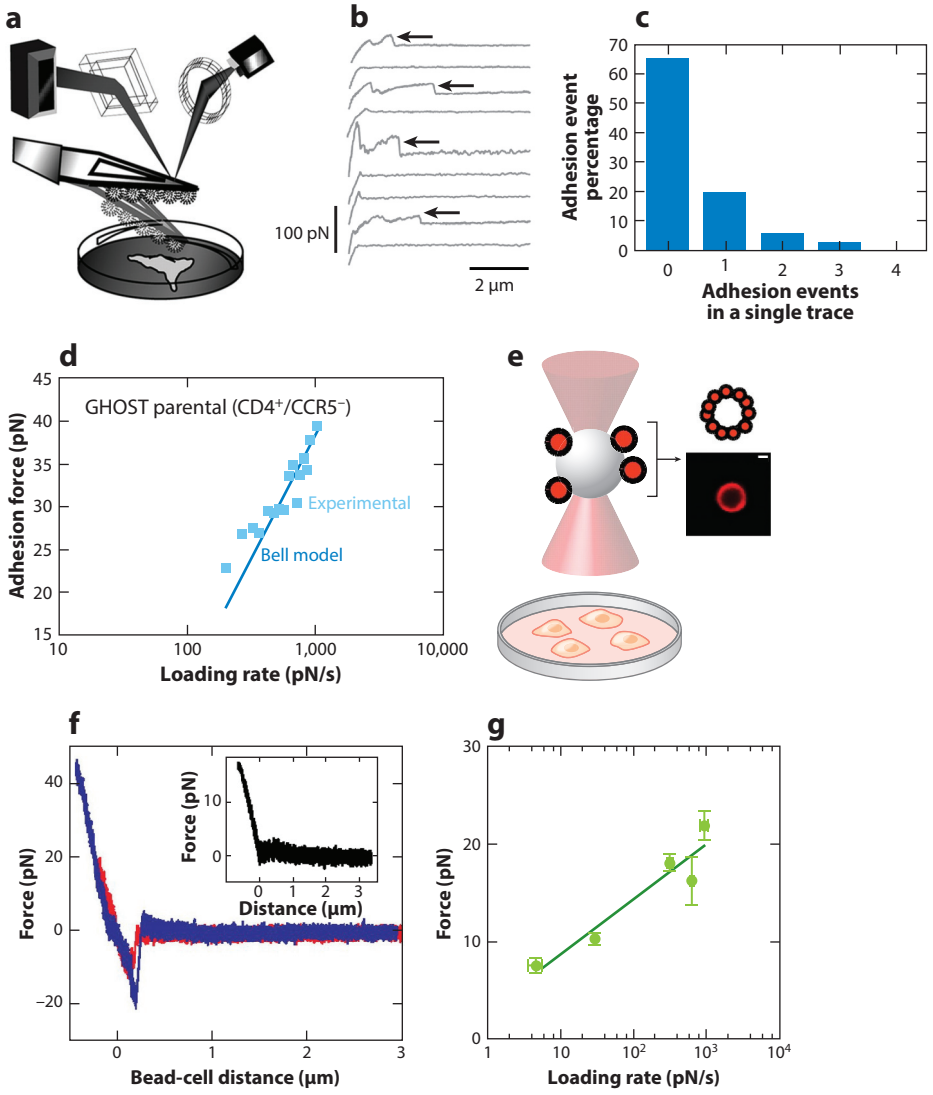
Super-resolution microscopy techniques such as structured illumination microscopy, single-molecule localization microscopy techniques such as PALM/(d)STORM, and stimulated emission depletion nanoscopy have lowered the effective resolution of fluorescence microscopy to enable visualization of subviral structures (135). Thus, these approaches have greatly expanded the possibilities for detailed investigation of virus-cell interactions. Although cellular glycans are difficult to label, traditional labeling using lectins or click chemistry has enabled a close-up view of the glycocalyx. Plant lectins provide an easy way to determine the presence and nanoscale organization of a specific glycan on cultured cells or tissue (59, 136). On A549 cells, sialylated influenza virus attachment factors form nanoscale clusters, which are linked to viral movement and receptor activation (59). As an alternative, feeding cells with a modified glycan precursor can lead to synthesis of fluorescently modified glycans without the need of an additional high-affinity probe (137). Combined with super-resolution microscopy, such bio-orthogonal labeling was used to study the lateral organization of glycans as well as the thickness of the cellular glycocalyx (138).

Spectroscopy based on fluorescence resonance energy transfer (FRET) allows studies of interactions in virus infection at a very small spatial scale (<10 nm). FRET is based on the capacity of the near-field energy transfer (low nanometer range) between two light-sensitive molecules (chromophores). This spectroscopy technique has been used to study virus-glycan interactions for HIV-1 (139) and norovirus (140). Fluorescence correlation spectroscopy correlates fluctuations of the fluorescence intensity of fluorescent particles or complexes (virus-glycan) in solution and has been used to demonstrate that the envelope glycoprotein mobility of HIV-1 depends on the viral maturation state (141).

Together these methods provide deep insights into the first binding steps of viruses to cell-surface glycans at high temporal and spatial resolution. Nevertheless, they have several disadvantages compared with other methods—for example, (*a*) a fluorophore must be attached to the molecule of interest, which can alter the structure of the molecule and in turn influence the binding behavior; (*b*) phototoxicity and photobleaching can cause difficulties in the interpretation of the data; and (*c*) there is a requirement for extensive processing of the experimental data sets.

## Single-Virus Force Spectroscopy Techniques

Compared with conventional ensemble methods (i.e., methods evaluating the average behavior of an ensemble of particles), single-virus techniques offer distinct advantages. First, conducting many sequential measurements allows determination of the distribution of molecular properties of nonhomogeneous systems. Second, because they represent direct records of the stochastic fluctuations of the system, single-molecule trajectories provide dynamic and statistical information, which is often hidden in ensemble-averaged results. Finally, these techniques allow real-time observation of rarely populated transients, which are difficult to capture using conventional methods (142). In this context, AFM (**Figure 2*a***) and optical/magnetic tweezers (**Figure 2*b***) enable measurement of binding forces at the single-molecule level and with high temporal resolution (143, 144). Thanks to grafting protocols, single virions can be attached to an AFM tip apex or on beads, which allows probing of virus interactions with specific receptors and glycans (115, 145, 146). Such approaches allowed studies of the binding behavior of intact virions with receptors to be conducted




## Figure 2

Single-virus force spectroscopy using AFM (*a–d*) and optical tweezers (*e–g*). (*a*) AFM-based force spectroscopy used to monitor HIV-1 interactions with cell-surface CD4 receptors. (*b*) Representative force-distance curves during retraction of the cantilever. Rupture events between a single virion and cell-surface receptors are marked by an arrow. (*c*) Probability of bond formation between a virion and a host cell receptor. (*d*) DFS plot showing binding forces as a function of the loading rate (i.e., velocity at which the force is applied). The fit of this curve using Bell's model yields the kinetic parameters describing the energy landscape of this interaction. (*e–g*) Optical tweezers used to analyze the attachment of influenza A virus to host cell receptors. (*e*) Schematic of the experiment showing virions adsorbed to a polystyrene bead and trapped in an optical field. Confocal microscopy image is shown to validate the virion adsorption (*right*, scale bar: 0.5 µm). (*f*) Binding forces measured between viruses on beads and adherent cells grown in glass-bottom Petri dishes. (*g*) DFS plot of a virus-receptor interaction on the surface of CHO cells. Abbreviations: AFM, atomic force microscopy; CHO, Chinese hamster ovary; DFS, dynamic force spectroscopy; HIV-1, human immunodeficiency virus type 1. Panels *a–d* adapted with permission from Reference 149. Panels *e–g* adapted with permission from Reference 115.

using purified receptors in vitro or in living cells (reviewed in 147). These technologies have been applied to characterize virus-host interactions for many viruses including herpesvirus, HIV-1, influenza virus, rhinovirus, and reovirus (115, 126, 145, 148–151). In terms of unraveling the first virus-binding steps to cell-surface exposed glycans, Chang et al. (148) and Dobrowsky et al. (149) performed single-molecule analysis of HIV-1 interacting with glycoprotein receptors on living cells and were able to monitor early fusion dynamics (**Figure 2*a–d***). In another study, Sieben et al. (115) elegantly combined optical tweezers, AFM-based single-virus force spectroscopy, and molecular dynamics simulations to study the binding of influenza virus to cells with varying surface distributions of α2,3- and α2,6-linked sialic acids (**Figure 2*e–g***).

## Mapping Virus Binding Sites Using Atomic Force Microscopy and Fluorescence Microscopy

Although AFM alone is an effective strategy for quantitatively defining the forces of virus-cell interactions, it lacks optical correlation to identify the cell-surface receptors involved. To address this challenge, correlative approaches using AFM and fluorescence microscopy have opened new avenues in the analysis of the first binding steps of viruses to cells.

As a proof-of-concept, we recently developed a versatile platform combining AFM and confocal microscopy enabling the mapping of single-virus binding sites on living cells using AFM while monitoring the cell-surface receptor distribution with confocal microscopy (96). Applied to a model virus [engineered rabies virus with the envelope glycoprotein of avian sarcoma leucosis virus (EnvA)], specific interactions with cells expressing viral cognate receptors [avian tumor virus receptor A (TVA)] were mapped on living cells, which allowed localization of the binding site at high resolution and quantification of the kinetic and thermodynamic parameters of the binding interactions. Simultaneously, confocal microscopy enabled differentiation between cocultured control cells and cells expressing fluorescently labeled TVA. This study demonstrated that the EnvA-pseudotyped rabies virus quickly ($\leq$1 ms) establishes specific interactions with single TVA receptors. The results also suggest that one TVA receptor on the cell surface binds one subunit of the trimeric EnvA glycoprotein with an intrinsic low affinity and that the affinity increases considerably with the binding of other TVA receptors to the same EnvA glycoprotein, suggesting a positive allosteric modulation of the EnvA-TVA bonds.

The same approach has been used to elucidate important aspects of the multivalent binding of a gammaherpesvirus to GAGs expressed on living cells during early steps of the infection (150). Using AFM, Delguste et al. (150) showed that gp150, the major envelope glycoprotein of murid herpesvirus-4, regulates GAG binding by other viral glycoproteins. By combining experiments using purified GAGs and living cells, they extracted quantitative information about the binding force of single viral particles to GAGs, and unprecedented insights were made about the tight regulation of virus attachment to cell surfaces (**Figure 3**). Defining the force of virus binding to GAGs revealed that interactions with a lower valency are preferred by wild-type virus particles, while gp150-deficient virions display a higher tendency to engage simultaneous, multiple interactions, suggesting a regulatory role of the glycoprotein gp150 in GAG binding. By minimizing multivalency between GAGs and viral glycoproteins, gp150 might facilitate lateral diffusion of the virus on the cell membrane, allowing the virus to search for its specific entry receptor. Moreover, by controlling the number of virus interactions with GAG moieties, gp150 also facilitates the release of newly assembled viral particles, as low valency interactions are more likely overcome during viral egress.

An important question in virology is the role of attachment factors in the context of virus infection. Using AFM-based single-virus force spectroscopy, reovirus binding to cell surfaces was

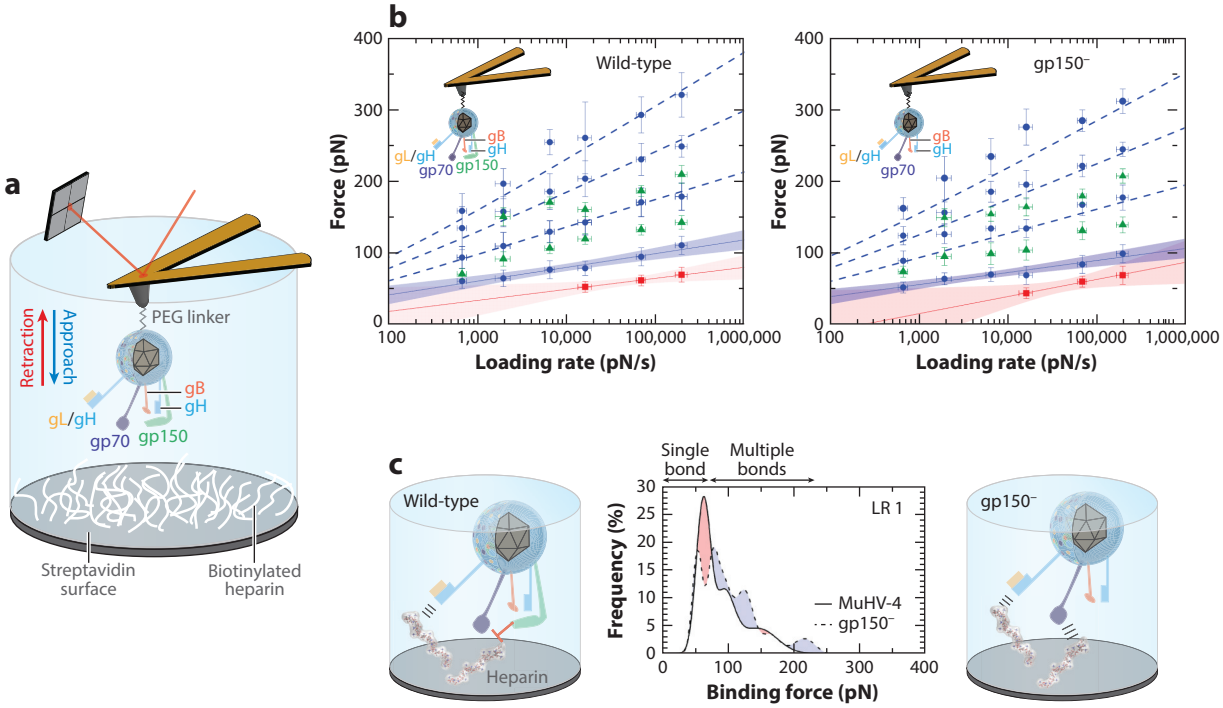



**Figure 3**

Murid herpesvirus-4 binding to heparin studied by AFM. (*a*) Wild-type virions and virions lacking glycoprotein gp150 were attached to AFM tips to probe the interactions with surfaces functionalized with heparin. (*b*) Comparison of DFS plots obtained for both virions (wild-type and gp150-negative) revealed no difference, suggesting that gp150 does not play a direct role in heparin binding. (*c*) Force distribution analysis reveals that single bonds occur more frequently with wild-type particles, while multiple bonds are more probable for gp150-negative virions, suggesting a regulatory role for the number of bonds established between the virions and GAGs. Abbreviations: AFM, atomic force microscopy; DFS, dynamic force spectroscopy; GAG, glycosaminoglycan. Figure adapted with permission from Reference 150.

investigated with a focus on the engagement of the σ1 viral attachment protein to α-linked sialic acid (α-SA) and junction-associated molecule (JAM)-A receptors (126). Combining in vitro approaches using both purified receptors and living cells, the respective contributions of the α-SA and JAM-A receptors were quantified. Based on the results obtained in experiments using purified receptors, the number of bonds established on living cells was determined for the early binding step. Surprisingly, the initial σ1 binding to α-SA was found to act as a trigger to enhance the overall avidity of σ1 for JAM-A (**Figure 4**). Additional experiments comparing binding properties of reovirus infectious subvirion particles, which have an altered conformer of σ1, and intact reovirus virions in the presence and absence of α-SA suggest that α-SA binding to reovirus virions triggers a conformational change in σ1 to a more extended form, underlying a direct interplay between attachment factors and specific receptors.

As described in this review, low-affinity interactions with glycans are essential for numerous viruses to ensure initiation of the viral life cycle. Identifying the binding partners involved together with the binding characteristics increases the opportunities to inhibit infection. New techniques to study virus attachment to receptors allow insights to be made about the molecular nature of these interactions and provide crucial information to develop new therapeutic approaches or identify new targets for vaccination.

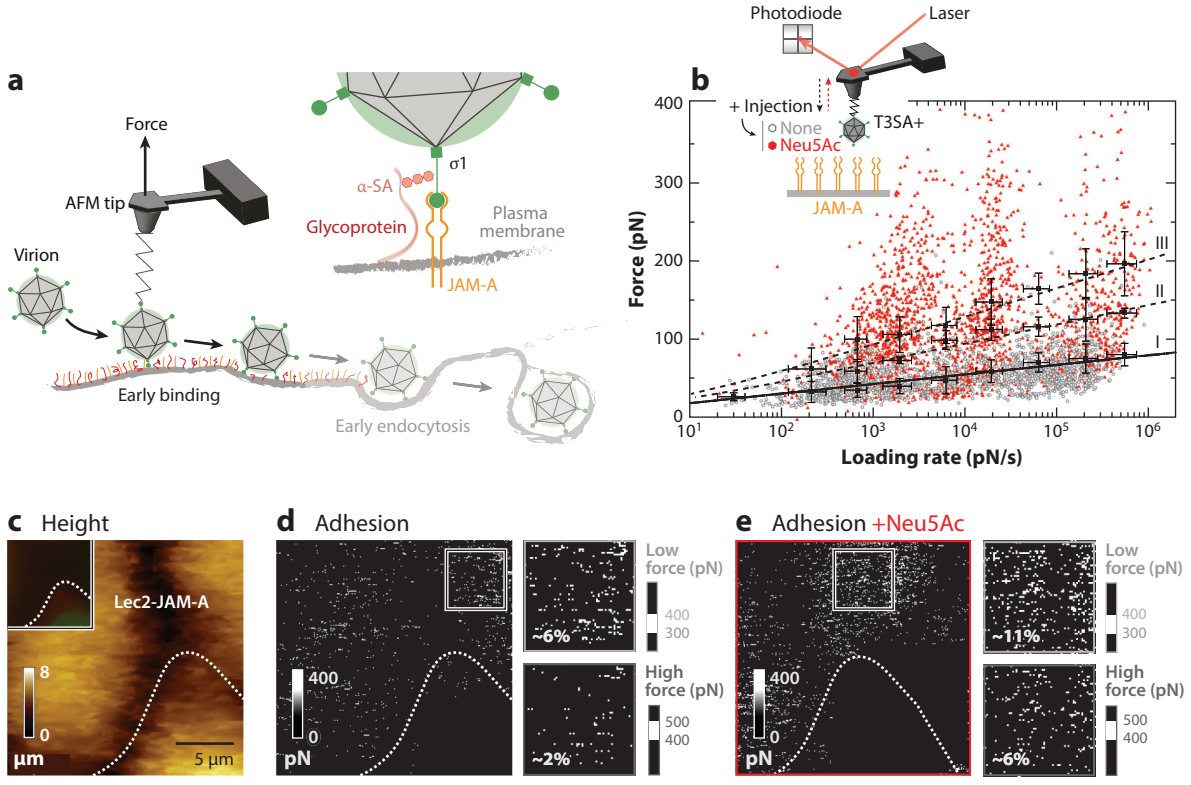



**Figure 4**

Interplay of glycan and proteinaceous receptors during reovirus binding to cells. (*a*) Schematic of probing reovirus binding to cell-surface receptors by AFM. (*b*) DFS plot before (*gray*) and after (*red*) injection of Neu5Ac, indicating a shift to higher binding forces (i.e., multivalent bond formation) between reovirus particles and JAM-A receptors. (*c–e*) Experiments using living cells reveal the same behavior. (*c*) AFM height image of Lec2-JAM-A and Lec2 cells deficient in JAM-A tagged with mCherry and GFP (*insert*: fluorescence image) and corresponding adhesion images before (*d*) and after (*e*) injection of Neu5Ac. The respective enlargements of the adhesion maps show an increase of higher interaction forces (in the range 400–500 pN), revealing that the initial σ1 binding to α-SA acts as a trigger to enhance the overall avidity of σ1 for JAM-A. Abbreviations: AFM, atomic force microscopy; DFS, dynamic force spectroscopy; JAM, junction-associated molecule; Neu5Ac, α5-N-acetylneuraminic acid; SA, sialic acid. Figure adapted with permission from Reference 126.

## SUMMARY POINTS

1. The glycocalyx is a dense network of glycans attached to lipids and proteins on cell surfaces, to which a wide variety of viruses bind to either enhance infectivity (attachment factors) or directly gain access to the cytoplasm (receptors).
2. Viruses mainly bind three types of cell-surface glycans: sialic acids, glycosaminoglycans, and histo-blood group antigens.
3. Interactions between single viral proteins and glycans are usually of low affinity, which allows viruses to diffuse on the plasma membrane, while the simultaneous engagement of multiple viral proteins stabilizes the binding interaction by enhancing the binding strength.

4. Synthetic compounds capable of forming multivalent interactions with virions have the potential to serve as antiviral drugs by blocking viral attachment to cell surfaces.
5. Virus-binding assays are relatively simple techniques to monitor the binding of viruses to specific molecules, while EM, X-ray crystallography, and nuclear magnetic resonance are methods that determine the fine structure of viral proteins involved in glycan binding, often in complex with the glycan.
6. Surface plasmon resonance and biolayer interferometry are simple yet powerful techniques to study the thermodynamic and kinetic parameters describing the binding properties of virions to their target molecules on cell surfaces, thus enhancing an understanding of interaction dynamics.
7. Fluorescence microscopy techniques provide insights into virus-binding events on cell surfaces at high temporal and spatial resolution as well as dynamic information about virus uptake.
8. Single-virus force spectroscopy techniques such as optical/magnetic tweezers and atomic force microscopy enable measurement of glycan-virion binding forces at the single-molecule level, providing unprecedented information about the dynamics of virus binding.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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**132. Overview about super-resolution microscopy methods to study virus-glycan interactions.**

**144. Comprehensive review about single-molecule force spectroscopy tools.**

**147. Review about the capability to unravel molecular details about virus infection using single-virus force spectroscopy.**