Chapter 10

Quantification of Receptor Association, Dissociation, and NA-Dependent Motility of Influenza A Particles by Biolayer Interferometry

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Abstract

We describe a method for real-time analysis and quantification of influenza A virus (IAV)–receptor interactions by biolayer interferometry (BLI). Biotinylated synthetic sialoglycans or sialoglycoproteins (biotinylated or Fc-tagged) were immobilized on the tip of biosensors (coated with streptavidin or protein A) that were subsequently dipped into IAV particle solutions in 96-well plates. Association and/or dissociation of IAV particles was recorded in consecutive steps in buffers of choice. From the association and dissociation curves, parameters can be derived that describe IAV particle–receptor interactions in absence or presence of neuraminidase activity. Overall, the method provides a quantitative description of the hemagglutinin-neuraminidase balance that determines the interaction kinetics of IAV with specific sialoglycan receptors.

Key words Influenza virus, Hemagglutinin, Neuraminidase, Association, Dissociation, Virus motility, Sialoglycan, Sialoglycoprotein, Biolayer interferometry, Binding kinetics

1 Introduction

Influenza A virus (IAV) binds to sialic acids (SIA) on glycan receptors present on the host cell surface by its envelope-embedded hemagglutinin (HA). The viral neuraminidase (NA) is a receptor-destroying enzyme that cleaves terminal SIAs from glycans to enable virus release from decoy receptors in mucus and on the cell surface and to prevent aggregation of newly budded virus particles at the end of the replication cycle. It has become clear that the HA binding affinity and specificity needs to be in balance with NA activity in order to support efficient infection of a specific host and cell type [1–4]. An essential component of this HA-NA balance is the multivalent binding mode of IAV particles to a receptor-coated surface. The intrinsically very low affinity of monovalent HA-SIA receptor interactions (K_D ~1–20 mM [5]) contrasts with

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the extremely high avidity observed for IAV particle–receptor surface interactions (K_D in picomolar range [2]). In absence of NA activity, this results in virtually irreversible virus binding, which is actually highly dynamic as individual HA-SIA interactions are rapidly formed and broken [6]. This provides the opportunity for NA to cleave temporarily unbound SIAs at the virus–receptor interaction surface. It was recently shown that NA activity causes virus motility resulting in viruses traversing the receptor-coated surface [6–8]. Only after gradually decreasing SIA density on the entire surface to a level that does not support virus binding anymore, virus dissociation takes place due to reduction of the number of simultaneous HA-SIA interactions. Recently developed biolayer interferometry (BLI) assays allowed direct analysis and quantification of the dynamic virus–receptor interactions. BLI is an optical label-free technology that, by analysis of changes in the interference pattern of white light reflected from a receptor-coated surface to which molecules or particles are binding in time, can record real-time binding and dissociation curves of IAV particles [5, 6, 9–13]. Unlike many endpoint assays that provide excellent insight into receptor specificity of HA and NA, BLI enables real-time analysis of the dynamics of virus association and dissociation in absence or presence of NA activity providing a quantitative assessment of the HA-NA balance. Here, we report protocols for BLI assays allowing quantification of HA and NA association and dissociation rates and HA-NA balance on synthetic glycans and glycoproteins.

2 Materials

2.1 Glycan Receptors

1. Biotinylated synthetic glycans and glycoproteins are available from several commercial resources (e.g., Sigma-Aldrich, Elicityl-oligotech, Sussex-research, Carbosynth, Dextra-UK) (*see* **Note 1**).
2. Expression plasmid encoding a recombinant soluble glycoprotein such as fetuin or the ectodomain of LAMP1 N terminally extended with a signal peptide and N terminally with a purification tag (e.g., Fc tag or His tag) and a biotin acceptor peptide (BAP; GGLNDIFEAQKIEWH [14]). Synthetic genes encoding such proteins can be acquired via several commercial vendors (e.g., GenScript) and cloned to a suitable expression vector (e.g., pCAGGS).
3. Expression plasmid containing a human codon-optimized gene encoding a BirA biotin ligase that is targeted to the secretory pathway by the addition of a N-terminal signal peptide [6]). Similar plasmids are commercially available (e.g., Addgene).
4. 293T cells (ATCC) or other cells useful for high-level expression of recombinant proteins (*see* **Note 2**).

5. Polyethylenimine (PEI, Polysciences).
6. Opti-MEM (Gibco) to be used as transfection medium.
7. Expression medium consisting of 293SerumFree medium (Gibco), Glutamax (Gibco, 100× stock diluted to 1×), Primatone (Sigma-Aldrich, 0.3% w/v), D-glucose monohydrate (Sigma-Aldrich, 0.2% w/v), NAHCO3 (Merck, 0.37% w/v), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), DMSO (Sigma-Aldrich, 1.5% v/v). Filter-sterilize the expression medium using a bottle-top vacuum filter (0.22-μM filter, Corning).
8. 1 M Tris–HCl, pH 8.0 (Invitrogen).
9. Protein A Sepharose Cl-4B (GE Healthcare).
10. Dulbecco's phosphate-buffered saline without Mg and Ca (DPBS, Lonza).
11. Elution buffer (0.1 M citrate acid pH 2.75, Merck).
12. Neutralization buffer (3 M Tris–HCl, pH 8.8).
13. Protease inhibitor: cOmplete, Mini Protease Inhibitor Cocktail (Roche).
14. Routinely used laboratory equipment and cell culture plastics.

2.2 Biolayer Interferometry Materials

2.2.1 Octet Equipment

1. Octet RED384 (Sartorius); 16 sensors can be run simultaneously in a 96-well or 384-well plate format; running temperature: ambient +4 °C to 40 °C. Other Octet types can be used as well.
2. A biosafety hood (e.g., a Chemcap Clearview™, customized by a local supplier to fit in the specific Octet equipment) in which the Octet is placed to allow analysis of BSL class II viruses.
3. Streptavidin (SA) biosensors and protein A (ProA) biosensors (Sartorius).

2.2.2 General Materials

1. 1 × PBS (+ calcium/magnesium; Lonza, Cat, No: BE17-513F) working solution.
2. Demineralized/distilled H_2O.
3. 10 mM oseltamivir carboxylate (Roche) stock solution.
4. 10 mM Glycine pH 2.0
5. Protease inhibitor: cOmplete, Mini Protease Inhibitor Cocktail (Sigma-Aldrich, 11836153001).
6. Black 96-well plates, half-volume (Greiner bio-one, Ref 675076).
7. Capto™ Core 700 (GE Healthcare Life Sciences, GE17-5481-01).
8. NanoSight NS300 (Malvern Panalytical) for Nanoparticle Tracking Analysis (NTA).

3 Methods

All procedures with viable viruses have to be performed according to the local biosafety level requirements. Propagation and handling of influenza viruses usually require a class II Biosafety cabinet and the OCTET machine needs to be placed in a (custom-made) class II safety cabinet. Alternatively, influenza viruses can be inactivated by UV irradiation (warning: overexposure will affect binding properties) before application in an OCTET machine.

3.1 Expression and Purification of Biotinylated Glycoprotein Receptors

1. Day 1. Seed the HEK293 T cells in appropriate cell culture flasks. The next day, the confluency should be 60–80%.
2. Day 2. Prepare the transfection mixture. For a T225 flask, pipet 2.0 ml Opti-MEM to a 10-ml tube, add 100 μl PEI, and mix rigorously, e.g., by using a Vortex Mixer, for at least 10 sec. Add DNA (20 μg of glycoprotein expression plasmid and 20 μg of ER-targeted BirA expression plasmid, optimal ratios may vary for different constructs). And mix rigorously for at least 10 s. Incubate the transfection mixture for 30 min at room temperature (*see* **Note 3**).
3. Day 2. Remove the medium from the flask with 293 T cells, but leave approximately 20 ml behind. Add the transfection mixture carefully (drop-by-drop) to the flask. Incubate for 5–6 h at 37 °C, 5% CO_2 or alternatively overnight.
4. Day 2 or 3. Remove the transfection medium completely and add 50 ml of expression medium.
5. Day 7. Tap the flask to detach all cells from the bottom and collect medium with cells in 50-ml tubes. Centrifuge the 50-ml tubes for 10 min at 1200 rpm (283 g) at 4 °C using an Eppendorf 5810R (or similar) centrifuge. Pour the supernatant to a new tube and centrifuge for 10 min at 3000 rpm at 4 °C. Pour the supernatant to a new 50-ml tube. Take a sample of the supernatant and store in the fridge or freezer for control analyses if desired.
6. Day 7. For protein A-bead purification, add 1 ml of 1 M Tris–HCl, pH 8.0. Add 200 ul of 50% protein A Sepharose beads for 1 T225 flask and incubate overnight while rotating the tube at 4 °C.
7. Day 8. Centrifuge the 50-ml tube for 10 min at 3000 rpm at 4 °C to spin down the beads. Remove the supernatant (and store for control analyses if desired).
8. Day 8. Resuspend the beads in 10 ml DPBS (beads from different tubes can be pooled if desirable), collect the beads by centrifugation, and remove the supernatant. Repeat this procedure once. Resuspend the beads in 1 ml DPBS and

transfer the mixture to a 1.5-ml Eppendorf tube. Centrifuge for 5 min at 5000 rpm at 4 °C and remove the supernatant. Repeat this washing step once more.

9. Day 8. Resuspend the beads in 1x elution buffer with approximately ½ of the bead volume (100 µl of elution buffer for 200 µl of beads). Mix well and within 5 min start centrifugation of the Eppendorf tubes with beads (5 min, 5000 rpm, 4 °C). Immediately transfer the supernatant to a new Eppendorf tube that contains neutralization buffer (35 µl neutralization buffer for 200 µl elution buffer) (*see* **Note 4**).
10. Day 8. Determine the protein concentration, for example by using NanoDrop Instrument (ThermoFisher) and an appropriate blank sample (mixture of neutralization and elution buffer) (*see* **Note 5**) Add protease inhibitor according to the manufacturer's instructions and store the purified protein in aliquots at 4 °C or in the freezer.
11. Use different amounts of the purified protein (e.g., 1-2-3-4 ug) to analyze glycoprotein receptor loading levels to protein A or streptavidin sensors as detailed below and to determine quantities needed to obtain equal receptor loading levels with different glycoprotein batches.

3.2 Virus Preparation

3.2.1 Virus Production

1. Virus particle preparations obtained by virus amplification in embryonated chicken eggs as well as MDCK cells can be used but contaminating materials like proteins, nucleic acids, and lipids can cause background signals during BLI and may need to be removed by subsequent virus purification (*see* Subheading 3.2.2).

3.2.2 Captocore Core Virus Purification (for Details, Also See GE Application Note 29-0003-34 AA)

1. Dilute beads slurry as provided by the manufacturer 10-fold in PBS, mix well, and centrifuge for 2 min at 2000 g. Remove supernatant and resuspend beads in same volume of PBS; repeat procedure 4 times.
2. After last centrifugation step, resuspend beads to obtain a 50% slurry in PBS.
3. Add slurry to virus sample at 1 (beads) to 50 (virus) ratio.
4. Incubate for 1 h on a rotating platform at 4 °C.
5. Centrifuge suspension at 1000 g for 5 min.
6. Collect and store virus supernatant at 4 °C.

3.2.3 NanoSight NS300 Virus Particle Counting

1. Take out the laser module and clean the optical flat with H_2O with a tissue.
2. Attach the O-ring Top Plate on the laser module and connect the tubing to the chamber.
3. Flush the system twice with Ultra-pure water and once with PBS.

4. Start the NTA software and make a measurement program according to the software instructions.
5. Make virus dilutions in PBS (dilutions optimal for measurement will contain around 5×10^8–5×10^9 virus particles/ml). At virus concentrations below 2×10^8 particles, quantification becomes less reliable (standard deviation larger than 50%).
6. Load 100 ul of virus sample into the chamber from a syringe filled with 500 ul virus sample.
7. Optimize camera settings for best particle detection and start the program. Repeat measurement 5 times with 100 ul virus sample.
8. Between different virus samples wash the system with 1 ml PBS.

3.3 Quantification of Virus Association Rate (Fig. 1)

3.3.1 Maximum Association Rate (See Fig. 1a, b)

1. Set up a 96-well plate (black, half-volume; 100 µl per well) containing the following solutions in separate columns numbered 1–8:
   1. 100 µl PBS (for recording baselines).
   2. 100 µl biotinylated receptor or Fc-tagged glycoprotein receptor (*see* **Note 6**).
   3. 100 µl PBS containing 10 µM oseltamivir carboxylate (for recording baselines).
   4. 85 µl of purified virus particles (*see* **Note 7**) plus 10 µl of 100 µM oseltamivir carboxylate and 5 µl protease inhibitor (1 µl trypsin inhibitor (1 mg/ml) and 4 µl protease inhibitor (cOmplete Mini, Roche, 1 tablet in 400 µl MilliQ).
   5. 85 µl of purified virus particles (*see* **Note 7**) plus 10 µl of 100 µM oseltamivir carboxylate and 5 µl protease inhibitor (1 µl trypsin inhibitor (1 mg/ml) and 4 µl protease inhibitor (cOmplete Mini, Roche, 1 tablet in 400 µl MilliQ). 85 µl of purified virus particles plus 10 µl of 100 µM oseltamivir carboxylate and 5 µl protease inhibitor (optional, *see* **Note 8**).
   6. 100 µl 10 mM glycine pH 2 (for regeneration of sensors).
   7. 100 µl PBS (for wash steps in regeneration of sensors)
   8. Transfer the plate to the analysis plate position in the OCTET machine (standard settings: 30 °C and plate shaking at 1000 rpm). Prior to starting, the experiment sensors need to be soaked in demineralized H2O in a 96-well plate at the sensor position for at least 10 min.
2. Set up the following assay steps in the Octet equipment software.
   1. Baseline; 600 s in column 1.
   2. Receptor loading; 900 s in column 2 (*see* **Note 6**).
   3. Baseline; 300 s in column 3.

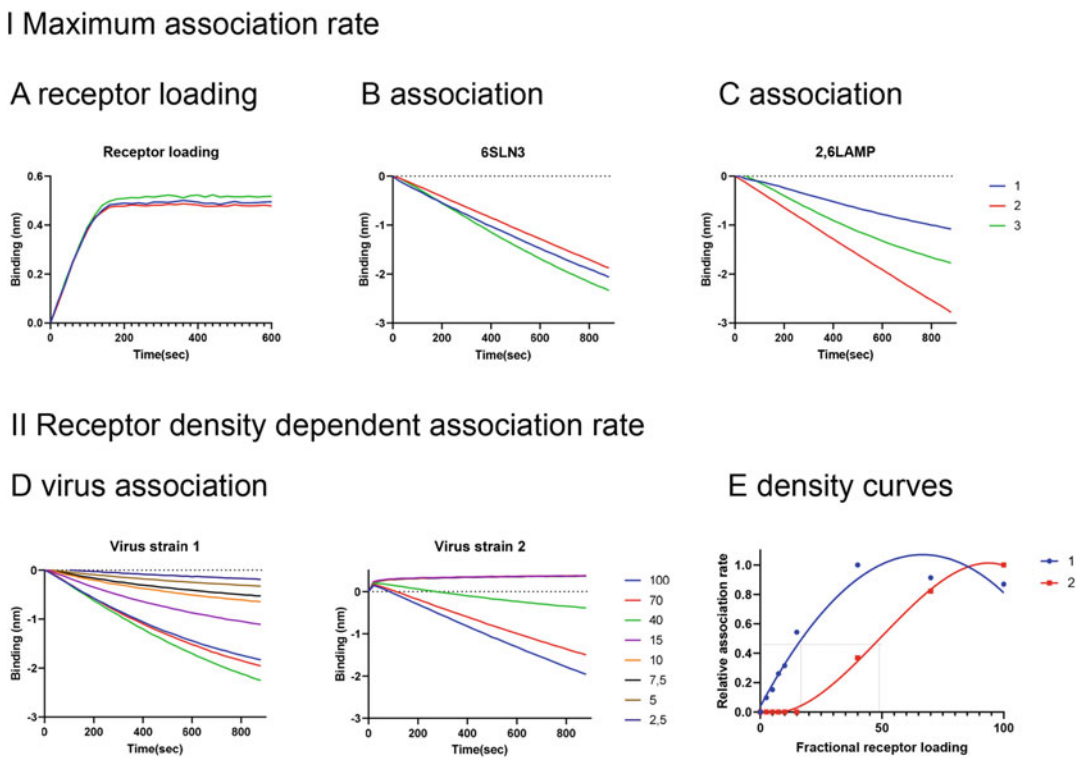

Fig. 1 Determination of maximum initial binding rate and receptor density-dependent binding rate. (**a**) Example of receptor loading curves for biotinylated synthetic glycan 6SLN3 (Neu5Acα2,6Galβ1,4GlcNAcβ1,3-Galβ1,4GlcNAcβ1,3 Galβ1,4GlcNAcβ1,3-LN2-Biotin). (**b**) Virus association curves of virus strains 1, 2, and 3 to 6SLN3. (**c**) Virus association curves of virus strains 1, 2, and 3 to 6'LAMP [12]. (**d**) Virus association curves of virus strains 1 and 2 to 6SLN3 loaded to 2.5–100% density. (**e**) Relative initial association rate of virus strains 1 and 2 plotted against receptor density

4. Virus association; 900 s in column 4.
5. Baseline; 300 s in column 3 (*see* **Note 9**).
6. Regeneration by dipping the sensor in pH 2; this can be programmed as a standard cycle consisting of a 3 times repeated cycle of 5 s in column 6 (10 mM glycine pH 2) and 5 s in column 7 (PBS).
7. Baseline; 300 s in column 3.
8. Fc-tagged receptor proteins need to be reloaded to the Protein A sensors after regeneration. Thus, for testing a second virus (column 5), **steps 2**–7 will be repeated. Biotinylated receptors remain associated with the streptavidin sensors. In this case for testing a second virus (column 5), **steps 4**–7 will be repeated. Additional viruses can be tested by adding extra columns.

3. Analysis and quantification of results.
    1. Determine the slope of the initial phase of binding. During the first 60 s of binding, the binding rate is often not yet linear. Therefore, the initial binding rate should be determined over a linear stretch of the binding curve from time-points 60–360 s.
    2. The binding rate should be normalized to a standard amount of virus particles (*see* **Note 10**) as determined by NTA particle counting.
    3. As an arbitrary unit, nm/600 s per 10^9 virus particles is recommended.

3.3.2 Receptor Density-Dependent Association Rate (See Fig. 1c, d)

1. Set up a 96-well plate (black, half-volume; 100 µl per well) containing the following solutions in separate columns 1–8:
    1. 100 µl PBS (for recording baselines).
    2. A dilution range of 100 µl glycan receptor from well 1 to well 8 (*see* **Note 6**, **11**, and **12**).
    3. Columns 3–8 as described under Subheading 3.3.1, **step 1**.
2. Set up the following assay steps in the Octet equipment software.
    1. Baseline; 600 s in column 1.
    2. Receptor loading; column 2 for 900 s or manual transition (*see* **Note 12**) to next step at required loading level range.
    3. Columns 3–8 as described under Subheading 3.3.1, **step 2**.
3. Analysis and quantification of results.
    1. Determine the slope of the initial phase of binding as described above under analysis of Subheading 3.3.1.
    2. Normalize the binding rate to the highest binding rate for each virus (usually observed for the highest concentration of receptor).
    3. Plot the relative association rate against receptor density.
    4. Determine the receptor density at which virus association rate is 50% of maximum rate (*see* **Note 13**).

3.4 Virus Dissociation (Quantifying the HA/NA Balance and Virus Motility) (Fig. 2)

3.4.1 Virus Dissociation Rate After Different Levels of Association (Fig. 2a–c)

1. Set up a 96-well plate (black, half-volume; 100 µl per well) containing the following solutions in separate columns 1–8:
    1. 100 µl PBS (for recording baselines).
    2. 100 µl biotinylated receptor or Fc-tagged glycoprotein receptor (*see* **Note 6**).
    3. 100 µl PBS containing 10 µM oseltamivir carboxylate (for recording baselines).

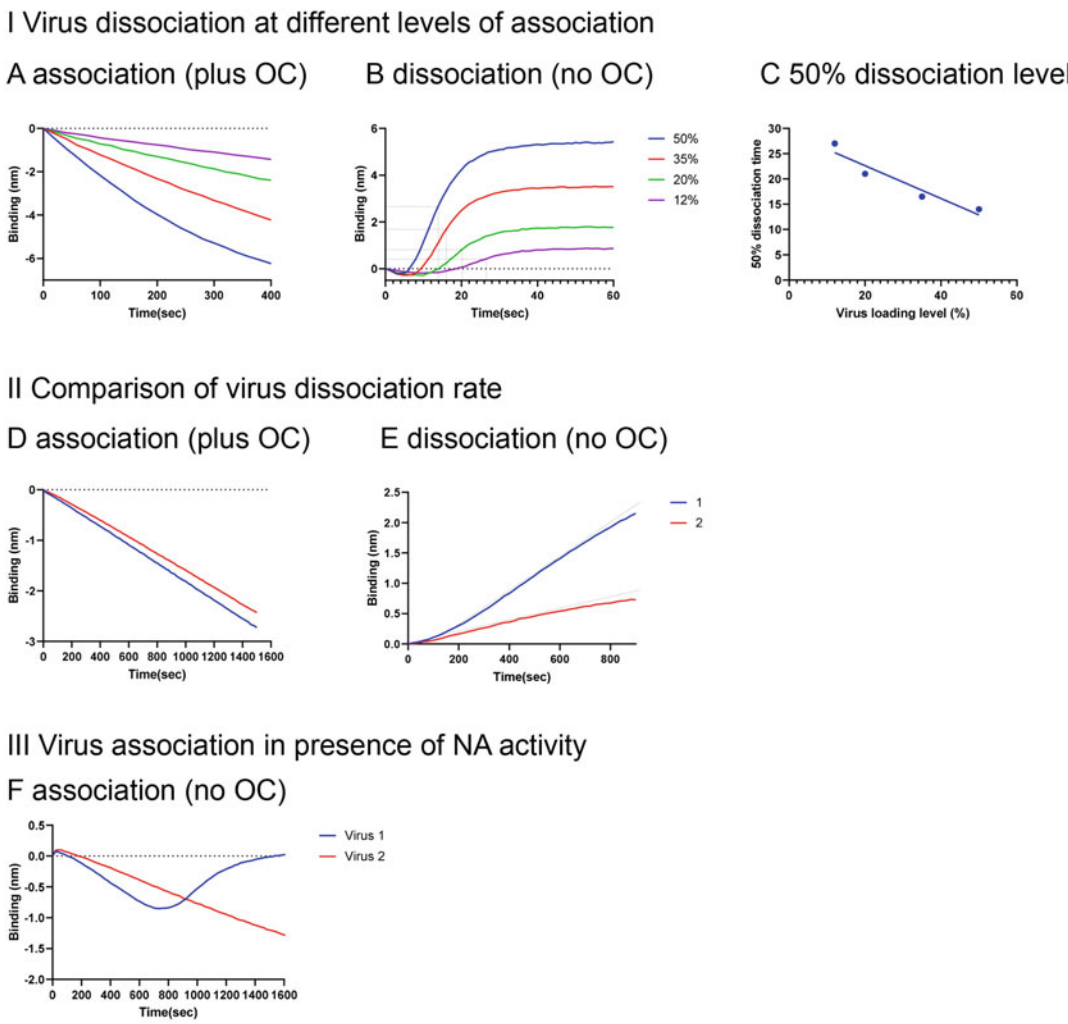

Fig. 2 Virus dissociation after association to different or equal levels. (**a**) Virus strain 1 was associated to 4 different association levels (12–50%) by adding different concentrations of virus. (**b**) Dissociation of virus in absence of oseltamivir carboxylate (dotted lines indicate time required to reach 50% dissociation from each sensor). (**c**) 50% dissociation time plotted against virus loading level. (**d**) Association of virus strains 1 and 2 to similar association levels. (**e**) Dissociation of virus strains 1 and 2 in absence of oseltamivir carboxylate. (**f**) Association of virus strains 1 and 2 in absence of OC

4. 85 µl of purified virus particles (*see* **Note 7**) at serial dilutions plus 10 µl of 100 µM oseltamivir carboxylate and 5 µl protease inhibitor (1 µl trypsin inhibitor (1 mg/ml) and 4 µl protease inhibitor (cOmplete Mini, Roche, 1 tablet in 400 µl MilliQ).
5. 100 µl PBS (for washing away oseltamivir carboxylate).
6. 100 µl PBS (for washing away oseltamivir carboxylate).
7. 100 µl PBS (for washing away oseltamivir carboxylate).
8. 100 µl PBS (for virus dissociation).

2. Set up the program in the Octet equipment
    1. Receptor loading; 900 s in column 2 (*see* **Note 6**).
    2. Receptor loading; 900 s in column 2.
    3. Baseline; 300 s in column 3.
    4. Virus association; 900 s in column 4.
    5. Baseline; 300 s in column 3.
    6. Wash; 5 s in column 5.
    7. Wash; 5 s in column 6.
    8. Wash; 5 s in column 7.
    9. Virus dissociation; 900–3600 s in column 8.
    10. Baseline; 600 s in column 3 (*see* **Note 14**).
3. Analysis and quantification of results.
    1. Determine the percentage of the sensor surface occupied by virus by dividing the binding level observed at the end of the virus association step (Fig. 2a) by 12 (the maximum shift observed at complete virus association for influenza virus particles).
    2. Determine the time required for 50% of virus dissociation for each sensor (Fig. 2b). Plot the required dissociation time against virus association levels (Fig. 2c).
    3. The result (Fig. 2c) clearly shows that the dissociation rate depends on virus association level which is caused by NA activity-driven virus motility making dissociation a cooperative event as extensively discussed in [6].

3.4.2 Dissociation Rate Comparison of Different Viruses (Fig. 2d, e)

1. Set up a 96-well plate (black, half-volume; 100 µl per well) containing the following solutions in separate columns:
    1. 100 µl PBS (for recording baselines).
    2. 100 µl biotinylated receptor or Fc-tagged receptor (*see* **Note 6**)
    3. 100 µl PBS containing 10 µM oseltamivir carboxylate (for recording baselines).
    4. 85 µl of purified virus particles of different strains plus 10 µl of 100 µM oseltamivir carboxylate and 5 µl protease inhibitor (1 µl trypsin inhibitor (1 mg/ml) and 4 µl protease inhibitor (cOmplete Mini, Roche, 1 tablet in 400 µl MilliQ) protease inhibitor at well-calibrated dilutions (*see* **Note 15**)). It is required to adjust the different virus strains to concentrations that result in equal association rates for each strain in order to be able to initiate the dissociation step at similar association levels.
    5. 100 µl PBS (for washing away oseltamivir carboxylate).
    6. 100 µl PBS (for washing away oseltamivir carboxylate).

7. 100 µl PBS (for washing away oseltamivir carboxylate).
8. 100 µl PBS (for virus dissociation).
9. Transfer the plate to the analysis plate position in the OCTET machine (standard settings: 30 °C and plate shaking at 1000 rpm). Prior to starting the experiment, sensors need to be soaked in demineralized H2O in a 96-well plate at the sensor position.

2. Set up the program in the Octet equipment
   1. Baseline; 600 s in column 1.
   2. Receptor loading; 900 s in column 2.
   3. Baseline; 300 s in column 3.
   4. Virus association; 900 s in column 4 (*see* **Note 16**).
   5. Baseline; 300 s in column 3.
   6. Wash; 5 s in column 5.
   7. Wash; 5 s in column 6.
   8. Wash; 5 s in column 7.
   9. Virus dissociation; 900–3600 s in column 8.
   10. Baseline; 600 s in column 3 (*see* **Note 14**).
3. Analysis and quantification of results.
   1. Determine the slope of the tangent (the maximal dissociation rate) for each dissociation curve (see dotted lines in Fig. 2e). Express maximal dissociation rate in nm/600 s.

3.4.3 Virus Association in Presence of NA Activity (Fig. 2f) (See ***Note 17***)

1. Set up a 96-well plate (black, half-volume; 100 µl per well) containing the following solutions in separate columns 1–8:
   1. 100 µl PBS (for recording baselines).
   2. 100 µl biotinylated receptor or Fc-tagged glycoprotein receptor (*see* **Note 6**).
   3. 100 µl PBS (for recording baselines).
   4. 95 µl of purified virus particles of different strains at well-calibrated dilutions (*see* **Note 18**) plus 5 µl of protease inhibitor (1 µl trypsin inhibitor (1 mg/ml) and 4 µl protease inhibitor (cOmplete Mini, Roche, 1 tablet in 400 µl MilliQ).
   5. 100 µl PBS containing 10 µM oseltamivir carboxylate (for recording baselines).
2. Set up the program in the Octet equipment.
   1. Baseline; 600 s in column 1.
   2. Receptor loading; 900 s in column 2 (*see* **Note 6**).
   3. Baseline; 300 s in column 3.
   4. Virus association; 900–3600 s in column 3.
   5. Baseline; 600 s in column 5 (*see* **Note 14**).

3. Analysis and quantification of results.
    1. Determine area under the curve (Fig. 2f, blue line; Unit is nm.sec) from 0 s to the timepoint where binding rate is zero again. This parameter quantifies the total number of particles of a virus strain that can bind to the receptor surface in presence of NA activity. Occasionally, for viruses with an HA/NA balance that results in a low dissociation rate (Fig. 2d, e, red line), the time required to return to zero binding rate may exceed the maximum assay time (~6 h). Lowering the receptor density will increase the dissociation rate and can in such instances be used to allow determination of the area under the curve.
    2. Alternative descriptive parameters to analyze the HA/NA balance are values like peak time (the timepoint at which the virus binding level is maximal) and peak height.

3.5 Inhibitor Assays of Virus Association and Dissociation (Fig. 3)

*3.5.1 Virus Dissociation Inhibitors (Fig. 3a–c) (See **Note 19**)*

1. Set up a 96-well plate (black, half-volume; 100 µl per well) containing the following solutions in separate columns:
    1. 100 µl PBS (for recording baselines).
    2. 100 µl biotinylated receptor or Fc-tagged receptor (*see* **Note 6**).
    3. 100 µl PBS containing 10 µM oseltamivir carboxylate (for recording baselines).
    4. 85 µl of purified virus particles plus 10 µl of 100 µM oseltamivir carboxylate and 5 µl protease inhibitor (1 µl trypsin inhibitor (1 mg/ml) and 4 µl protease inhibitor (cOmplete Mini, Roche, 1 tablet in 400 µl MilliQ)
    5. 100 µl PBS (for washing away oseltamivir carboxylate).
    6. 100 µl PBS (for washing away oseltamivir carboxylate).
    7. 100 µl PBS (for washing away oseltamivir carboxylate).
    8. 100 µl PBS with 7 wells for twofold dilution series of oseltamivir carboxylate in PBS starting at 100 nM and one well with PBS (for virus dissociation).
    9. Transfer the plate to the analysis plate position in the OCTET machine (standard settings: 30 °C and plate shaking at 1000 rpm). Prior to starting the experiment, sensors need to be soaked in demineralized H2O in a 96-well plate at the sensor position.
2. Set up the program in the Octet equipment.
    1. Baseline; 600 s in column 1.
    2. Receptor loading; 900 s in column 2.
    3. Baseline; 300 s in column 3.
    4. Virus association; 900 s in column 4 (*see* **Note 16**).

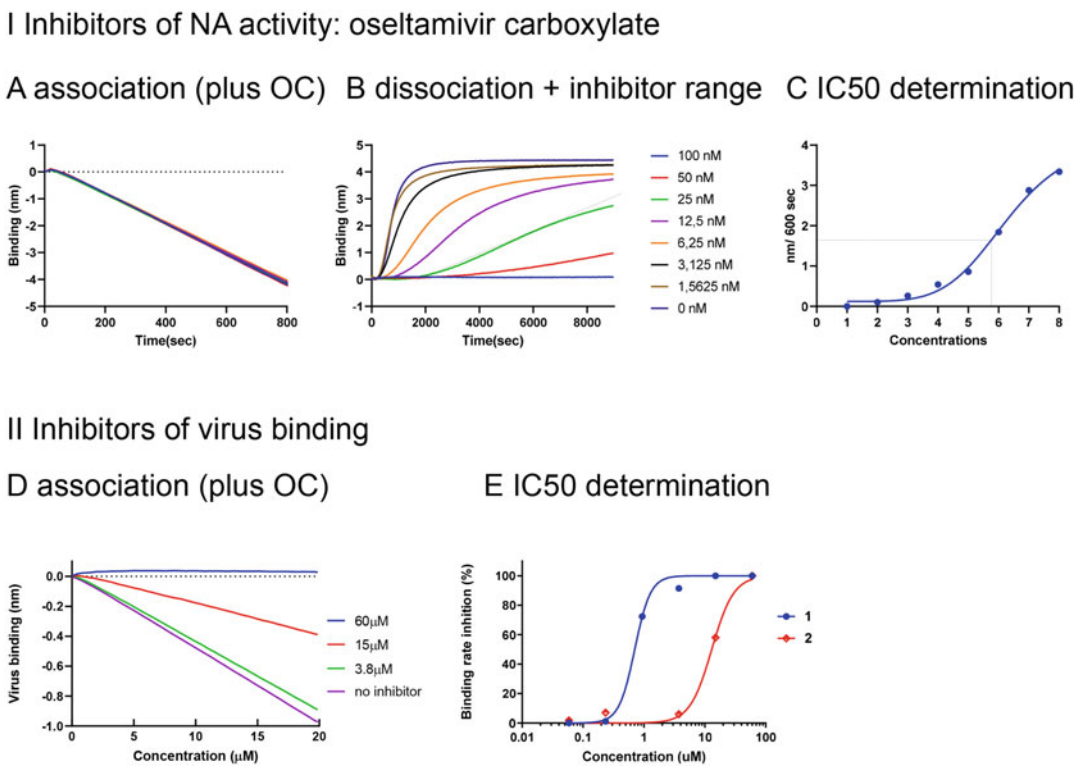

Fig. 3 IC50 determination of inhibitors of virus binding and dissociation. (**a**) Virus association curves. (**b**) Virus dissociation curves in presence of a twofold dilution series of oseltamivir carboxylate (dotted line is the tangent of the dissociation curve at 25 nM). (**c**) Virus dissociation rate plotted against oseltamivir carboxylate dilutions (1 is 100 nM; 8 is no oseltamivir carboxylate). (**d**) Virus association rate after pre-incubation (4 h at 4 °C) of virus with the indicated amount of trivalent sialic acid analog [15]. (**e**) Inhibition of virus binding rate plotted against inhibitor concentration

5. Baseline; 300 s in column 3.
6. Wash; 5 s in column 5.
7. Wash; 5 s in column 6.
8. Wash; 5 s in column 7.
9. Virus dissociation; 900–3600 s in column 8.
10. Baseline; 600 s in column 3 (*see* **Note 14**).

3. Analysis and quantification of results
   1. Determine the slope of the tangent (the maximal dissociation rate) for each dissociation curve (see dotted line in Fig. 3b). Express maximal dissociation rate in nm/600 s.
   2. Plot the maximum dissociation rates against inhibitor dilution factor (Fig. 3c).
   3. Determine the IC50 of the inhibitor by calculating the concentration at which the dissociation rate is 50% of maximum (dotted lines Fig. 3c).

*3.5.2 Virus Binding Inhibitors (*See ***Note 20****)*

1. Set up a 96-well plate (black, half-volume; 100 μl per well) containing the following solutions in separate columns:
    1. 100 μl PBS (for recording baselines).
    2. 100 μl biotinylated receptor (*see* **Note 6**).
    3. 100 μl PBS containing 10 μM oseltamivir carboxylate (for recording baselines).
    4. 85 μl of purified virus particles (*see* **Note 7**) pre-incubated with a fourfold dilution range of oseltamivir carboxylate.
    5. Transfer the plate to the analysis plate position in the OCTET machine (standard settings: 30 °C and plate shaking at 1000 rpm). Prior to starting the experiment, sensors need to be soaked in demineralized H_2O in a 96-well plate at the sensor position for at least 10 min.
2. Set up the following assay steps in the Octet equipment software.
    1. Baseline; 600 s in column 1.
    2. Receptor loading; 900 s in column 2 (*see* **Note 6**).
    3. Baseline; 300 s in column 3.
    4. Virus association; 900 s in column 4.
    5. Baseline; 300 s in column 3 (*see* **Note 9**).
3. Analysis and quantification of results.
    1. Determine the slope of the initial phase of binding. During the first 60 s of binding, the binding rate is often not yet linear. Therefore, the initial binding rate should be determined over a linear stretch of the binding curve from timepoints 60–360 s.
    2. The binding rate should be normalized to a standard amount of virus particles (*see* **Note 10**) as determined by NTA particle counting.
    3. As an arbitrary unit, nm/600 s per 10^9 virus particles is recommended.

4 Notes

1. Biotinylated receptors should essentially be devoid of free biotin, as this will reduce biotinylated receptor binding to streptavidin sensors.
2. The glycosylation pattern obtained is specific for the cell line in which the protein is expressed. Extensive differences between different cell lines are known to exist.
3. It is not necessary to transfect the BirA expression plasmid when protein A rather than streptavidin sensors will be used.

4. Prolonged incubation of glycoproteins in the elution buffer may result in their denaturation.
5. NanoDrop protein quantification is more reliable at high than at low protein concentrations.
6. The receptor loading rate is concentration dependent and receptor loading should preferably reach maximum loading levels within 900 s for optimal reproducibility of virus association rate. Maximum receptor loading level is reached when the reflection (recorded as nm shift on the y-axis) does not change any more (*see* Fig. 1a). If necessary. the loading time can be elongated. A final concentration of 100 nM for biotinylated synthetic glycans or 4–15 μg/ml for biotinylated glycoproteins or Fc-tagged glycoproteins is recommended.
7. Virus concentration needs to be sufficiently high for determination of binding rate. This differs between viruses, also depending on the receptor used. Concentrations ranging from 2 to 500 pM can result in a linear binding curve allowing determination of the initial virus binding rate. The 8 wells of a column can be used to compare up to 8 different virus strains side by side in a single step or 2 or 4 concentrations of 4 or 2 viruses, respectively, for a more precise comparison of viruses that may display quite different binding rates to different receptors. Alternatively, the binding of a virus strain to different receptors can be analyzed side by side in a single run by loading different receptors in **step 2**. The example of Fig. 1 shows the flexibility of the system. Here, three viruses were analyzed side by side for their binding to a synthetic glycan and a glycoprotein.
8. Additional viruses can be analyzed for binding to the same receptor set by adding extra columns containing virus particles. After each virus binding step, a cycle for regenerating the sensors is required.
9. Virus binding is virtually irreversible to sensors loaded to maximum receptor density. During this step, a flat line should therefore be observed.
10. The initial binding rate has been shown to be directly proportional to virus concentration up to 40% occupation of the sensor surface (~5 nm) [6]. Thus, standardization of initial binding rate to virus particle number is sufficient for comparing different virus strains and batches. Calculation of the binding affinity constant K_D from BLI curves [5] is complicated by the fact that equilibrium binding levels can usually not be reached at low virus concentrations and dissociation rate is virtually zero due to the extremely high avidity of IAV particle binding (K_D in picomolar range, *see* Subheading 1).

11. Receptor dilution range needs to be fine-tuned depending on the virus. An initial 100% to 12.5% linear dilution range (step size 12.5%) will indicate the sensitivity to receptor density for a particular virus. A second, more narrow, dilution range can then be chosen for precise determination of the density curve of a particular virus.
12. To establish a receptor gradient, two methods can be applied. Biotinylated sialoglycans can be precisely loaded to the required density by complementing them with the corresponding non-sialylated glycan (e.g., 25% 3S(LN)2 plus 75% (LN)2). In this case, the loading step can be run for 900 s to maximally load the sensors with the glycan mixture. Alternatively, loading of a dilution series containing only sialylated glycans (synthetic glycan or glycoprotein) can be watched during the loading. The loading step can be manually switched to the subsequent baseline step at the timepoint at which the diluted glycans have loaded to the required range of loading levels. Figure 1c shows an example of the later method. The loading level can then be directly read on the y-axis for each sensor.
13. This parameter further characterizes virus binding as it differs considerably between virus strains. Notably, initial virus binding rate is not necessarily correlated to receptor density dependency of the binding rate (see Fig. 1c, d, two viruses with the same initial binding rate differ remarkably in their density-dependent binding rate). For Octet SA sensors, the SIA density at maximal loading (here indicated as 100%) of a monoantennary synthetic glycan will be 0.6 pmol/cm^2. For a biotinylated glycoprotein like LAMP1, the SIA density will be approximately 5–40-fold higher depending on the degree of branching and sialylation of the attached glycans.
14. A baseline in PBS plus 10 μM oseltamivir carboxylate will block NA activity-dependent dissociation. In most cases, this will almost directly result in a flat line indicating that no further virus particles are dissociating in absence of NA activity. The main reason for including this step is to record potential cases where virus particles keep dissociating due to the lowered receptor density.
15. Virus dissociation is a cooperative process in which virus transverse over the receptor surface while cleaving receptors (*see* Subheading 1, [6–8]). For comparison of the dissociation rate of different viruses, it is therefore essential to load them to the same level. This can be accomplished by diluting the viruses on basis of a prior association rate experiment. Virus association rate has been shown to be directly proportional to virus concentration and can therefore be easily adjusted to equal

rates. As virus dissociation depends on HA and NA characteristics, it reflects the HA/NA balance for a particular receptor [6, 12, 13].

16. Association should be switched manually to the next baseline step when virus association levels are at a pre-determined level in order to be able to compare the results of different experiments. An association level of 2–4 nm (sensor surface occupied for ~15–30% with virus particles) will provide reproducible results. For comparison of results from different runs, a fixed loading level should be chosen.
17. Determining virus association in presence of an active NA (no addition of OC) describes the natural condition under which IAV binding takes place. The obtained binding curves result from the simultaneous effects of receptor cleavage by NA and receptor association by HA and therefore reflect the HA/NA balance for a particular receptor.
18. For standardizing the assay, different virus strains tested side by side in the same run should be calibrated to either equal particle numbers as determined by NTA particle counting or to the same association rate as explained in **Note 13**.
19. Virus dissociation inhibitors are inhibitors of NA activity. Inhibitor sensitivity is often quantified by assays measuring the cleavage of small soluble NA substrates like 2-′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid. However, NA activity-dependent release of virus particles is depended on the binding strength of HA. The BLI assay described here therefore determines the effect of neuraminidase inhibitors on the HA/NA balance of a virus strain on the particular receptor that is loaded.
20. This assay requires pre-incubation of virus with inhibitors that bind with high affinity to the HA receptor binding site, thereby blocking subsequent binding of virus particles to receptor-coated BLI sensors [15].

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