Application of virus-like particles (VLP) to NMR characterization of viral membrane protein interactions

Aleksandar Antanasijevic1 · Carolyn Kingsley1 · Arnab Basu2 · Terry L. Bowlin2 · Lijun Rong3 · Michael Caffrey1

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Abstract The membrane proteins of viruses play critical roles in the virus life cycle and are attractive targets for therapeutic intervention. Virus-like particles (VLP) present the possibility to study the biochemical and biophysical properties of viral membrane proteins in their native environment. Specifically, the VLP constructs contain the entire protein sequence and are comprised of native membrane components including lipids, cholesterol, carbohydrates and cellular proteins. In this study we prepare VLP containing full-length hemagglutinin (HA) or neuraminidase (NA) from influenza and characterize their interactions with small molecule inhibitors. Using HA-VLP, we first show that VLP samples prepared using the standard sucrose gradient purification scheme contain significant amounts of serum proteins, which exhibit high potential for non-specific interactions, thereby complicating NMR studies of ligand-target interactions. We then show that the serum contaminants may be largely removed with the addition of a gel filtration chromatography step. Next, using HA-VLP we demonstrate that WaterLOGSY NMR is significantly more sensitive than Saturation Transfer Difference (STD) NMR for the study of ligand interactions with membrane bound targets. In addition, we compare the ligand orientation to HA embedded in VLP with that of recombinant HA by STD NMR. In a subsequent step, using NA-VLP we characterize the kinetic and binding properties of substrate analogs and inhibitors of NA, including study of the H274Y-NA mutant, which leads to widespread resistance to current influenza antivirals. In summary, our work suggests that VLP have high potential to become standard tools in biochemical and biophysical studies of viral membrane proteins, particularly when VLP are highly purified and combined with control VLP containing native membrane proteins.

Keywords Antiviral agent · Drug resistance · Influenza · STD · Virus entry · WaterLOGSY

Introduction

The membrane proteins of enveloped viruses, which include Ebola, HIV, influenza, and SARS Coronavirus, play critical roles in viral entry and thus they are attractive targets for antiviral therapeutics (Kuhn et al. 2007; Lagoja and De Clercq 2008; Caffrey 2011; Yermolina et al. 2011). For example, hemagglutinin (HA) found in the viral membrane of influenza mediates virus receptor binding and entry; neuraminidase (NA) also found in the viral membrane of influenza mediates virus egress (Skehel and Wiley 2000; von Itzstein 2007). The study of viral membrane proteins by biochemical and biophysical methods is challenging due to the presence of transmembrane domains, glycosylation, multiple disulfide bonds, and limitations of detergent usage (Skehel and Wiley 2000; Garavito and Ferguson-Miller 2001; von Itzstein 2007; Caffrey 2011). To date most efforts to study viral membrane proteins have utilized solubilized versions of recombinant proteins produced in mammalian or insect cells, which often require the addition of stabilization domains (Stevens et al. 2006;
Lee et al. 2008; Li et al. 2010; Julien et al. 2013). However, the preparation of recombinant constructs is tedious and there are concerns that the recombinant protein structure may not accurately reflect the native structure due to the absence of transmembrane domains, membrane components (i.e., lipids, carbohydrates, cholesterol and cellular proteins), and presence of stabilization domains. An alternative approach is to prepare infectious virus containing their native membrane proteins; however, there are clearly biosafety concerns with the preparation and study of infectious virus (e.g. pandemic strains of influenza, HIV or Ebola). Recently, the use of virus-like particles (VLP) has become a very powerful tool to study the membrane proteins of many dangerous viruses without the necessity for biosafety level 3 or 4 facilities (Yonezawa et al. 2005; Zhang et al. 2008; Garcia and Lai 2011; Qian et al. 2013). In these experiments VLP are prepared by co-transfection of plasmid encoding the desired viral membrane protein with a plasmid encoding a capsid background (e.g. that of HIV or influenza), often with the addition of a reporter construct (e.g. GFP or luciferase). Current applications of viral membrane proteins embedded in VLP include studies of processing, kinetics and activity (Jacobs et al. 2005; Jalaguier et al. 2011; Ku et al. 2013; Antanasijevic et al. 2014a; Tedbury et al. 2015), drug screening trials (Wang et al. 2011; Ku et al. 2013; Antanasijevic et al. 2014b). In the present work, we show the importance of VLP purity for studies of viral membrane proteins using influenza HA and NA as model systems. Moreover, we demonstrate that WL is significantly more sensitive than STD NMR for the study of VLP interactions. In addition, we compare the interaction of an inhibitor with membrane-bound viral protein versus recombinant protein. Finally, we demonstrate the utility of VLP for the study of mutant viral membrane proteins by NMR-based kinetic methods.

Materials and methods

Recombinant HA production and purification

SF9 insect cells were grown in SF-900 II serum free media (Life Technologies). The cells were co-transfected with a pAcGP67 plasmid containing a H5 HA (A/Vietnam/1203/04 (H5N1), BEI Resources) expression construct and BD BaculoGold linearized baculovirus DNA (BD Biosciences). The H5 HA protein construct has an altered C-terminus, where the transmembrane and cytosolic regions of the protein were removed and replaced with an artificial trimerization domain (the foldon from T4 fibritin), and a His-tag as previously described (Stevens et al. 2006). Cell handling, transfection and protein expression were performed as recommended by the BD BaculoGold starter package kit (BD Biosciences). Viral titers were monitored using the BacPAK™ qPCR Titration Kit (Clontech Laboratories). For expression, fresh SF9 cells at 80 % confluence were infected with H5 HA containing baculovirus solution at MOI between 3 and 6. 4 days later the suspension was collected and the cells were removed by centrifugation. H5 HA is secreted into the insect cell media and purified by Ni-affinity chromatography. The protein concentrate was then subjected to Sephacryl S300 gel filtration column with phosphate buffer (50 mM sodium phosphate/pH 8.1 and 50 mM NaCl) as a running buffer. Protein fractions were pooled and concentrated and the final yield was ~4 mg of H5 HA per liter of insect cell culture.

VLP production and purification

HEK293T cells were grown in DMEM media with 10 % FBS and 1X penicillin/streptomycin. Cells at 70–80 %
confluency were co-transfected with plasmids encoding HIV core (pNL4-3.Luc.R-E, NIH AIDS Reagents Program) and H5 HA (strain A/Vietnam/1203/04 (H5N1), BEI Resources) and N1 NA (strain A/Puerto Rico/8/1934 (H1N1), BEI Resources). For the production of VLPs with only NA on the surface (NA-VLP) or without any viral membrane protein (native-VLP), the cells were co-transfected with HIV core and NA plasmids or just the HIV core DNA, respectively. PEI (Polysciences, Inc) was used as a transfection reagent. 48 h post transfection the cell supernatant was collected (60–80 ml), cleared through a 0.45 μm filter, and concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore) with a 100 kDa cutoff. The concentrate was then run through a Sepharose 4 Fast Flow column (GE Healthcare) with phosphate buffer (20 mM sodium phosphate/pH 7.5 and 150 mM NaCl) used as running buffer. VLP containing fractions were combined and subsequently VLP were precipitated over a 30% sucrose cushion (33,000 rpm for 50 min). The VLP pellet was then re-suspended in 0.5 ml phosphate buffer, concentrated further if necessary, and dialyzed overnight against phosphate buffer to remove residual sucrose. The relative purity of VLP fractions was determined by SDS-PAGE on Novex 4–12 % Bis–Tris gels (Life Technologies).

ELISA assays

VLP samples were lysed with Triton X-100 (1 % final concentration) and the VLP samples (with or without dilution) were loaded onto ELISA plates pre-coated with anti-p24 or anti-H5 HA antibodies. Further manipulations were performed according to the HIV-1 p24 antigen capture assay kit (SAIC Frederick) and Hemagglutinin (H5N1)(A/Anhui/1/2005) ELISA Development Kit (Immune Technology Corp) protocols.

WaterLOGSY and STD NMR Experiments

NMR experiments were performed on Bruker 800 or 900 MHz AVANCE spectrometers equipped with room temperature or cryogenic triple resonance probes, respectively, at 25 °C. WL experiments were performed as previously described (Antanasijevic et al. 2014b). To saturate water $^1$H, 2 ms square shaped pulses were used and the total saturation time was 2 s with a relaxation delay of 2.5 s. The number of scans was set to 1024, which corresponds to an ~2 h experiment. Saturation transfer difference (STD) experiments were performed as previously described (Antanasijevic et al. 2014b). A train of 50 ms Gaussian pulses was used to selectively excite the protein $^1$H. Total saturation time was 1 s and the relaxation delay was set to 2.5 s. “On” resonance frequency was set to −0.5 ppm and the “off” resonance acquisition was set to 300 ppm. The total length of each STD experiment was ~13 h. Buffer conditions were 50 mM phosphate/pH 8.2, 50 mM NaCl and 10 % $^2$H$_2$O for WL or 100 % $^2$H$_2$O for STD. 3 mm NMR tubes were used in all studies. Data were processed and analyzed using NMRDraw (Delaglio et al. 1995). Relative % STD was calculated as described previously (McCullough et al. 2012). Briefly, % STD = 100 × STD$_{obs}/$STD$_{max}$-STD signal was calculated based on the equation: STD = ΔI/Ioff, where ΔI = Ioff − Ion and Ioff and Ion are the resonance intensities after the “off” and “on” presaturation of the protein/VLP target. Errors in STD experiments were estimated as ΔI/Iref[(N$_{AI}$/ΔI)$^2$ + (N$_{Iref}$/Iref)$^2$]^{1/2}.5 where N$_{AI}$ and N$_{Iref}$ are the noise levels calculated by NMRDraw in the appropriate spectrum.

Simulations

To calculate the relative STD signal as a function of correlation time and target protein concentration, we used the program CORCEMA-SX 3.8 (Jayalakshmi and Krishna, 2002) and our previously published model of the HA-MBX-2329 complex (Antanasijevic et al. 2014a). HA concentrations examined were 30 nM (the concentration of HA in the VLP experiments), 300 nM, 3000 nM and 30,000 nM (the concentration of HA in the recombinant HA experiments) with 100 μM MBX2329 (ligand). The other input parameters included: 10 Å cutoff distance, 100 % $^2$H$_2$O, $K_d$ = 5 μM, $k_{on}$ = 1 × 10$^9$ s$^{-1}$, free ligand $\tau_c$ = 0.5 ns, 900 MHz field strength, saturation time = 1 s, and instantaneous irradiation of Ile, Leu and Val methyl groups.

NA activity assays

NA catalyzed reactions were followed by measuring the line intensities for the substrate MUNANA resonance at 2.50 ppm. Experimental conditions were ~10 nM NA or H274Y-NA in VLP, 20 mM phosphate/pH 7.4, 150 mM NaCl, and 1 mM CaCl$_2$ at 25 °C in the presence and absence of 10 μM MUNANA (substrate). OriginPro (OriginLab Corp) was used to determine the kinetic and inhibitory constant $K_m$. 

Results

Importance of VLP purification

Recently, we characterized the interaction of a small molecule inhibitor of influenza entry, MBX2329, with recombinant H5 HA by WL and STD NMR (Basu et al. 2014). MBX2329 is a fusion inhibitor that selectively
disrupts the entry of Group 1 HA influenza viruses, like H1 and H5 HA, with IC50 values in the low µM range by binding to HA and inhibiting a critical pH dependent conformational change (Antanasijevic et al. 2014a; Basu et al. 2014). In the present work we wanted to extend our studies using HA embedded in VLP to ascertain the potential use of VLP for drug discovery and optimization studies; however, we had concerns about potential small molecule interactions with contaminants present in VLP preparations. For example, standard cell media contains 10 % Fetal Bovine Serum (FBS), which results in ~4 mg/ml of total protein added (Lindl 2002). The relatively high levels of added protein could potentially form detectable non-specific interactions with the small molecule of interest. Consequently, we first tested whether MBX2329 exhibits binding to proteins present in FBS. For this assay we chose to use WL NMR, which we have shown to be significantly more sensitive than STD NMR (Antanasijevic et al. 2014b). In this experiment, the presence of positively phased signals of the small molecule, in the presence of large molecular weight biomolecules, is due to binding. As shown in Fig. 1, MBX2329 exhibits detectable binding to serum proteins at 2 and 10 % levels. Consequently, the relative purity of VLP preparations and the potential presence of contaminating soluble proteins is a significant concern for biochemical and biophysical studies.

In the next step, we prepared VLP containing H5 HA using a HIV core background by co-transfection of plasmids containing H5 HA and the HIV core, as previously described (Antanasijevic et al. 2014a). Note that in these preparations, a small amount of plasmid containing N1 NA was included (final protein ratio of ~1:10 NA to HA) to enhance production of VLP containing HA (HA binds to sialylactose present on the cell surface, which inhibits virus egress, Chen et al. 2007). Subsequently VLP were harvested by collecting the cell supernatant from transfected cells and concentrated using ultrafiltration. Samples were then subjected to purification by sucrose gradient centrifugation, the standard protocol employed for the purification of VLP samples used in biochemical and biophysical studies (Haselhorst et al. 2008; Mohan et al. 2010; Garcia et al. 2014). We then analyzed the proteins present by SDS-PAGE as shown in Fig. 2. First, a large amount of protein is present in the starting cell culture media. Moreover, the VLP proteins present in the initial purification step are undetectable at this point and thus are a very small fraction of the total protein. In contrast after the sucrose gradient step, the VLP proteins become detectable after this step; however, they remain a relatively small fraction of the total protein present (typically <15 %). As a consequence, we added gel filtration chromatography as an additional purification step. As shown in Fig. 2, the viral proteins have now become predominant, consisting ~70 % of the total protein, and thus this preparation of VLP is clearly more appropriate for biochemical biophysical and biochemical characterizations. Using the 3-step purification protocol (concentration, sucrose gradient centrifugation, and gel filtration), we collect 0.5 ml of purified and concentrated stock from ~80 ml of starting VLP-containing supernatant. In the final preparation, the average p24 levels (i.e. the HIV core protein)

![Fig. 1 WL NMR detection of small molecule binding to serum proteins present in cell culture media. For reference the 1D NMR spectrum of the downfield region of MBX2329 is shown. Arrows denote ligand peaks used as reporters of ligand binding to serum proteins. Experimental conditions were 100 µM MBX2329 ±2 or 10 % Fetal Bovine Serum (FBS) in 50 mM phosphate/pH 8.2, 50 mM NaCl, 10 % 2H2O at 25°C. WL spectra were acquired for ~2 h on an 800 MHz spectrometer equipped with a room temperature probe](image1)

![Fig. 2 SDS PAGE stained by Coomassie Blue showing purification of HA-VLP. Lanes correspond to: MW molecular weight markers; 1 cell media, 2 VLP in supernatant, 3 VLP after sucrose gradient centrifugation, 4 VLP present after sucrose gradient centrifugation and gel filtration chromatography. For reference the positions of HA composed of HA1 and HA2 and the HIV core components composed of p24, MA and NC are shown to the right](image2)
were 10 µg/ml (~400 nM) and the amount of HA was estimated to be 2 µg/ml (~30 nM). One VLP on average has ~3000 p24 molecules (Jennings et al. 2005) and thus these numbers we calculate that there are approximately ~75 HA trimers per VLP (and ~6 NA tetramers), which is in good agreement with the literature values reported for different types of VLP with the HIV background (ranging between 10 and 100 viral membrane proteins per VLP (Crooks et al. 2000; Berkower et al. 2004; Zhu et al. 2006)). In the next step, we purified VLP containing only NA for use as controls and subsequent characterization of membrane embedded NA. In this case the final concentration of NA was 4 µg/ml (~60 nM), which corresponds to ~120 tetramers of NA per VLP. Finally, we note that the relative numbers of viral membrane proteins per VLP may be adjusted, at least to a certain degree, by changing the ratio of the viral membrane protein plasmid with respect to that of the HIV core plasmid.

**WL NMR studies of MBX2329 binding to HA-VLP**

As noted above, based on studies of recombinant HA, the small molecule MBX2329 binds to HA and inhibits influenza entry by stabilizing the neutral pH conformation (Antanasijevic et al. 2014a; Basu et al. 2014). Consequently, we tested the potential to detect MBX2329 binding to HA embedded in the membrane using HA-VLP by WL NMR. For reference, the 1D NMR spectrum of the ligand is shown at the top of Fig. 3. As shown in Fig. 3, binding of MBX2329 to recombinant HA is evident from the positively phased peaks of ligand in the presence of 10 µM recombinant protein. In a similar manner, binding of MBX2329 to HA-VLP is clearly detected (Fig. 3). Note that specificity of the binding to HA is established by performing a control experiment using NA-VLP, as opposed to HA-VLP, normalized to the same concentration of VLP (Fig. 3, this control is critical in our case because our HA-VLP contain a small amount of NA, as noted above). In the case of the HA-VLP and NA-VLP experiments the concentrations of HA and NA were ~10 nM. As shown in Fig. 3, the S/N is similar for identical experimental times using recombinant HA and VLP-bound HA, despite the 1000 fold decrease in HA concentration present in VLP, thereby demonstrating the potential high sensitivity of VLP-based NMR experiments.

**STD NMR studies of MBX2329 binding to HA-VLP**

STD NMR is generally less sensitive than WL NMR (Antanasijevic et al. 2014b); however, STD NMR offers unique information that can be used to characterize the orientation of the ligand and guide chemical optimization (Mayer and Meyer 2001; Antanasijevic et al. 2013). Thus, we tested the potential to characterize MBX2329 binding to HA embedded in the membrane (i.e. HA-VLP) by STD NMR. As shown in Fig. 4a, binding of MBX2329 to HA-VLP is clearly detected by STD NMR and, importantly, no detectable binding is observed to NA-VLP (in both cases the VLP concentrations were normalized using p24). We next quantified the relative intensities of the ligand 1H, which reflects the relative proximity of the ligand 1H to the HA surface (Mayer and Meyer 2001; Bhunia et al. 2012; Antanasijevic et al. 2013; Basu et al. 2014). In Fig. 4b, we compare the relative orientation of MBX2329 to membrane bound HA with the relative orientation of MBX2329 to recombinant HA. Note that the recombinant HA experiments were performed at 30 µM and the HA-VLP experiments were performed at 30 nM. Strikingly, the relative proximities of the ligand 1H are similar, which suggests that the mode of binding is similar between recombinant and membrane bound HA. For example aromatic region of the molecule is in closer contact to hydrophobic residues in the binding pocket and is more involved in binding than the azepane moiety. Accordingly, the linker and azepane groups may represent attractive regions for introducing...
chemical changes to improve the inhibitory or pharmacokinetic properties of this compound.

As mentioned above, we observe similar STD signals for recombinant HA at 30 μM and VLP-bound HA at 30 nM. In the next step, we used the program CORCEMA (Jayalakshmi and Krishna 2002) and our previously published model of the HA-MBX-2329 complex (Antanasijevic et al. 2014a) to calculate the relative STD signal as a function of correlation time and target protein concentration (Fig. 5). Based on the estimated correlation time of the recombinant HA trimer (≈100 ns), the relative STD signal is expected to be ≈90% (Fig. 5). On the other hand, the estimated correlation time VLP-bound HA is estimated to be ≈1 ms and the relative STD signal is expected to be ≈70% (Fig. 5). Thus, our observation of similar STD signal for HA under very different physical states is in relatively good agreement with the CORCEMA simulations. In summary, STD NMR studies performed with VLP based samples suggest that the mode of binding of MBX2329 to HA is similar in recombinant and membrane bound forms. Importantly, HA-VLP provide the same type and quality of information as the recombinant HA based experiments, using significantly less material.

NMR Kinetic studies of wild-type and mutant NA-VLP

Current treatments for influenza include inhaled zanamivir (Relenza), oral oseltamivir (Tamiflu) and intravenous peramivir (Rapivab), which were developed as substrate analogs targeting viral NA (von Itzstein et al. 1993; Lagoja and De Clercq 2008; Cairo 2014). Previous studies have shown that NA-VLP purified using sucrose gradient centrifugation exhibit enzymatic activity (Mohan et al. 2010; Garcia et al. 2014). Accordingly, we tested whether our NA-VLP, purified with the additional gel filtration step discussed above (Fig. 2), were suitable for kinetic and biophysical studies. First we used 4-Methylumbelliferyl-a-D-N-acetylneuraminic acid (MUNANA), a fluorescent substrate analog of NA (Potier et al. 1979), to measure the activity of NA present in NA-VLP by NMR detection of product formation. As shown in Fig. 6a, product is uniquely formed in the presence of NA-VLP and the enzyme activity is reversed by the addition of the inhibitor DANA (Neu5Ac2en), a transition state analog and the parent compound of FDA-approved NA inhibitors (von Itzstein et al. 1993; Lagoja and De Clercq 2008; Cairo 2014). Importantly, control measurements using VLP prepared in the absence of NA expression (native-VLP), in which p24 levels were used to normalize the amount of VLP in the samples, exhibited no activity (Fig. 6a). As shown in Fig. 6b, the Michaelis–Menten plot of VLP-bound NA activity yields \( K_m \approx 22 \mu M \), which is in good agreement with the expected value.
agreement with previous fluorescence-based kinetic studies (Rameix-Welti et al. 2006; Collins et al. 2008; Marathe et al. 2013) and support the functionality of the VLP-bound NA. In the next, step we prepared H274Y-NA-VLP, in which the NA possesses the H274Y mutation responsible for resistance to Tamiflu (Bloom et al. 2010; McKimm-Breschkin 2013). As shown in Fig. 6c, the Michaelis–Menton plot of H274Y-NA-VLP catalyzed reaction. The product formation was detected by the

WL NMR studies of NA-VLP

In the next step, WL NMR was applied to detect the interaction between the inhibitor DANA and NA-VLP. In control measurements we used the same amount of VLP prepared in the absence of NA expression (native-VLP), with p24 levels used to normalize the amount of VLP in the samples. As shown in Fig. 7a, binding of DANA to NA-VLP is easily detected by WL NMR. Importantly, the DANA binding is NA-specific as shown by the absence of signal in the native-VLP. We then compared the amount of WL signal from the NA-VLP experiment in the absence and presence of 2.5 mM MUNANA, the substrate analog, using WL NMR competition assay previously developed for recombinant proteins by our group (Antanasijevic et al. 2013; Basu et al. 2014). As shown by Fig. 7b, the presence of substrate in high excess causes a decrease in the DANA WL signal, which is consistent with the competitive inhibition model previously described for this family of compounds (Meindl et al. 1974).

Discussion

In this work we demonstrated the high potential of using VLP-based systems to study viral membrane proteins by NMR methods. As the model system we chose VLP prepared with the influenza proteins HA or NA on the surface, potential targets for novel antivirals, and the HIV core. The HIV core used (pNL4-3.Luc.R-E-) allows the expression of VLP with different combinations of viral membrane proteins, as well as VLP containing the native proteins of the host cell (Jacobs et al. 2005; Yermolina et al. 2011; Antanasijevic et al. 2013, 2014a). Importantly, the native-VLP offer the potential to serve as powerful control samples to demonstrate the specificity of small molecule interactions with membrane bound targets.

For our study of HA embedded in VLP, we characterized the HA interaction with MBX2329, a small molecule antiviral that binds to the HA stem loop region and prevents a critical change in conformation (Antanasijevic et al. 2014a, b; Basu et al. 2014). However, non-specific binding of a ligand to a target biomolecule, which may be due to the promiscuity of the ligand and/or target, is a significant concern for ligand based NMR techniques. Consequently, we first tested the importance of VLP purity for the reduction of non-specific interactions by testing binding of MBX2329 to proteins present in FBS, a routine additive to cell cultures and potential contaminant due to its high protein concentration. For example, 10% FBS contains ~4 mg/ml total protein (Lindl 2002) and a purification scheme in which 90% of the serum proteins would be removed would still contain 400 µg/ml compared to target

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proteins in VLP at concentrations <1 μg/ml. As shown in Fig. 1, non-specific interactions between MBX2329 and proteins present FBS were indeed observed. Therefore, we made an effort to further purify our VLP preparations from serum proteins, as well as cellular components. Historically, sucrose gradient centrifugation was the primary method to concentrate and purify viruses and VLP used in biochemical and biophysical studies (Haselhorst et al. 2008; Mohan et al. 2010; Garcia et al. 2014). In the present work, we showed that this treatment alone is not enough to achieve an acceptable level of purity required for NMR studies (Fig. 2). Indeed, after the sucrose gradient centrifugation step, viral proteins accounted for only ~15% of the total protein present. However, the combination of sucrose gradient centrifugation and gel filtration helped remove the majority of serum (as well as cellular) proteins from the samples, with the viral proteins accounting for more than 60% of the total protein content in the purified VLP preparations. The quality may be further improved with the application of additional steps (e.g., ion exchange or affinity chromatography); however, additional steps are expected to result in losses in VLP amount and activity. Moreover, many of the remaining proteins present in the VLP are expected to be due to those of the native membranes from which the VLP form and thus they will always remain. Importantly, the activity of the remaining native proteins may be accounted for by preparing native-VLP (Figs. 6, 7), or alternatively with VLP containing another membrane protein (Figs. 3, 4), as discussed below.

After demonstrating the utility of the additional purification step, we characterized the interaction of MBX2329 with HA embedded in VLP, which corresponds to full length HA containing the native C-terminal transmembrane and intracellular domains, by WL NMR. Interestingly, the significantly greater size of the VLP particle (~200 MDa) compared to recombinant HA (~210 kDa) resulted in a much higher sensitivity in the experiments performed with VLPs. For example, ~10 nM HA embedded in VLP was necessary to obtain WL NMR spectra with optimal S/N ratio in a 2 h experiment. In order to collect data of comparable quality (i.e., S/N) with recombinant HA as the target, 10 μM concentration was necessary. Consequently, by switching from a recombinant viral membrane protein without its transmembrane domain to HA embedded in VLP, ~1000 times more sensitivity was observed, supporting the high sensitivity of WL NMR for very large biomolecular systems (Antanasijevic et al. 2014b). Furthermore, as noted above, the control versions of VLP are readily generated. For example, MBX2329 exhibits non-detectable interaction with NA-VLP (Figs. 3, 4), which strongly supports the specificity of this compound for HA in the context of a membrane environment. In the next step, we compared the binding mode of MBX2329 with recombinant HA and HA embedded in VLP by STD NMR

Fig. 7 Characterization of inhibitor DANA binding to NA-VLP by NMR. a 1D NMR spectra of DANA binding to NA-VLP (middle spectrum) and not to native VLP (bottom spectrum). For reference the 1D NMR spectrum of DANA is shown (top spectrum). b WL NMR competition assay to demonstrate competitive binding between inhibitor DANA and substrate analog MUNANA. Experimental conditions for the WL NMR were ±80 nM NA in VLP, 200 μM DANA, and ±2.5 mM MUNANA in 20 mM PO₄/pH 7.4, and 150 mM NaCl, with WL spectra acquired for ~2 h on an 900 MHz spectrometer equipped with a cryogenic probe.
(Fig. 4). As noted above, STD NMR gives insight into the relative contact of the ligand \(^1\)H with the target surface and thus may be exploited to guide chemical optimization. In agreement with our previous work (Antanasijevic et al. 2014b), we observed that STD NMR is significantly less sensitive than WL NMR (the WL and STD spectra were acquired in 2 and 13 h, respectively). Nonetheless, similarly to the WL studies we achieved an increase in sensitivity by a factor of \(~1000\) when performing the experiments with HA-VLP with respect to recombinant HA, which we show is in good agreement with CORCEMA simulations (Fig. 5). Strikingly, the recombinant HA and HA-VLP datasets gave almost identical STD signatures (Fig. 4), suggesting very similar binding modes. In both cases, the relatively low STD signals of the \(^1\)H in the linker region and the azepane group suggest that they are attractive sites for chemical optimization of this compound. Finally, the absence of measurable STD signal in the control measurement (NA-VLP) once again confirms the specificity of MBX2329 towards HA. Importantly, to our knowledge this work represents the first comparison of the binding mode of a small molecule inhibitor to recombinant and membrane-bound versions of a membrane protein.

We next demonstrated the utility of VLP applied to NA, a high priority target for influenza antivirals (von Itzstein et al. 1993; Lagoja and De Clercq 2008; Cairo 2014). In a first step we showed that NA embedded in VLP exhibits specific enzyme activity and that this activity is inhibited by DANA (Fig. 6), a parent compound of current influenza antivirals (von Itzstein et al. 1993; Lagoja and De Clercq 2008; Cairo 2014). Subsequently, a full analysis of NA activity and inhibition was performed by NMR (Fig. 6). In addition, we demonstrated the ability to characterize the H274Y mutant of NA, which is commonly occurring mutation responsible for resistance to Tamiflu (Bloom et al. 2010; McKimm-Breschkin 2013). Notably, the H274Y-NA in VLP exhibited \(K_m\) increased by \(~1.6\times\), which is in good agreement with previous studies of recombinant and influenza virus H274Y N1 NA for which \(K_m\) of the mutant ranged from 2 to 4X higher than wild-type (Rameix-Welti et al. 2006; Collins et al. 2008). Note that, with respect to recombinant membrane proteins, mutations are more readily generated in VLP (Caffrey 2011; Yermolina et al. 2011; Antanasijevic et al. 2014a). For example, the production of a mutant recombinant protein in a mammalian or insect expression system requires weeks of effort as opposed to VLP, which are routinely produced in a few days.

Finally, to further demonstrate the utility of VLP for biophysical studies we characterized the interaction of the inhibitor DANA with NA-VLP by WL NMR. As shown in Fig. 7, we observed binding of DANA to NA-VLP but not native-VLP, further underscoring the importance of native-VLP controls. In addition we performed a competition WL NMR assay to demonstrate that DANA is a competitive inhibitor of the NA substrate, as previously observed by structural and kinetic studies. Thus, we clearly show that VLP can be used in combination with NMR to detect the binding of different inhibitors, as well as their binding sites and type of inhibition.

In summary, our work suggests that VLP have high potential to become standard tools in biochemical and biophysical studies of viral membrane proteins, particularly when they are highly purified and combined with control VLP containing native membranes. Importantly, the VLP constructs contain the entire protein sequence and are comprised of native membrane components including lipids, cholesterol, carbohydrates and cellular proteins. To date biochemical and biophysical studies have been limited to the viral membrane proteins from influenza (Haselhorst et al. 2008; Mohan et al. 2010; Garcia et al. 2014, work reported herein); however, the VLP containing many other viral membrane proteins, including Ebola, HIV, and SARS-CoV are readily generated for virology studies (Jacobs et al. 2005; Kuhn et al. 2007; Yermolina et al. 2011) and thus we are confident that these systems will also be amenable for study by biochemical and biophysical methods to discover, characterize, and optimize ligand interactions.

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