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Initial step of virus entry: virion binding to cell surface glycans

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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 viruses 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, to define atomic-level structures of virus-glycan complexes and to study these interactions at the single-virion level. This review gives 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 offering new perspectives to better understand virus-glycan interactions in physiologically relevant conditions.

INTRODUCTION

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. On the other hand, mammalian cells are very dynamic and provide various structures and processes allowing the uptake of macromolecular assemblies. Throughout the evolution, viruses developed various strategies to take advantage of various 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 underrated, 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 *i.a*. 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 will decipher the role of glycans during the initial step of virus-cell binding. We will discuss different techniques available to study virus-glycan binding as well as recent advances to understand their role during virus infection.

ROLE OF GLYCANS IN VIRAL INFECTION

To ensure replication and avoid immune recognition within the extracellular medium, it is essential for viral particles to enter susceptible cells. Furthermore, conditions within the extracellular medium can somehow 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 caps 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 mucous). Using those cell surface

oligosaccharides as first attachment factors drastically enhances viral infectivity (5), mainly by concentrating viral particles on the cell surface and by facilitating the subsequent binding towards specific virus receptors for internalization (6). In addition to that, some cell surface carbohydrates can also be used by viruses directly as entry receptors (7).

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 they are attached by. Glycoconjugates consisting in 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 made 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 species is, among other aspects, influenced by its very first binding step, which is frequently involving glycans present at the surface of susceptible cells (2). Therefore, the tropism of a viral specie for a tissue or a particular cell type is primarily affected by the type of glycans displayed on their surfaces and the ability of viral proteins to recognize them. In addition to the presence of a specific glycan, the type of glycosidic linkage can also influence the host range specificity, as it is the case for influenza viruses (12, 13).

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

Glycan types

***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 residue through 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 typically found to be terminating branches of *N*-glycans and *O*-glycans (14) (**Figure 1a**). 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 through acetylation, methylation, hydroxylation and sulfation (3). Those modifications can be crucial in the context of virus tropism as for example the preferred ligand for influenza A and B viruses is Neu5Ac while they do not bind to 9-*O*-acetyl-Neu5Ac residues, whereas the reverse is true for the influenza C virus (15). In addition to that, 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 *via* α2,3- or α2,6-glycosidic linkages to galactose or *N*-acetylgalactosamine at the tail end of glycan chains (16). This allows the protruding functional groups (hydroxyl, glycerol, carboxylate and N-acetyl) to be easily accessible for interactions with viral particles (17). A number of viruses therefore 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 was often suggested as a species barrier between avian and human hosts. While these specific glycans are certainly important, recent glycomics studies started to unravel a more complex diversity of sialylated attachment factors and sialic acid derivatives (18-20). Further examples of sialic acid-dependent viruses are parainfluenza viruses (13), reoviruses (21), polyomaviruses (22) and coronaviruses (23) (**Figure 1b**).

Histo-blood group antigens 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 1a**). These terminal structures on glycan chains were shown to be used as attachment factors by human noroviruses (25) and by a rotavirus switching its specificity from sialylated glycans (26) (**Figure 1b**).

Glycolipids containing terminal sialic acids can also play the role of attachment factor and/or receptor for viruses binding to sialylated moieties, as exemplified by Simian virus 40 binding to ganglioside GM1 (27)

Glycoaminoglycans (GAGs). 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 very abundant on the surface of practically all mammalian cells (28) and constitute the main form of glycans within the glycocalyx (**Figure 1a**). 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 carry a high overall negative charge density. Even though their structure consists of repeating disaccharide units, GAG chains are highly heterogeneous. This is mainly due to the postsynthetic addition of sulfate groups to the constituents of the chains. The sulfation patterns on the GAG chains is of main importance and interest, as sulfation motifs and their location on the chains are tissue-specific and related to the development state of the cell, as well as disease-induced physiological conditions (29). It has also been shown that the sulfation pattern of GAG chains influences their interactions with viruses (30). Binding studies of herpes simplex virus 1 (HSV-1) to different GAGs showed that the degree of sulfation of the GAG directly influences the binding affinity of HSV-1 (31).

Proteoglycans can be classified based on their cellular and subcellular localization as well as genetic homologies (32). Among the ones localized at the cell surface, two main proteoglycans are involved in the virus attachment to cellular surfaces: 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, transferrin, growth factors…). Therefore virus binding to these receptors serve as an entry ticket to the interior of the cell through 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 called glycosylphosphatidylinositol (GPI). Glypicans are therefore exclusively extracellular proteins that take a globular shape (6). In contrast to syndecans, the anchorage region of GAG chains on the proteoglycan is located near the juxtamembrane domain (32), bringing virus particles in close vicinity 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) bear a high amount of negative charges and are the main GAGs used by viruses as attachment factors (2). HS and CS are both made of 10 to 100 repeating units (36) but differ in their type of disaccharides, their sulfation pattern and their location on the cell surface. HS chains are mainly attached to syndecans and are therefore situated relatively close to the cell surface. On the other hand, while CS chains can also be found attached to syndecans, the vast majority of them 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 have been shown to serve as receptors for several viruses, including HIV-1 (37), herpes simplex virus (38) and human papillomavirus (39) (**Figure 1b**).

As viral membrane proteins are produced using the host cells machinery and travel through the secretory pathway, they also receive host-dependent glycan modifications. Depending on their location, these can have a strong impact on viral reproduction and pathogenicity. Besides their influence on immune recognition (40), protein folding and conformation (41, 42), glycosylations where shown to be important modulators for virus-cell binding. Glycosylations of the influenza virus hemagglutinin were shown to affect folding and pH stability when near the stalk and cleavage site of the protein (41, 42). When near the binding site, they alter the affinity for sialylated attachment factors (43) as also shown for avian coronaviruses (44).

Virus cell binding and entry

Due to their ubiquitousness, viruses have evolved to use glycans as attachment factors. Examples include hepatitis C virus (6), influenza virus (13), human immunodeficiency virus (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 glycoproteins have evolved to recognize specific glycan patterns on the cellular surface of target cells. As the constituents of the glycocalyx bear a substantial amount of negative charges, 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 and/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 their glycan-binding proteins (54). This allows viruses to form multiple simultaneous interactions with cell surface sugars, *i.e.* multivalent interactions (55). Engaging simultaneously multiple viral proteins allows to compensate for the inherently weak affinity of the glycan-protein interactions and to strengthen the binding of viral particles towards cellular 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 cellular surfaces. Multivalent binding was reported for a wide variety of viruses indicating a principal mode of cell attachment (55, 56). Studies on adenovirus ad-37 showed that the affinity of virus binding towards sialic acids was increased 250-fold when bivalent interactions were involved, compared to the monovalent binding (51, 57). As another example, studies performed with recombinant influenza A hemagglutinin could show how multiple glycans can be bound by individual hemagglutinin trimers depending on their origin host (58). This study indicates the requirement of a dense glycan organization on cellular surfaces. Interestingly, super-resolution microscopy could recently visualize the presence of dense sub-micrometer glycan nanodomains, which could indeed provide a multivalent virus-binding platform. Following this hypothesis, single-virus tracking supported 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 very location where the initial interaction took place. This involves spatial confinement of the virions waiting on the cell surface for endocytosis or the recruitment of a (co-)receptor (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 in order to reach a specific location on the plasma membrane enabling virus internalization (61, 62). Studies showed that this strategy is used by influenza A virus, mouse polyomavirus and simian virus 40 for example (63-65). The structural basis for this explorative motion often remains elusive due to the nanoscale size of the virus-cell interface. Here, super-resolution microscopy could 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 can also be achieved thanks to cell surface glycans. For example, stable binding of Simian virus 40 to cellular surfaces is highly dependent on the motion of virions bound to the glycosphingolipid GM1, in order to gather a sufficient amount of receptors (66). Motion of influenza A virus was also studied *in vitro* and shown to be highly dependent on the modulation of the number of sialylated receptors engaged by hemagglutinin viral proteins and on the action of the cleaving enzyme neuraminidase (67, 68). Recently, the polarized intravirion organization of hemagglutinin and neuraminidase was suggested to be important for viruses to avoid immobilization and provide the ability 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 diffusivity 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 herpes simplex virus 1, which displayed different diffusion coefficients depending on the GAG sulfation pattern (31). In this context, the number of physical bonds

between the viral particles and the receptors directly influences its diffusion potential. While multiple parallel interactions would allow strong virus attachment, a low number of bonds could be preferred to allow diffusion towards specific entry receptors.

Glycan-based antiviral strategies

Since attachment of viral particles to glycans is usually the very first step of infection for a large number of virus families, interfering with this process could be of prime interest to 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 just those molecules to physically interfere with virus-cell interactions (72). Therefore, antiviral compounds that compete with cell surface glycans for virus-binding have great potential as therapeutic agents in the fight against viral pathogens (73). As stated above, 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, a number of multivalent inhibitors have been developed and studied to interfere with attachment of different viral families. Various polyvalent assemblies targetting the hemagglutinin protein have shown potency as inhibitors of influenza A virus attachment, with dramatic enhancement of efficiency as compared to their monovalent counterparts. These structures include functionalized polymers (74), liposomes (75) as well as solid and 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 have been shown to decrease GAG-mediated virus attachment *in vitro*. Recently, Cagno *et al.* introduced heparan sulfate-coated nanoparticle structures that showed virucidal activities also *in vivo* (80). Moreover, Munoz *et al.* successfully synthesized giant multivalent glycofullerene structures inhibiting the infection by pseudotyped Ebola viral particles (81). Unfortunately, despite these promising results, antiviral effects remain rarely observed *in vivo* (82, 83) and often correlated with low biocompatibility and high cytotoxicity. Some of these issues have been successfully adressed recently, revealing that an optimal scaffold size and geometry play important roles in modulating the efficacy and host compatibility of synthetic compounds also *in vivo* (76, 84).

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

EXPERIMENTAL APPROACHES TO STUDY VIRUS BINDING

Many techniques have already been successfully applied to study virus binding to cell surface glycans on purified systems. The current challenge is to address this fundamental question under physiologically relevant conditions. New developments include imaging and tracking techniques, such as (cryo-)electron microscopy (cryo-EM), fluorescent-based techniques as well as biophysical techniques, including optical tweezers (OT) and atomic force microscopy (AFM). In the frame of this review, we will give an overview of these techniques with their strengths and weaknesses.

Virus-binding assays

Early virus-binding assays relied on the virus' ability to agglutinate (*i.e*. clump) cells such as heavily glycosylated erythrocytes [*i.e*. hemagglutination (85)]. While this type of assay is still used to quantify viruses and probe their specificity (*e.g*. influenza), more recent approaches take advantage of presenting synthetic glycans. In general, a virus-binding assay relies on monitoring viral adsorption to a known molecule of interest (*i.e.* specific receptor, antibody, glycan…). A detection method is used to determine the presence and extent of virus binding towards the molecule. Many virus binding assays following this principle that differ in the presentation of the molecule and the detection method used to monitor virus binding exist (*e.g.* liquid or solid phase, radioactive or fluorescent assays). We will here give an overview of the most popular virus-binding assays.

Solid-phase binding assays are a widely used approach to study interactions *in vitro* and have the advantage to be fast and relatively simple. These assays are based on testing binding of virions in solution to an immobilized receptor on a 'solid phase' (e.g. protein, glycan…). Binding is usually monitored using fluorescence-based detection methods (immunofluorescence analysis, ELISA …)

or radioactive methods (86). 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 binding to glycans, glycan arrays have been recently developed and 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 (87). In such screens, recombinant viral proteins expressed soluble or immobilized on nanoparticles as well as whole viruses are analyzed for binding to a wide distribution (> 600) of synthetic glycans that have been robotically printed on glass slides (88). Glycan arrays differ in their glycan composition (89) and the mode of glycan immobilization (90), which have been previously compared in-depth (91). In the past decade, glycan arrays have become the standard for many virus-glycan studies, *e.g.* for adenovirus (57), influenza virus (92), reovirus (21) and polyomavirus (93). 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 to determine the 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 towards the presentation of such natural glycan libraries, by harvesting glycans from cells or tissues and imprinting them in a glycan array format (94).

To better mimic physiologically relevant conditions, virus-binding assays can also be performed directly on (living) cells. In these assays, viruses are directly incubated with a confluent layer of cells (preferably at 4°C to avoid endocytosis) and virus binding is monitored through fluorescent labeling of viruses (95). Labeled viruses are usually obtained *in vitro* by incorporating radioactive nucleotides (96), radioactive lipids for enveloped viruses (97) or by chemical modification of exposed surface glycoproteins with fluorescent dyes (98). However, in the latter case, the labeling of the virus can alter its binding due to changes of 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.

Structural insights into virus-glycan interactions

Structural studies provide a relatively fine knowledge of the structure of viral glycoproteins involved in binding to host attachment factors as well as insights into their entry mechanisms such as snapshots on membrane fusion mechanisms. A wide variety of methods enables to address structural insight ranging from recent techniques such as electron microscopy (EM) or cryo-EM techniques to more classical approaches like nuclear magnetic resonance and X-ray crystallography.

Electron microscopy is an important tool in virology. The first pictures of viruses go back to the late 1930's (99) thanks to the transmission electron microscope (TEM). The high resolving power of TEM permits studies at the nanometer scale, providing direct images of viruses for diagnosis and research. Since then many improvements of the technique were achieved in terms of resolution but also to maintain the biological sample in their native-like conditions. In this context, the development of cryo-EM has been particularly remarkable with a resolution approaching nowadays that of X-ray crystallography without the need to resort to crystallization, fixation, or large amounts of biological material. Single-particle cryo-EM reconstruction is typically applied to purified viral proteins or whole viruses and macromolecular complexes with the aim of resolving the organization of multi-component assemblies. Being mostly applied to resolve structures of a variety of viruses in the sub-nanometer range [reviewed in (100)], it has also 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 (101); the discovery of a glycan shield of a coronavirus spike protein together with its epitope masking (102); the description of the structural basis for human coronavirus attachment to sialic acid receptors (103). In order to gain insights into complex specimens or viruses undergoing dynamic processes, cryo-electron tomography (cryo-ET) is another electron-beam based method. Cryo-ET extends imaging in 2 (2D) to 3 dimensions (3D) where the specimens are tilted typically by 1 or 2 degrees from about −70° to +70° along an axis perpendicular to the electron beam. A series of 2D projection images are collected, aligned with each other and then back-projected to generate 3D images (104). This technique has been applied to characterize a variety of viruses, as

366 reviewed in (105) and has provided structures of glycoproteins *in situ* on the surface of virions,
367 including HIV-1 Env [reviewed in (106)] and influenza hemagglutinin (54). Since these approaches
368 depend on the detection of morphologically recognizable structures or on crystals of purified
369 material, the application of these techniques is mainly restricted to the analysis of free
370 extracellular virions, or of particles attached to or being released from a host cell.
371 Down to the atomistic level, interactions between viral glycoproteins and glycans have been
372 captured in numerous X-ray co-crystal structures of such complexes. While not being a new
373 technique, X-ray crystallography has undertaken a substantial development in nearly every step
374 of the crystallographic and protein purification process, accelerating structural determination
375 (107). X-ray crystallography has been successfully employed to show that reovirus sigma1 capsid
376 protein binds differently to sialylated glycans depending on the studied serotype (T1 or T3) (21,
377 108), or to study influenza virus binding to differentially linked sialic acids (109, 110). Other
378 examples include identifying the structural basis of adenovirus (51, 57), coronavirus (103) and
379 rotavirus (111) binding to glycans. An interesting application of crystallographic data is their use
380 in simulated force spectroscopy experiments (112, 113). Knowing the exact molecular
381 coordinates of a protein-ligand complex, allows to apply a specific pulling force and then visualize
382 the complex unbind. By recording a wealth of structural and quantitative data, this method allows
383 to study unbinding pathways bond-by-bond. As recently applied to an influenza hemagglutinin –
384 glycan complex, simulated force spectroscopy revealed a variety of unbinding pathways and
385 dynamic binding/un-binding behavior of the interaction (114). However, X-ray crystallography
386 requires working on isolated proteins and thus represent only a single picture of many
387 conformations accessible to the protein.
388 To overcome this issue and to map virus-glycan interactions in real-time and in solution, nuclear
389 magnetic resonance (NMR) spectroscopy appears as a good alternative. This technique provides
390 additional information on dynamics of glycan motion in the free and protein-bound states. In its
391 basic form, saturation transfer difference (STD) NMR, experiments require no isotope labeling
392 and only relatively small amounts of virus or viral receptor-binding protein. However, the virus
393 (protein) - glycan complex has to have clear and detectable chemical shift distinct from the
394 unbound material, and often NMR techniques work best for relatively low affinity interactions. In

the field of studying virus-glycan interactions, NMR analysis of different glycan receptors bound to different viral binding proteins gave complementary information on glycan-protein contacts from the X-ray co-crystal structures (21, 51, 110) or served as a stand-alone technique to reveal the molecular basis of glycan recognition by calicivirus (115).

Finally, modern mass spectrometry (MS) techniques enable to work under native conditions opening the possibility to study the dependence of glycan-binding on virus assembly state (116). Also conformational changes can be mapped with hydrogen/deuterium exchange MS (HDX MS) as exemplified for hepatitis B virus (117).

These techniques lack insights into the dynamics yielding snapshots rather than capturing the whole dynamic information essential for a full understanding of the binding and subsequent infection process. Approaches to elucidate the thermodynamics and kinetics of virus binding to cell surface glycans in a dynamic context are discussed in the next section.

Insight into the thermodynamics and kinetics of virus binding

Besides the structural studies of virus-glycan interactions, there is a crucial need to study the thermodynamic and kinetic parameters regulating virus binding to their receptors. Especially in the context of drug design and protein mutability, a better understanding of the interplay between thermodynamics and kinetics will translate to better decision making for selecting drug candidates (118). Several techniques are discussed below enabling to study the kinetics and thermodynamics of virus-glycan interactions.

Surface plasmon resonance (SPR) is a label-free biosensor technique for studying interactions in real time. SPR exploits the fact that the binding of biomolecules on the sensor surface will result in a change of 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 device enables to determine association and dissociation kinetics. This methodology was used to explore *e.g.* influenza virus – glycan interactions (119), and to further monitor the inhibition of influenza virus infection by sialic acids (120). The main limitation of this method is the poor control of glycan density and orientation, which was suggested to 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 lead to an underestimation of the dissociation rate due to the formation of multiple parallel bonds. Besides SPR, isothermal titration calorimetry (ITC) is one of the most rigorous means of defining the equilibrium-binding constant between a glycan and a viral binding partner and is based on the detection of the heat released or absorbed during a biomolecular binding interaction down to the nanomolar range. ITC has been applied as a complementary technique *e.g.* to determine the binding stoichiometry and equilibrium dissociation constant between HIV-1 and different glycoproteins (121) or for the identification of a trisaccharide containing α2,3-linked sialic acid as a receptor for mumps virus (122).

Biolayer interferometry (BLI) as well as microscale thermophoresis (MST) are two more recent affinity-based techniques, increasingly used for analyzing virus-glycan interactions in the past years. BLI is a label-free optical and analytical technique that allows to study interactions between biomolecules on a 2D biosensor surface. This analytical 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 upon laser-induced heating (123). Both techniques allow monitoring interactions of intact virions or isolated glycoproteins in real time, providing the ability to monitor binding specificity, rates of association and dissociation, or concentration, with high precision and accuracy. Recently, BLI has been used to analyze the kinetics of the interplay between influenza A virus hemagglutinin and neuraminidase during infection (124) or to validate the findings on glycan-mediated enhancement of reovirus-receptor binding (125).

Optical microscopy and spectroscopy towards dynamic studies

To address glycan organization and dynamic processes on living cells, fluorescence microscopy is a method of choice. In the last decade, different fluorescence-based techniques have empowered virologists 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. Moreover, study of post-entry steps revealed how the cytoskeleton and its motors support virion trafficking and uncoating, leading to the delivery of its genetic cargo (126).

High-resolution optical techniques such as confocal laser scanning microscopy (CLSM) and spinning-disc confocal microscopy enable to localize and even co-localize virions during their interaction with cells. In a recent study, CLSM was used to show that influenza A virus co-localizes with GM1-based lipid rafts during virus attachment to the cell surface (127). Furthermore, dynamic studies at high temporal resolution (~ms) allow single-virus tracking, as applied to image early infection steps of Sindbis virus (128), influenza A virus (129), MLV (130) and many other viruses, as reviewed in (77). For an in-depth review on the different microscopy methods please refer to (131), and on imaging, tracking and computational analyses of different viruses and their cellular attachment, entry and replication please refer to (132, 133).

With its high sensitivity, total internal reflection microscopy (TIRF) enables to obtain high-contrast images, at low background, reduced cellular photo-damage and rapid exposure times; therefore compatible with live-cell imaging at high-temporal and high-spatial resolution. Due to the intrinsic properties of the evanescent field, the TIRF excitation of fluorophores decreases exponentially with distance from the coverslip, meaning that only fluorophores close to the coverslip (*e.g.* within ~100 nm) are selectively illuminated. Thus, TIRF appears as a useful tool to track single virions movement on artificial as well as cellular membranes *in vitro*. It has been extensively used to study influenza A virus interacting with specific glycans, which in turn determine virus binding, infectivity, transmissibly, pathogenicity and host specificity (67, 68).

More recently, super-resolution microscopy like structured illumination microscopy (SIM), single molecule localization microscopy (SMLM) techniques such as PALM/(d)STORM and stimulated emission depletion (STED) nanoscopy have lowered the effective resolution of fluorescence microscopy to the level of subviral structures (134). Thereby, they greatly expanded the possibilities for detailed investigation of virus-cell interactions. Although cellular glycans remain difficult to label, traditionally labelling using lectins or click chemistry has enabled a close-up view of the cells glycocalyx. Plant lectins provide an easy way to determine the presence and nanoscale organization of a specific glycan on cultured cells or tissue (59, 135). On A549 cells, sialylated influenza virus attachment factors could be shown to form nanoscale clusters, which could be linked to viral movement and receptor activation (59). As an alternative, feeding the cells with a

modified glycan precursor allows to fluorescently modify glycans without the need of an additional high-affinity probe (136). Combined with super-resolution microscopy, such bio-orthogonal labelling was used to study the lateral organization of glycans as well as the thickness of the cellular glycocalyx (137).

Spectroscopy based on fluorescence resonance energy transfer (FRET) allows to study interactions in virus infection at a very small spatial scale (<10 nm). FRET is based on the capability of the near-field energy transfer (low nm range) between two light sensitive molecules (chromophores). This spectroscopy technique has been applied to study glycan-virus interactions for norovirus (138) or HIV-1 (139). Fluorescence correlation spectroscopy (FCS) is a correlation analysis of fluctuations of the fluorescence intensity of fluorescent particles or complexes (virus-glycan) in solution and has been used to prove that the envelope glycoprotein mobility on HIV-1 depends on virus maturation state (140).

Taken together, all these methods are able to provide deep insights into the first binding steps of viruses to cell surface glycans at high temporal and spatial resolution. Nevertheless, they bear also several disadvantages compared to other methods, *e.g.*: (i) a fluorophore needs to be attached to the molecule of interest, which can alter the structure of the molecule and in turn influence their binding behavior, (ii) phototoxicity and photobleaching can raise to some difficulties in the interpretation of the data, (iii) high demand on data post processing.

Single-virus force spectroscopy techniques

Compared to conventional ensemble methods, single-virus techniques offer distinct advantages. First, conducting many sequential measurements enables to determine the distribution of molecular properties of inhomogeneous systems. Second, being direct records of the stochastic fluctuations of the system, single-molecule trajectories provide dynamic and statistical information, which are often hidden in ensemble-averaged results. Finally, they permit real-time observation of rarely populated transients, which are difficultly captured using conventional methods (141). In this context, atomic force microscopy (AFM) (**Figure 2a**) and optical/ magnetic tweezers (**Figure 2b**) enable to measure binding forces at the single-molecule level and with high-temporal resolution (142, 143). Thanks to grafting protocols, single virions can be attached to the AFM tip apex or on beads enabling to probe the interaction with specific receptors and/or glycans

(114, 144, 145). Such experiments allowed the characterization of the binding behavior of intact virions with receptors to be performed in vitro on purified receptors or even directly on living cells [reviewed in (146)]. These technologies have been already applied in the context of virus-host interactions for many viruses including *i.a.* HIV-1, rhinovirus, influenza virus, herpesvirus and reovirus (114, 125, 144, 147-150). In terms of unravelling first virus binding steps to cell-surface exposed glycans, Chang *et al*. and Dubrovski *et al*. performed single-molecule analysis of HIV-1 interacting with glycoprotein receptor on living cells and were able to monitor early fusion dynamics (**Figure 2a-d**) (147, 148). In another study, Sieben *et al*. (114) elegantly combined optical tweezers, AFM-based single-virus force spectroscopy, and molecular dynamics simulations to study the binding of influenza to cells presenting a different surface distribution of α2,3- and α2,6-linked sialic acid (**Figure 2e-g**).

Mapping virus binding sites using AFM and fluorescence microscopy

Although AFM alone has proven to be an effective tool for quantitatively characterizing the forces involved in virus-cell interactions, it lacks optical correlation to identify the host cell surface receptors involved. To address this challenge, correlative approaches of AFM with fluorescence microscopy have recently opened new avenues in the elucidation of the first binding steps of viruses to animal cells, which will be introduced in detail together with selected examples in the next section.

As a proof-of-concept, Alsteens *et al.* 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 receptors distribution thanks to the confocal microscope (95). Applied to a model virus (engineered rabies virus carrying the envelope protein of avian sarcoma leucosis virus), specific interactions with cells expressing viral cognate receptors [avian tumor virus receptor A (TVA)] were mapped on the living cells enabling to localize at high-resolution the binding site and quantify the kinetic and thermodynamic parameters of the interactions. Simultaneously, confocal microscopy enabled to differentiate between co-cultured control cells and cells expressing fluorescently labeled TVA. The study demonstrated that the EnvA pseudotyped rabies virus quickly (≤1 ms) establishes specific interactions with single TVA receptors. The results also suggested that one TVA receptor of the cell surface binds one subunit

of the trimeric viral glycoprotein EnvA with an intrinsic low affinity and that the affinity increases considerably with other TVA receptors binding to the same EnvA suggesting a positive allosteric modulation of the EnvA–TVA bonds.

The same approach has been very recently used to elucidate important aspects of the multivalent binding of a gammaherpesvirus to glycosaminoglycans exposed on living cells during the early step of the infection (149). Using AFM, Delguste *et al*. showed that gp150, the major envelope glycoprotein of Murid herpesvirus-4, plays a regulating role upon GAG binding by other viral glycoproteins. By combining experiments on purified GAGs and on living cells, they extracted quantitative information related to the binding force of single viral particles towards GAGs, and gained unprecedented insights into the tight regulation of virus attachment to cell surfaces (**Figure 3**). Force probing of virus binding to GAGs revealed that interactions with a lower valency were preferred by WT virus particles, while gp150-deficient virions showed a higher tendency to engage simultaneous multiple interactions suggesting a putative regulatory role of the glycoprotein gp150 towards GAG-binding. By minimizing multivalency between GAGs and viral glycoproteins, gp150 could facilitate virus lateral diffusion on the cell membrane, allowing the virus particle to search for its specific receptor for entry. 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 virion egress.

A salient question in virology is the role of the attachment factors in the context of virus infection. Using AFM-based single-virus force spectroscopy, reovirus binding to cell surfaces has been investigated with a focus on the attachment of σ1 viral glycoprotein to both α-linked sialic acid and JAM-A receptors (125). Combining *in vitro* approaches on both purified receptors and on living cells, the respective contributions of the α-SA and JAM-A receptors were quantified. Based on the results obtained on purified receptors, the number of bonds established on living cells can be determined for the early binding step. Surprisingly, the study put in evidence that the initial σ1 binding to α-SA acts as a trigger that enhances the overall avidity of σ1 for JAM-A (**Figure 4**). Comparison with infectious subvirion particles (ISVPs) in the absence of α-SA suggested that α-SA binding to intact reovirions triggers a conformational change of the σ1 glycoprotein to a more

extended conformation, underlying a direct interplay between attachment factors and specific receptors.

As described in this review, low affinity interactions with glycans are essential for numerous virus species to ensure the achievement of their viral life cycle. Clearly identifying the binding partners involved together with their characteristics would increase the chances to precociously inhibit infection. New characterization techniques allow gaining insights into the molecular nature of these interactions and could provide crucial information to implement new therapeutic approaches or identify new targets for vaccination.

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 virus displacement on the plasma membrane, while the simultaneous engagement of multiple viral proteins allows stabilizing and enhancing the binding strength of a virus towards cell surfaces.
4. Synthetic compounds capable of performing multivalent interactions with virions are of primary interest to develop antiviral drugs blocking viral attachment to cell surfaces.
5. Virus-binding assays are relatively simple techniques allowing to monitor the binding of viruses to specific molecules, while electron microscopy, X-ray crystallography and nuclear magnetic resonance are methods that allow determining the fine structure of viral proteins involved in glycan binding.
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 towards their target molecules on cell surfaces, allowing to gain insights into the dynamics of the interactions.

7. Fluorescence microscopy techniques provide deep insights into the virus binding events on cell surfaces at high temporal and spatial resolution, as well as dynamic information upon virus uptake.
8. Single-virus force spectroscopy techniques such as optical/magnetic tweezers and atomic force microscopy enable to measure binding forces of virions towards glycans at the single-molecule level providing unprecedented information on 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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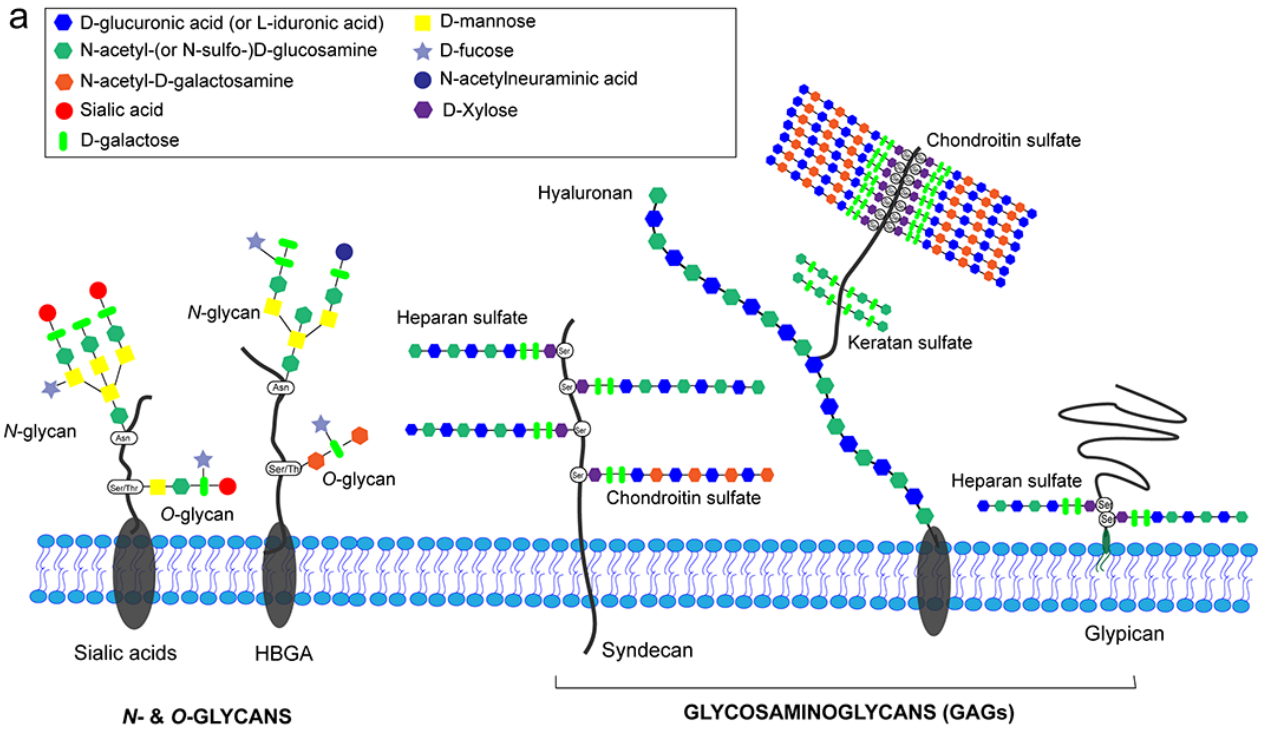
Reference Annotations

- (12) Overview of sialic acid receptors of different virus families and their influence on viral tropism.
- (32) Classification of proteoglycan gene families based on cellular location, protein homology and protein cores.
- (55) Description of inherent characteristics of polyvalent interactions mainly regarding their strength compared to monovalent interactions.
- (60) Review of the first virus binding events to cell surfaces and subsequent diffusion and uptake.
- (80) Presentation of heparan sulfate mimicking antiviral nanoparticles capable of irreversibly altering the structure of virions.
- (91) Comparison of different glycan microarray formats.
- (118) Review on the current understanding of thermodynamic and kinetic data used in drug discovery.
- (131) Overview about super-resolution microscopy methods to study virus-glycan interactions.
- (143) Comprehensive review about single molecule force spectroscopy tools.
- (146) Review about the capability to unravel molecular details about virus infection using single-virus force spectroscopy.

Definition list

- Glycans: Carbohydrate structure made of mono- or polysaccharides that can be free or covalently linked to a core molecule.
- Attachment factors: Cell surface structures that allow virus attachment and promote binding to specific entry receptors.
- Entry receptors: Cell membrane structures that trigger virus entry inside permissive cells upon virus binding.
- Binding affinity: Strength at which a ligand binds its receptor. Expressed as the ligand concentration that occupy half the receptors at equilibrium.
- Random diffusion: Random movement of a particle driven by a concentration gradient and that depends on particle random walk mechanisms.
- Directional displacement: Constrained movement of a particle along specific cellular structures or pathways.
- Antiviral drugs: Medication used to treat viral infections by blocking the viral cycle at a specific step (cell binding, genome replication,…).
- Multivalent inhibitors: Molecules interfering with virus binding to cell surfaces using structures displaying multiple copies of glycans.
- Glycofullerene: Spherical macromolecular carbonaceous structure capped with glycan moieities.

- Cryo-electron microscopy: Electron microscopy technique applied to frozen samples allowing high-resolution imaging of biomolecules.
- X-ray crystallography: Technique to determine the three-dimensional structure of (macro-) molecules down to atomic resolution.
- 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.
- Fluorescence microscopy: Optical imaging technique relying on the spontaneous emission of light by specific fluorescent dyes upon excitation.
- Atomic force microscopy (AFM): Microscopy technique using a fine tip attached to a cantilever to image various surfaces and force-probe their constituents.
- Optical/ magnetic tweezers: Precision biophysical tools allowing the measurement of the mechanical properties of biomolecules thanks to an optical/magnetic trap.
- Force spectroscopy: Technique allowing the study of interactions and binding forces between individual molecules down to the single molecule level.



b

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

Figure 1. Glycans used as attachment factors or entry receptors by different viral species. (a) Mostly used glycoconjugates from the cell surface are sialic acid terminated *N*- and *O*-glycans, glycosaminoglycans (heparan sulfate, chondroitin sulfate, hyaluronan and keratin sulfate) and histo-blood group antigens. (b) Viruses from different families use one or two types of glycans as attachment factors and/or receptors depending on the virus type. Example of viruses are provided in brackets. For some viruses, it is still not clear whether binding to sialylated glycans is sufficient to induce virus internalization (ex: influenza A virus for which other specific receptors were identified, such as epidermal growth factor receptor).

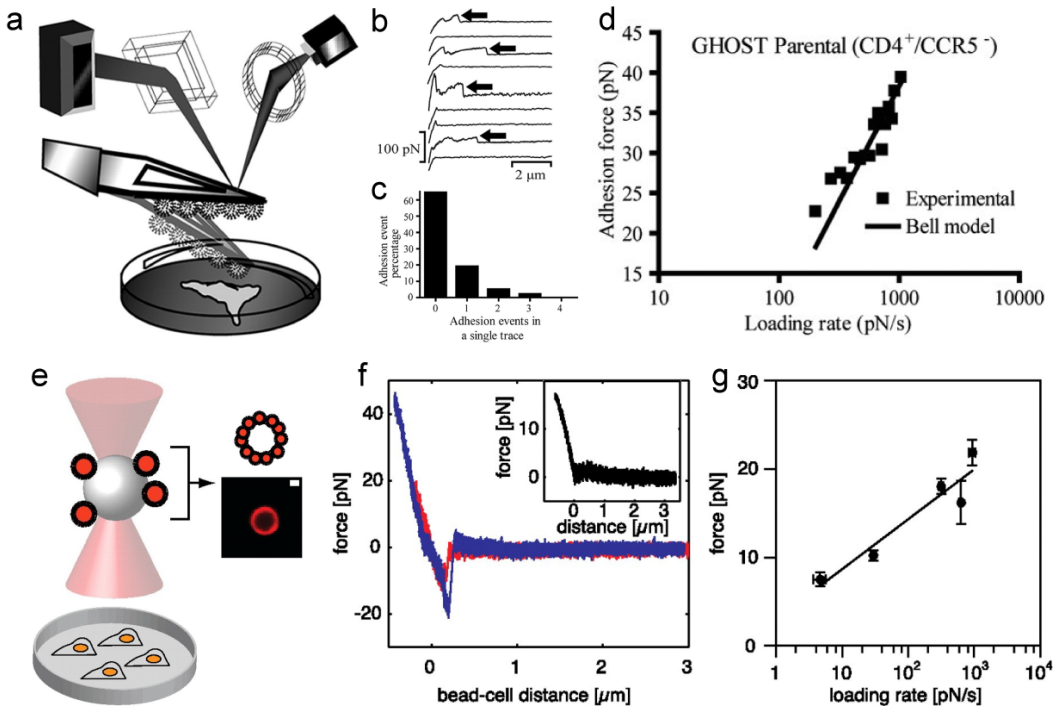


Figure 2. Single-virus force spectroscopy using AFM (a-c) and optical tweezer (d-f). (a) AFM-based force spectroscopy is used to monitor HIV-1 interactions with cell surface CD4 receptors. (b) Representative force-distance curves during retraction of the cantilever. Rupture events between a single virion and cell surface receptor is marked by an arrow. (c) Probability of bond formation between a virion and a host cell receptor. (d) Dynamic force spectrum (DFS) showing binding forces as a function of the loading rate (i.e. velocity at which the force is applied). Fit of this curve using Bell's model yields the kinetic parameters describing the energy landscape of this interaction. (e-f) Optical tweezers are used to characterize the attachment of influenza virus to host cell receptors. (e) Schematic of the experiment showing influenza virions adsorbed on a polystyrene bead and trapped in an optical field. Confocal microscopy image is shown to validate the virions adsorption (right, scale bar: 0.5 µm). (f) Binding forces were 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. [panels adapted with permission from (148) and (114) respectively].

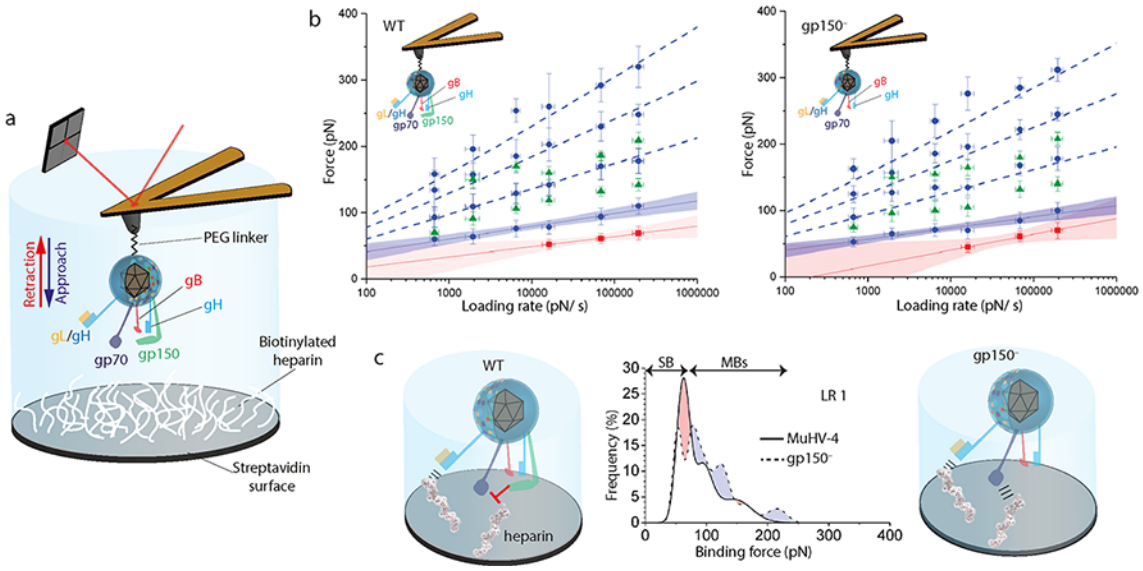


Figure 3. Murid herpesvirus-4 binding to heparin studied by AFM. (a) Wild-type (WT) virions and virions lacking the glycoprotein gp150 were grafted to AFM tips to probe the interaction with surfaces functionalized with heparin. (b) Comparison of DFS plots obtained for both virions (WT and gp150-) revealed no difference, suggesting that gp150 does not play a direct role in heparin binding. (c) Force distribution analysis reveals that single bonds (SB) occur more frequently with WT particle, while multiple bonds (MBs) are more probable for gp150- virions, suggesting a role of regulation in the number of bond established between the virions and GAGs. (panels adapted with permission from 136).

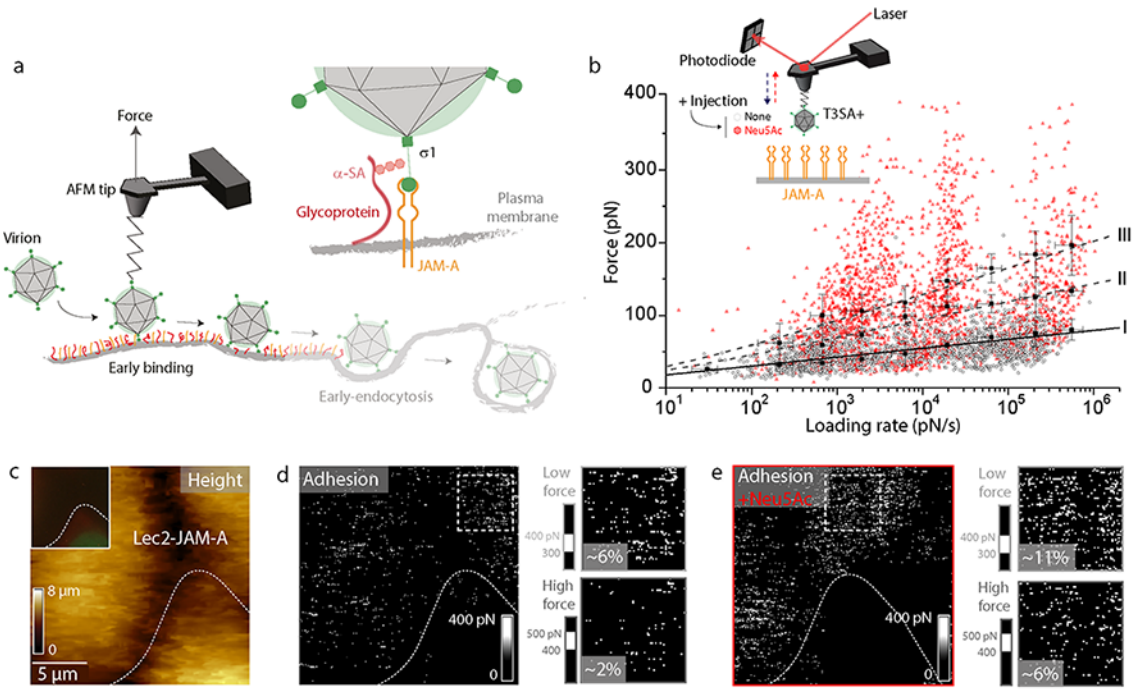


Figure 4. Interplay of attachment factor and specific receptors during reovirus binding to cells surface. (a) Schematic of reovirus probing by AFM to cell surface receptors. (b) DFS plot before (grey) and after injection of Neu5Ac (red) indicating a shift towards higher binding forces (i.e. multivalent bond formation) between reovirions and JAM-A receptors. (c-e) Experiments on living cells reveal same behavior tendency. (c) AFM height image of Lec2-JAM-A and Lec2 cells deficient in JAM-A tagged with mCherry and GFP (fluorescence image shown *in insert*) and corresponding adhesion images before (d) and after (e) injection of Neu5Ac. The respective zooms of the adhesion maps show an increase of higher interaction forces (in the high range 400-500 pN), revealing that the initial σ1 binding to sialic acid acts as a trigger that enhances the overall avidity of σ1 for JAM-A.