Universal label-free in-process quantification of influenza virus-like particles

Sofia B. Carvalho1,2, Mafalda G. Moleirinho1,2, David Wheatley3, John Welsh3, René Gantier4, Paula M. Alves1,2, Cristina Peixoto1,2 and Manuel J. T. Carrondo1,5

1 iBET, Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal
2 Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal
3 Pall Life Sciences, Portsmouth, UK
4 Pall Life Sciences, Westborough, MA, USA
5 Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Monte da Caparica, Portugal

Virus-like particles (VLPs) are becoming established as vaccines, in particular for influenza pandemics, increasing the interest in the development of VLPs manufacturing bioprocess. However, for complex VLPs, the analytical tools used for quantification are not yet able to keep up with the bioprocess progress. Currently, quantification for influenza relies on traditional methods: hemagglutination assay or single radial immunodiffusion. These analytical technologies are time-consuming, cumbersome, and not supportive of efficient downstream process development and monitoring. Hereby we report a label-free tool that uses biolayer interferometry (BLI) technology applied on an Octet platform to quantify influenza VLPs at all stages of bioprocess. Human (α-2,6-linked sialic acid) and avian (α-2,3-linked sialic acid) biotinylated receptors associated with streptavidin biosensors were used to quantify hemagglutinin content in several mono- and multivalent influenza VLPs. The applied method was able to quantify hemagglutinin from crude samples up to final purified bioprocessing VLP product. BLI technology confirmed its value as a high throughput analytical tool with high sensitivity and improved detection limits compared to traditional methods. This simple and fast method allowed for real-time results, which are crucial for in-line monitoring of downstream processing, improving process development, control and optimization.

Keywords: Biolayer interferometry technology · Downstream process · In-process HA quantification · Multivalent VLPs · Octet

1 Introduction

Influenza virus infections in humans remain a worldwide concern, resulting in significant health and economic burdens [1]. During seasonal epidemics, 5 to 10% of the adult population and 20 to 30% of children are affected, resulting in 3 to 5 million cases of severe illness and up to 500,000 deaths annually worldwide [2]. Pandemic strains are also a significant global threat and can lead to millions of deaths [1].

Hemagglutinin (HA) and neuraminidase (NA) are the two influenza glycoproteins on which virus subtype classification is based. HA, the major envelope protein, contains the epitopes for neutralizing antibodies and is responsible for virus binding to host cell receptors, glycans that contain sialic acids. NA is a sialidase that removes the sialic acid receptor from the host cell surface playing an important role in the release of the virus progeny [3–5].

Host cell receptors contain α-2,6-linked or α-2,3-linked sialic acids moieties. Depending on the HA proteins pre-
sent on the virus surface, the binding specificity for the two linkages is different. Human influenza virus engages preferentially to α-2,6-linked sialic acids whereas avian virus primarily binds to α-2,3-linked sialic acid receptors [5]. Binding preferences of different strains are an important determinant for species barrier, restraining human infections with avian influenza virus. However, virus ligand preferences change: some mutations in avian HA proteins can lead to the emergence of new pandemic strains with different binding preferences, acquiring human receptors’ specificity. The same can happen for mutations in human HA proteins leading to changes in binding specificity to avian receptors [5–7].

Vaccination is a key strategy for prevention of influenza infections for both seasonal and pandemic virus. Due to the genetic processes of antigenic drift and shift, the content of the influenza vaccine needs to be reviewed annually [8]; furthermore seasonal vaccines do not provide protection against novel pandemic strains [9]. Most current licensed influenza vaccines are still produced using egg-based manufacturing, limiting vaccine supply, critical in the case of pandemics. This is driving a need for faster vaccine development and more effective vaccines against influenza. New vaccines have recently reached the market using both mammalian and insect cell lines [10].

Virus like particles (VLPs) display native virus proteins, triggering a protective immune response. Since they lack genetic material, VLPs are not infectious nor replicative, making them safer alternatives to killed or attenuated virus [11] and have been long established for hepatitis B virus and human papillomavirus [12, 13]. Several platforms, including VLPs, are under development as candidates for both seasonal and pandemic Influenza virus [9, 10, 14–17].

These trends increase the need for improved characterization and quantification tools: fast, reliable and easy to set up, applicable for use with in-process samples, from upstream crude extract to downstream purified product. Single radial immune diffusion (SRID), the approved method by regulatory authorities for potency determination, and hemagglutination assay (HA assay) are the main methods to quantify HA protein. HA assay has been used for evaluation of production and purification yields of influenza-VLPs. However, there are several disadvantages associated with these assays. SRID is a time consuming assay, taking two to three days to perform, which is impractical for process development. It has a low sensitivity (3–5 μg/mL) and there are several limitations regarding presence of aggregates and non-aqueous components that interfere with HA diffusion in the gel. Importantly, it requires HA antigen references and specific monoclonal antibodies that need to be constantly updated, delaying the release of new vaccines. The HA assay, although it is relatively fast compared to SRID, requires fresh red blood cells to obtain reproducible results. Due to the different cell origin and different cell types, an external standard is necessary to use for each assay. Since VLPs are more heterogeneous than the influenza virus particles, the ratio of red blood cells to VLPs can differ from 1. Usually, for normal Influenza virus, this ratio is approximately 1. Changing this ratio, which happens for VLPs, can lead to an erroneous estimate of total particles [18, 19].

The regulatory authorities recommend using alternative influenza vaccine potency assays [20]. There are several emerging methods that attempt overcome the issues described above: receptor-based ELISA (enzyme linked immunosorbent assay) that uses synthetic sialic acid receptors to HA quantification [20], Flu-Toc immunosassay for HA quantification using subtype specific but broadly reactive monoclonal antibodies [21], reversed-phase HPLC (high-performance liquid chromatography) [22–24], NA activity assays, qPCR (quantitative real time PCR) [18, 19], surface plasmon resonance assays using antibodies or sialic acid receptors [25–27]. These new methods have to become simple, cost and time effective, therefore the need for antibody or antigen update, the lack of proper standards and the capacity to quantify in-process samples and from different strains at the same time have to be evaluated.

Here we report a VLP influenza label-free quantification tool based on biolayer interferometry applied on an Octet platform. It constitutes a step forward to the related methods already reported because takes advantage of HA interaction with human (α-2,6-linked sialic acid) and avian (α-2,3-linked sialic acid) receptors and eliminates the need for antibodies. The method is able to analyze in-process samples from crude bulk to final purified bioprocessing product with improved detection and quantification limits, compared to the approved method. This simpler and faster tool facilitated the measurement of results in real-time, which is crucial for the monitoring and optimization of the bioprocess. It is universal in the sense that it is suitable for different influenza groups and strains; it is not just for monovalent but also for multivalent vaccine candidates.

2 Materials and methods

2.1 Cell culture

High Five cells (Trichoplusia ni derived BTI-Tn-5B1-4 cell line) (B855-02, Invitrogen Corporation, Paisley, UK) were routinely cultured in Insect-XPRESS™ medium (Lonza, Basel, Switzerland) and kept in a humidified incubator at 27°C and 110 rpm. Cells reach a concentration of 2 to 3 × 10^6 cells mL^{-1} every two to three days and were reinoculated at 3 × 10^6 cells mL^{-1}. Cell concentration and viability were determined by using haemocytometer cell counts (Brandt, Wertheinmain, Germany) and trypan blue exclusion dye method (Merck, Darmstadt, Germany).
2.2 VLP production and harvest

For VLP production, High Five cells were infected with recombinant baculovirus (kindly provided by Redbiotec AG) encoding different strains of Influenza HA and M1 proteins (described in Samples section). Cell concentration at infection (CCI) was $2 \times 10^6$ cells mL$^{-1}$, and the multiplicity of infection (MOI) was 1 IP cell$^{-1}$ (infectious particles per cell). Baculovirus titers were determined using the MTT assay [28, 29].

High Five infected cells were harvested at a viability of 50–60%, corresponding to approximately 48 hpi, by centrifugation at 200 × g for 10 min (JA10 rotor, Avanti J25I centrifuge, Beckman Coulter, USA). The pellet was discarded and Benzonase® (101654, Merck Millipore, Germany) was added to the supernatant at a final concentration of 50 U mL$^{-1}$ and incubated at room temperature (RT) (22°C) for at least 15 min.

2.3 VLP Downstream processing

The clarification of VLP-containing bulk was carried out by dead-end filtration using a Sartopore® filter with 0.45 + 0.2 µm pore size (SART5445307H7-SS-A, Sartorius, Germany). The filtration module was previously conditioned with 50 mM HEPES, pH 7.4, 300 mM NaCl (working buffer). The clarification was performed at a constant flow rate of 100 mL min$^{-1}$ using a Tandem 1081 Pump (Sartorius Stedim Biotech, Germany) and the pressure was monitored by an in-line pressure transducer (080-699PSX-5, SciLog®, USA) to control possible over-pressure.

Clarified bulk was concentrated using tangential flow filtration (TFF). For ultrafiltration flat sheet Pellicon 2 Mini Ultrafiltration Module Biomax 300 kDa 0.1 m² (P2C300C01, Merck Millipore, USA) was used. The membrane module was set up accordingly with the manufacturer’s instructions. A fixed feed flow rate of 500 mL min$^{-1}$ was set up. Transmembrane pressure (TMP) was controlled by adjusting the retentate flow rate using a flow restriction valve. The pressure was monitored at feed inlet, retentate outlet and permeate outlet by in-line pressure transducers. The feed/retentate and the permeate outlet were adjusted manually. For each sample 60-seconds videos were acquired and particles between 70 and 150 nm were considered.

using a 13 mL capillary loop, at a constant flow rate of 3 mL min$^{-1}$. Working buffer was used as eluent and the eluted fractions were collected for further analyses.

VLPs’ corresponding fractions were further concentrated and diafiltered with working buffer using a flat sheet Pellicon XL Ultrafiltration Module Biomax 300 kDa 0.005 m² (PXC300C50, Merck Millipore, USA) at a constant flow rate of 40 mL min$^{-1}$. Final product was sterile filtered using a 0.2 µm syringe filter unit (10462960, Whatman - GE Healthcare, USA).

2.4 Samples

All Influenza VLP samples were produced and purified as described above. Influenza vaccine Influvac® (Abbott, USA) was used as positive control. Different Influenza strains from distinct subtypes and groups were selected (H1, B, and Group2 – H3, H4, H7, H10, H14, and H15) to replicate genetic diversity. VLPs produced contained HA and M1 influenza proteins. Multivalent particles are composed of a combination of multiple HA proteins.

2.5 Nanoparticle tracking analysis

Particles presence, concentration and size distribution were measured using the NanoSight NS500 (Nanosight Ltd, UK). The samples were diluted in D-PBS (14190-169, Gibco®, UK) so that virus-like particles concentration would be in the $10^8$–$10^9$ particles mL$^{-1}$ – the instrument’s linear range. All measurements were performed at 22°C. Sample videos were analyzed with the Nanoparticle Tracking Analysis (NTA) 2.3 Analytical software – release version build 0025. Capture settings (shutter and gain) were adjusted manually. For each sample 60-seconds videos were acquired and particles between 70 and 150 nm were considered.

2.6 Hemagglutination assay

Hemagglutinin protein detection and quantification was performed by hemagglutination assay for all the samples and for all stages of bioprocess evaluated. The assay was carried out according to the procedure described in the literature [30]. The predictive range of concentrations calculated for the Hemagglutination assay was determined based on the error associated with the 1.2 serial dilution used between plate wells. For VLPs’ hemagglutination assay, the concentrations are typically in µg mL$^{-1}$, which does not happen for virus hemagglutination, where a measure of activity is obtained, typically in HAU (hemagglutination units).

2.7 Biolayer interferometry quantification assay

Influenza VLPs’ binding to sialic acid receptors was measured by biolayer interferometry (BLI) using Octet
RED96 and Octet RED384 systems (fortéBIO, Pall Corp., USA). Data were acquired (kinetics mode) and analyzed using the Data acquisition software v9.0 (fortéBIO, Pall Corp., USA) or Data Analysis software v9.0 (fortéBIO, Pall Corp., USA). When necessary, data were exported as a Microsoft Excel file for further analysis in other software packages. Binding was calculated from the response amplitude (wavelength shift in nm) obtained in the first 100 s of each step. Avian 3'-SLN (3’SiaLacNAc-PAA-biot, 0036-BP) and human 6'-SLN (6’SiaLacNAc-PAA-biot, 0997-BP) receptors (Lectinity, Moscow, Russia) containing 20 mol% receptor analogue and 5 mol% biotin on a 30-kDa polyacrylamide backbone were resuspended in 50 mM HEPES, pH 7.4, 300 mM NaCl (working buffer) with 3 mM EDTA and 0.005% Tween-20. High Precision Streptavidin (SAX) Biosensors (18-0037, fortéBIO, Pall Corp., USA) were hydrated and blocked with Sample diluent (18-5028, fortéBIO, Pall Corp., USA). Unless otherwise stated all the samples were diluted with working buffer. To prevent cleavage of the receptors by influenza neuraminidase, Influvac® vaccine samples were incubated for 1 h at RT with 100 μM Oseltamivir phosphate (CAS number 204255-11-8, Sigma-Aldrich) and 100 μM Zanamivir (CAS number 139110-80-8, Sigma-Aldrich) inhibitors.

The quantification assay was set up by diluting biotinylated receptors with sample diluent and loaded into SAX Biosensors. Influenza VLP samples were then associated to the biosensors and association and dissociation profiles were measured. The method was defined with five steps: Baseline, Loading, Baseline, Association and Dissociation. Experiments were performed at 25°C and sample plates (microplate 96 well, F-bottom, black, 655209, from Greiner bio-one and microplate 96 well half-area, F-bottom, black, 3694, from Corning Costar) were agitated at 1000 rpm.

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the FDA Guidelines (FDA, ICH Guidance for Industry, Q2B Validation of Analytical Procedures: Methodology, 1996). The approach used for determining both limits was based on the standard deviation of the response and the slope of the calibration curve:

\[ \text{LOD} = \frac{3.3\sigma}{S} \]  
\[ \text{LOQ} = \frac{10\sigma}{S} \]

where \( \sigma \) is the standard deviation of the response and \( S \) the slope of the calibration curve. Quantifications were made taking into consideration the initial values (0 to 100 s) of the binding responses.

3 Results

3.1 Design of a quantification assay for influenza VLPs

The influenza VLPs quantification assay is based on biolayer interferometry analysis and performed on an Octet RED system. The approach takes advantage of the binding of influenza virus hemagglutinin to sialic acid recep-

![Figure 1. Schematic representation of influenza quantification assay. Biotinylated α-2,3 and α-2,6-linked sialic acid receptors, hydrated and blocked (baseline), are loaded into high precision streptavidin biosensors (loading). After a second baseline step, HA protein from influenza VLPs bind to the receptors, giving a response (association). Possible process impurities or non-bound VLPs are removed in the dissociation step.](image-url)
tors present in host cells. The method uses high precision functionalized streptavidin (SAX) biosensors and biotinylated α-2,3 and α-2,6-linked sialic acid receptors. Figure 1 schematizes the quantification assay. Briefly, biosensors are hydrated and blocked for non-specific binding with sample diluent at the initial baseline step. Then, the loading of the biotinylated sialic acid receptors is performed; a new baseline step with working buffer guarantees that the receptors that are not correctly linked are removed. Influenza VLPs are then associated to the receptors. Depending on the sample stage of purification, there are impurities that can be removed during the dissociation step. For the cases studied, the dissociation was negligible when compared to association (data not shown). Non-specific binding was evaluated by VLP association to naked biosensors, i.e. without loading of sialic acid receptors. Having the biosensors blocked, the binding magnitude to naked biosensors is significantly lower than the one observed for the receptor, which was confirmed by subtracting both binding curves (Supporting information, Fig. S1).

3.2 Criteria definition and optimization for the assay implementation

Human influenza VLP strains were quantified using BLI technology. As the results obtained with BLI analysis were compared with chicken erythrocyte agglutination assay, it was necessary to evaluate influenza strains’ binding to α-2,3, α-2,6 or a mixture of both receptors. It is expected that strains with higher affinity for α-2,6 present a higher binding response for that receptor. The same should occur for α-2,3 strains. The binding to the different sialic acid receptors of different influenza strains (monovalent or multivalent VLPs from different groups) was measured (Fig. 2A–E). Both receptors, alone or in a 1:1 ratio mixture,
were loaded. It was expected that different strains would present different binding responses, according to their specificity for the receptors [31]. The binding profiles were different for all analyzed strains. As predicted, depending on the strain type, higher binding responses for $\alpha-2,6$ receptor or for the mixture of both receptors were observed. The mixture of both receptors gives more reliable results to compare the binding responses calculated with BLI and HA assays. Thus, the quantification assay was implemented using a mixture of both receptors in a 1:1 ratio.

Receptor concentrations ranging from 0.5 mg mL$^{-1}$ to 4 mg mL$^{-1}$ were evaluated to define the optimal loading. At concentrations above 2 mg mL$^{-1}$ biosensor saturation occurred (data not shown) thus those values were not considered. Figure 2F presents the fine tuning optimization for $\alpha-2,6$, $\alpha-2,3$ and mixture receptor loading. Optimal receptor concentration was chosen at 1.2 mg mL$^{-1}$, just before the saturation plateau for $\alpha-2,6$ receptor and the mixture, although not for the $\alpha-2,3$ receptor, to avoid overcrowding of the biosensor and consequently VLP binding interference (fortéBIO, PALL Life Sciences, Biomolecular Binding Kinetics Assays on the Octet Platform, Application Note 14, 2013). Commercial influenza vaccine Influvac$^\text{®}$ was used as HA assay positive control. This vaccine has both HA and NA influenza proteins. As described in the literature [5] NA catalyzes the cleavage of sialic acid so the binding of Influvac vaccine to the receptors is affected by sample incubation with NA inhibitors (Zanamivir, Oseltamivir) [32]. In the absence of NA inhibitors $\alpha-2,6$ receptor binding curve suffers a significant decrease due to enzyme activity (Fig. 3A). However, even with NA inhibitors incubation, vaccine binding behavior is quite different from VLP samples, presenting two binding transitions (Fig. 2A–E, Fig. 3A). Moreover, the vaccine does not significantly bind $\alpha-2,3$ sialic acid receptor (with and without NA inhibitors), as com-

Figure 3. Calibration curves for influenza VLPs and Influvac vaccine. Representative binding curves (n = 3) of Influvac vaccine at a HA concentration of 45 µg mL$^{-1}$ to $\alpha-2,3$ and $\alpha-2,6$ sialic acid receptors. Incubation with NA inhibitors was evaluated. Blue line corresponds to $\alpha-2,6$ receptor incubated with NA inhibitors and orange line to $\alpha-2,6$ receptor without inhibitors. Grey line corresponds to $\alpha-2,3$ receptor with inhibitors and yellow line to $\alpha-2,3$ receptor without inhibitors (A). Representative calibration curves (n = 3) of Influvac vaccine binding to $\alpha-2,3$ and $\alpha-2,6$ sialic acid receptors. Circle marker corresponds to $\alpha-2,6$ and square marker to $\alpha-2,3$ (B). Representative calibration curves of H1 strain influenza VLP binding to $\alpha-2,3$ and $\alpha-2,6$ receptor mixture. Ultrafiltration retentate sample without trehalose (C) and with trehalose (D). The standard error of the estimation associated with the linear regression is 0.02 nm (which corresponds to -0.53 µg mL$^{-1}$) (C) and 0.006 nm (which corresponds to -0.26 µg mL$^{-1}$) (D).
pared with α-2,6 receptor binding curves; this is due to different affinities for the receptors, as it is well established that human strains bind preferably to α-2,6 receptor [5]. Nevertheless, to establish a calibration curve for the assay, vaccine at different concentrations was loaded onto α-2,6 and α-2,3 receptors. As can be observed in Fig. 3B, initial response does not present a linear behavior, in contrast to what is observed for VLP samples (Fig. 3C–D). These results show that Influvac vaccine is unsuitable for assay calibration. A recombinant HA protein was also evaluated, presenting extremely low binding values (below 0.03 nm), even at high concentrations (20 μg mL–1) as well as non-specific binding and high levels of dissociation (Supporting information, Fig. S2).

Ultrafiltrate (UF) retentate samples of each VLP were used to construct the calibration curves. Figures 3C and 3D show representative calibration curves for one of the evaluated strains. The linear behavior is identical to the other strains in the concentration range analyzed (Supporting information, Fig. S3). Limit of detection (LOD) was estimated as ~0.5 μg mL–1 for all the group 1 monovalent strains, 0.6 μg mL–1 for multivalent and 0.9 μg mL–1 for group 2 strains. Limit of quantitation (LOQ) was estimated as ~1.6 μg mL–1 for the monovalent strains, 1.8 μg mL–1 for multivalent and 2.7 μg mL–1 for group 2 strains (Supporting information, Table 1). The addition of trehalose to improve long-term product stability was investigated. The sugar was found to interfere with the assay, drastically changing the response values of otherwise identical samples. Even for samples measured using calibration curves that also contained trehalose, the results were inconsistent.

3.3 BLI quantification method enables in-process samples’ quantification

To evaluate the BLI quantification method, samples of mono and multivalent strains and different steps in the purification process were analyzed (Fig. 4, Supporting information, Table 2). For comparison samples were also quantified by HA assay. As an example, Fig. 4A confirms the methodology for a B monovalent strain for the entire purification process, from bulk samples to final purified bioprocessing product (before formulation): concentration values correlate well between methods. A reduced number of samples present differences in HA concentrations; this may be due to errors associated with the hemagglutination assay. Considering the errors associated with the hemagglutination assay, the majority of the calculated values with BLI assay fall between the predictive range of concentrations (Fig. 4B). The same results were observed for the other evaluated strains, including multivalent ones (Supporting information, Fig. S4).
Samples have different purity levels, depending on the DSP (Downstream Processing) step, leading to slightly changes in binding response behavior. Grouping all the samples per DSP step confirms that the majority of quantifications are within the predictive range of concentrations in accordance with HA assays (Fig. 4C–D). The overview of the results shows that samples from the clarification stage contain culture media compounds, possibly interfering with the analyses, as routinely observed in laboratory HA assays; thus small differences between quantification methods for these early stage samples are to be expected. As the DSP advances, purity increases and HA concentrations calculated with BLI assay became closer to those acquired with HA assay. Samples with trehalose at the storage concentration used here (15% w/v) do not allow robust quantifications.

### 3.4 BLI quantification method is specific for subtype and group VLP strains

To evaluate specificity, several strains from the same subtype or group were analyzed against the same calibration curve; experiments were performed for H1, H3 and B subtypes for several mono and multivalent strains. Strains from H1 subtype group were quantified using only one H1 strain as calibration curve; a UF retentate sample was used as calibration and three monovalent, one trivalent and one pentavalent VLP samples were quantified (Fig. 5). Results confirm subtype and group quantification is possible, with only one sample out of the error limits. This was also observed for H3 and B group samples containing tri and pentavalent VLPs (Supporting information, Fig. S5).

### 4 Discussion

Constant influenza antigenic shift and drift drives rapid development of vaccines and more efficient production and purification processes, requiring fast and reliable analytical tools to characterize final bioprocessing product, but also all stages of the upstream and downstream processes. As mentioned before, the main limitations of current methodologies, SRID and hemagglutination assays were reviewed [18, 19]. These influenza quantitation methods were designed for purified virus samples and are not suitable for crude samples or for VLPs bioproduction assays.

Here we report a HA quantification assay permitting the analysis of in-process samples of influenza multivalent VLPs involving sialic acid receptors interaction with the virus.

As a proper standard was not available, a commercially available influenza vaccine (Influvac) already implemented as HA assay positive control was evaluated. However, initial responses obtained with this “standard” were not linear, precluding the design of a calibration curve. Moreover, the vaccine association to the sialic acid receptors does not present the expected behavior, when compared to the association curves reported for the BLI technique. This is probably due to the presence of NA that catalyzes the cleavage of sialic acid receptors. However, even when vaccine samples were incubated with NA inhibitors, the binding response continues to be partially affected, meaning that vaccine formulation, in particular the stabilizers, could also have a role.
Searching for a new standard with similar protein content and the same production conditions as the evaluated samples, ultrafiltrate (UF) retentate samples of each VLP were used for calibration curves. Recombinant HA protein was also evaluated as a standard but no significant binding was observed, mainly because the protein was not in the native conformational trimeric structure, required for receptor interaction [27, 33].

The HA assay was performed for comparison to BLI technology. Erythrocytes used in HA assays should be chosen depending on the receptor selectivity of the targeted virus. However, it is not feasible to use human erythrocytes for process development quantification assays. Human influenza virus agglutinates chicken erythrocytes and, due to their characteristics they are routinely used in HA assays. These erythrocytes contain both α-2,3 and α-2,6-linked sialic acid receptors, although in different proportions [31]. Therefore, to improve the comparison of both assays a 1:1 ratio between both receptors was used. This ratio represents a broader condition to quantify different samples because different strains have different affinities for the sialic acid receptors.

The developed method was designed for in-process VLP samples and to be able to quantify HA for the entire bioprocess. As a proof of concept, several influenza VLPs from different strains, groups, stages of the process, mono and multivalent were evaluated. Most of the quantified values are within the predictive range of concentrations, stipulated based on the HA assay associated errors. However, there are several factors that can influence the binding response and, therefore, the method robustness, as reported elsewhere [25]. Our samples contained different levels of impurities such as DNA and total protein, different concentrations of cell culture medium and NaCl, and presence or absence of trehalose. Only samples with trehalose fall slightly out of the predictive range of concentrations.

The method is able to quantify different samples from the same subtype, using a single calibration curve. The binding affinities of strains from the same subtype are close enough to give concentration values that are within the predictive range.

This quantification method is an appealing tool for bioprocess development, from time of harvest control to final bioprocessing product quantification. Leading with broad spectra of samples from bulk to final product, residual amounts of baculovirus and/or exosomes can be present. Analogously to the traditional and approved methods, our tool is not able to distinguish HA that is present on VLPs or in other particles. This issue is not completely addressed due to the lack of proper DSP processes and analytical tools. It is also worth to note that recent reports showed that residual baculovirus, present in VLP samples, can trigger an innate immune response. This raises the question whether baculovirus presence is harmful or an advantage [34, 35]. Nevertheless, this method presents several advantages compared to the methods already settled for influenza virus. The method is an advance over the SPR-based assays that use microfluidics and is not compliant with bulk samples. Moreover, both the sialic acid receptors and the samples can be recovered, and the biosensors can be regenerated, in contrast with the SPR-methods. Being antibody-independent it eliminates the constant update, required for SRID and other methods. Moreover, the replacement of erythrocytes by sialic acid receptors solves the issue of influenza strains that do not agglutinate chicken erythrocytes and makes the assay more robust, when compared with HA assay, eliminating the use of fresh cells and decreasing the variations associated with user operation and host-origin. Importantly, it is possible to quantify a plethora of strains and multivalent influenza VLPs with an improved LOD and LOQ [18], a step further in the development of a universal quantification tool suitable for bioprocess development.

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S. C., D. W., J.W. and C. P conceived the study. S. C. and M. M. designed and performed the cell culture, VLPs’ production and the downstream processing studies. S. C., D. W. and J. W. designed and S. C. performed the biolayer interferometry experiments. C. P. and M. C. directed the project. S. C. wrote the manuscript with contributions from all authors.

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