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SPRi-based hemagglutinin quantitative assay for influenza vaccine production monitoring

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ABSTRACT

Influenza vaccine manufacturers lack tools, whatever the involved production bioprocess (egg or cell-based), to precisely and accurately evaluate vaccine antigen content from samples. Indeed, the gold standard single-radial immunodiffusion (SRID) assay, which remains the only validated assay for the evaluation of influenza vaccine potency, is criticized by the scientific community and regulatory agencies since a decade for its high variability, lack of flexibility and low sensitivity. We hereby report an imaging surface plasmon resonance (SPRi) assay for the quantification of both inactivated vaccine influenza antigens and viral particles derived from egg- and cell-based production samples, respectively. The assay, based on fetuin-hemagglutinin interactions, presents higher reproducibility (<3%) and a greater analytical range (0.03–20 μg/mL) than SRID for bulk monovalent and trivalent vaccine and its limit of detection was evaluated to be 100 times lower than the SRID's one. Finally, viral particles production through cell culture-based bioprocess was also successfully monitored using our SPRi-based assay and a clear correlation was found between the biosensor response and total virus particle content.

1. Introduction

The influenza vaccine manufacturing field is presently strongly lacking updated, rapid and reliable quantification tools to evaluate both vaccine lot potency and support new production process developments via the in-process quantification of bioactive virus particles.

Presently, influenza vaccine lots release relies on the off-line gold standard single-radial immunodiffusion (SRID) assay [1]. SRID requires strain-specific reference sera and antigen reagents to determine the μg amount of influenza hemagglutinin (HA) antigen from fragmented virus preparations. The annual preparation of SRID reagents takes up to 6 months and constitutes critical bottleneck for the release of vaccine lots [1–3]. This is why, WHO and regulatory agencies are strongly supporting the development of improved influenza vaccine potency assays [4,5] to speed up the vaccine lot release especially in the case of a pandemic outbreak.

A large set of analytical technologies has been evaluated to replace or complement SRID. Various immunoassays including ELISA, latex bead agglutination or slot-blot were developed with either specific monoclonal antibodies or reference sera to target influenza antigen epitopes [6–8]. Alternative chromatography-based strategies, like RP-HPLC and LC-MS, were also proposed to directly quantify HA content in vaccine preparations [9,10].

On the other hand, current influenza vaccine production mainly relies on embryonated egg-based technology. Such manufacturing process is challenged every year by vaccine reformulation, the reduced production yields of several influenza strains in eggs as well as the recurrent threat of a new pandemic outbreak [4,5]. Alternative production processes based on cell culture have been successfully developed in recent years to complement egg-based technology. Thus several mammalian cell lines were evaluated and the MDCK cell is now the sole mammalian cell line validated for the production of FluceIVax® vaccine [11–14]. Nevertheless, when it comes to optimization and monitoring of such cell-based processes, manufacturers are presently lacking in-process analytical tools to follow the virus particle production level. This time, analytics aims to monitor virus particle release, both infectious

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and non-infectious. Unfortunately, although non-infectious particles could represent 10–1000 times the number of infectious viral particles [15,16], standard methods are only quantifying infectious particles thanks to infectivity assays. Such assays are not appropriated for in-process and on-line monitoring.

As non-infectious particles appear to have a major contribution in final HA antigen content, several quantification technologies were evaluated to assess the total viral particles (VP) content for production process monitoring. Among physical quantification techniques proposed for VP evaluation, one can cite Nanoparticle Tracking Analysis (NTA), Tuneable Resistive Pulse Sensing (TRPS), Flow Virus counter and Field-Flow Fractionation combined to Multi-Angle Light Scattering (FFF-MALS) [17]. These techniques present several advantages such as reduced analysis time (minutes-hours vs. days) and increased reproducibility. Their weaknesses stand on a low specificity and/or on the absence of information regarding HA bioactivity. The only assays reported in the literature able to combine both advantages are quantitative surface plasmon resonance (SPR) assays [18,19].

SPR is commonly used to characterize biomolecular interactions and has been extensively used to determine affinity constants of influenza hemagglutinins toward a large panel of ligands. However, while SPR has already proven its potential for label-free quantitative evaluation of bioactive antibodies and recombinant proteins produced in cell culture [19,20], very few studies were performed on the use of SPR-based assay for quantitative evaluation of influenza hemagglutinin antigens or virus particles [2,21].

The present work is dedicated to the development of a SPRi-based assay for the rapid and label-free quantification of both influenza virus particles and hemagglutinin antigen content in influenza vaccine preparations. Based on immobilized fetuin, a protein containing generic sialic acid ligand of hemagglutinin influenza viruses, we developed a quantitative assay harboring high specificity and reproducibility with both egg-based influenza inactivated vaccine and virus particles produced by cell-based systems. Moreover, attention has been given to the regeneration capability of the biosensor surface, leading to the further development of an on-line real-time monitoring system for cell-based virus bioproduction processes [22].

2. Materials and methods

2.1. Reagents

Fetuin from fetal bovine serum, bovine serum albumin, ethyl dimethyl carbodiimide (EDC), N-hydroxysuccinimide (NHS), sodium dodecyl sulfate (SDS), polysorbate 20, Triton-X100, bacterial neuraminidase from *C. perfringens* and trypsin acetylated from bovine pancreas were purchased from Sigma (Saint Quentin Fallavier, France). Sodium acetate buffer and ethanolamine-HCl were acquired from Sierra sensors (Hamburg, Germany). Zwittergent 3–14 and Phosphate Buffer Saline (PBS) tablets were acquired from Merck (Darmstadt, Germany). All solutions were prepared using milliQ water. Oseltamivir carboxylate was purchased from Roche (Bale, Switzerland). Trivalent inactivated vaccine was purchased from Sanofi Pasteur (Vaxigrip® 2016/2017, lot #N2A381M, Sanofi Pasteur, Lyon, France) and recombinant A/California/04/2009 hemagglutinin from Sinobiological (Beijing, China). Madin–Darby canine kidney cells (MDCK, ATCC CCL34), serum-free Ultra-MDCK and EMEM media, L-glutamine and penicillin/streptomycin solutions were from Lonza (Amboise, France). Optipro, SFM4Transfx-293 and 2X MEM media, Nobel agar, Coomassie blue and BCA assay reagents were from Thermo Fisher (Dardilly, France). Bio-beads SM2 were purchased from Bio-RAD (Les Ulis, France). SRID standard antigen (NIBSC 09/196 and NIBSC 12/114) and polyclonal serum (NIBSC 14/134) for H1N1 A/California/7/2009 and H3N2 A/Victoria/361/2011 were obtained from NIBSC (http://www.nibsc.org).

2.2. SPR imaging system

SPR imaging experiments were performed using a SPR-2 instrument (Sierra Sensors, Hamburg, Germany). The sensor is based on Kretschmann configuration and consists in a microfluidic flow cell and a gold-coated prism functionalized with carboxylic acid groups (Amine coupling sensor, Sierra Sensors) useful for ligand immobilization. Biomolecular interactions at the surface of the sensor lead to changes in local refractive index. Recorded images of the flow cell are processed in order to obtain a numerical signal and generate sensorgrams expressed in response unit [RU].

Raw SPR data expressed in RU were processed through a custom MATLAB (The Mathworks, Natick, USA) script for interpolating and smoothing/despiking. Sensorgrams were obtained by first subtracting the signal from the reference surface to the signal from sensing surface for each sample in order to obtain standardized data. Initial binding rates [RU/s] were calculated based on the slope of the referenced sensorgrams for the first 30 s of the association phase, while response level was determined as the maximum response at the end of the association phase.

The limits of detection (LOD) and quantification (LOQ) were subsequently determined based on the following equations, where σ was the standard deviation of 3 consecutive injections of negative control sample (0 μg HA/mL):

$$LOD = \frac{3.3\sigma_{BLANK}}{slope}; \quad LOQ = \frac{10\sigma_{BLANK}}{slope}$$

The negative control consisted of SPR buffer (PBS Tween 20 0.05%) containing oseltamivir at a concentration of 10 mM for the analyses of vaccine samples, and EMEM cell culture medium containing oseltamivir at a concentration of 10 mM for influenza virus analyses.

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA).

2.3. Proteins immobilization

For immobilization purpose, proteins were used in a concentration of 200 μg/mL in 10 mM sodium acetate buffer, pH = 4.5. Immobilization protocol was performed directly within the flow cell of the SPR-2 apparatus. Briefly, a surface activation solution composed of 0.2 M EDC and 0.05 M NHS was applied at a flow rate of 25 μL/min on the surface of a SPRi sensor for 4 min. Then, fetuin solution was injected for 4 min (25 μL/min). In parallel, sensor reference surface (see Fig. 1B) was functionalized with control proteins, either BSA or truncated fetuin (without terminal sialic acid moiety). Remaining activated carboxylic acid functions of the sensor surface were then deactivated using 1 M ethanolamine injection for 3 min.

2.4. Protein surface density

According to the average density of proteins (i.e. $\rho_{prot} \approx 1.35 \times 10^{-21}$ g·nm^{-3}) the minimum volume of a globular protein can be calculated based on the following formula [23]:

V_{prot} [nm^3] = $MW_{prot}/(\rho_{prot} \times N_A)$, where N_A being Avogadro number.

Thus, assuming fetuin can be modeled as a globular protein of 48.4 kDa, calculated V_{fetuin} is 59.5 nm^3 and fetuin diameter 4.8 nm, leading to a maximum theoretical fetuin surface density

of 5.43×10^{10} molecules/mm^2. Similarly, assuming bovine serum albumin can be modeled as a globular protein of 66 kDa, calculated V_{BSA} is 81 nm^3 and BSA diameter 5.3 nm, leading to a maximum theoretical BSA surface density of 4.42×10^{10} molecules/mm^2.

The mean SPRi signal obtained during fetuin and BSA immobilization were 1340 RU and 870 RU, respectively. The measured responses can be converted into protein surface density using the classical correspondence where 1 response unit (RU) corresponds to approximately 1 pg/mm^2 [24]. Therefore, surface densities of 1.66×10^{10} molecules/mm^2 and 7.9×10^9 molecules/mm^2 were calculated for fetuin and BSA, respectively.

2.5. SPRi assay

Running buffer for all experiments was composed of PBS containing 0.05% (v/v) of Tween 20. A constant running buffer flow rate of 25 μL/min was used during all experiments while temperature in the measurement chamber was maintained at 15 °C. Sample injection flow rate was fixed to 5 μL/min for 6 min. This low flow rate favors conditions of mass transport limitation (MTL) enabling the determination of initial binding rates (dR/dt), which are proportional to analyte concentration [25]. Regeneration of the biosensor surface was performed through the injection, at a flow rate of 25 μL/min, of 25 μL of PBS containing 0.25% (v/v) of SDS. The neuraminidase inhibitor oseltamivir carboxylate was added to all samples at a concentration of 10 μM, to prevent cleavage of sialic acid residues from the fetuin-functionalized sensor.

2.6. Preparation of split-inactivated virus from ovoculture

Pandemic Influenza viral strain NYMC X-179A (reassortant A/H1N1/California/07/2009, NIBSC 09/216) was used for the production of split-inactivated virus. A stock production of Triton-fragmented H1N1 influenza virus was realized by ovoculture on 11-day-old embryonated hen's eggs. Allantoic fluid was harvested 72 h post-infection, concentrated by ultracentrifugation (UC) and purified through 25–60% sucrose gradient UC. Visible virus band was collected and layered onto PBS buffer before a final UC. Pellets were re-suspended before virus fragmentation with 0.5% Triton-X100 detergent for 1 h at 37 °C and dialyzed against PBS. Removal of detergent was achieved thanks to Bio-beads SM2 resin. After beads removal, samples were inactivated with 0.01% formaldehyde for 72 h at 20 °C. This preparation was calibrated using SRID assay and constituted our reference samples. Split-inactivated virus stored at 2–8 °C remain stable for several months [26].

2.7. Preparation of influenza virus from cell culture

Cell-based production of influenza virus was performed using Madin–Darby Canine Kidney cells (MDCK Lonza, ATCC CCL34). Cells were grown in T-flasks 175 cm^2 in serum-free Ultra-MDCK medium supplemented with L-glutamine and penicillin/streptomycin until reaching 70–80% confluence (approximately 10^5 cells/cm^2). For infection, influenza viruses from H1N1 pandemic NYMC X-179A strain (reassortant A/H1N1/California/07/2009, NIBSC 09/216)) were inoculated at a multiplicity of infection (MOI) of 10^{-4} virus/cell determined by $TCID_{50}$ assay (see next paragraph). Infection was performed in serum-free EMEM medium supplemented with L-glutamine, penicillin/streptomycin at a concentration of 100 U/mL and 1 μg/mL Trypsin. Virus production were harvested and clarified by 10 min low-speed centrifugation at 2000g before SPRi analyses. Control cell cultures were treated using identical protocol except for virus infection.

For the evaluation of viral production kinetics, cultures were sampled daily, clarified by 10 min low-speed centrifugation at 2000g and stored at −80 °C before analysis.

2.8. Titration of infectious viral particles (IVP)

Infectious titer (IVP/mL) of influenza virus particles were quantified by tissue culture infectious dose at 50% ($TCID_{50}$) and plaque forming units (PFU) assays. For PFU assay, confluent MDCK cells were plated in 6-well tissue culture plates, inoculated with 800 μL of cell culture supernatant serially diluted (1:10) in EMEM medium, and incubated 1 h at 37 °C under continuous shaking. Cells were washed with EMEM medium and overlaid with 1.1% Nobel agar previously mixed 1:1 with 2x MEM containing in addition 200 U/mL of penicillin-streptomycin and 2 μg/mL Trypsin. The plates were then incubated for 3 days at 37 °C, 5% CO_2 before visual examination of cytopathic effect. Infectious titer protocol was already decribed in Petiot et a. 2017 [12].

2.9. Quantification of total viral particles (VP)

Total particles were quantified by Tunable resistive pulse sensing technique (TRPS) using qNano Gold (IZON Science, Lyon, France). Analyses were performed using TPU membranes with a tunable nanopore of 150 nm (NP150). Prior analyses, samples were diluted at 1/5 in PBS buffer.

2.10. Quantification of hemagglutinin (HA) antigen content

HA concentration in split-inactivated lot of H1N1 virus has been determined by Single Radial Immunodiffusion assay (SRID) according to standard protocol with calibrated standard antigen (NIBSC 09/196) and polyclonal serum (NIBSC 14/134) of A/California/7/2009 [27]. Standard antigen and samples were incubated with a final concentration of 1% Zwittergent 3–14 for 30 min at room temperature, diluted at 1:1; 3:4; 1:2; 1:4 and loaded in triplicate into wells. Gels were incubated for 20 h at 22 °C to enable

Table 1
Detailed description of the reference samples used in the present study. * TIV: Trivalent Inactivated Vaccine Vaxigrip® 2016–2017 (lot #N2A381M). † HA content of influenza vaccine based on vaccine SRID titer provided by the manufacturer. ‡ HA content provided by NIBSC.

Technique	TIV Vaccine* *Trivalent, purified, inactivated and fragmented virus*	Split-inactivated H1N1 virus *H1N1 X-179A produced by ovoculture, purified and fragmented*	NIBSC influenza reference reagents 09/196 (H1N1) and 12/114 (H3N2) *Partially purified, inactivated, freeze-dried virus*	H1N1 virus *H1N1 X-179A produced in MDCK cell culture*
SRID [μg HA·mL^{-1}]	90†	239	37 (H1N1) ‡ 79 (H3N2) ‡	N.A.
Neuraminidase assay [nmol 4-Me·h^{-1}·mL^{-1}]	900	3000	N.A.	80
Total Particles [particles·mL^{-1}]	1.15×10^{11}	1.58×10^{12}	N.A.	1.71×10^{10}
Infectious titer [PFU·mL^{-1}]	Non-infectious	Non-infectious	Non-infectious	4.5×10^7

antigen migration, then washed with PBS, dried and stained in Coomassie blue. Precipitating rings were measured using ProtoCol 3 (SYNBIOSIS), and HA content was determined in μg/mL with the parallel line bioassay method by comparison of obtained dose-response curves of the samples with those of the standard antigen.

3. Results and discussion

The present study aimed at developing and validating the use of a SPRi-based assay for the characterization and quantification of influenza virus and antigen production (egg or cell-based process). In order to fully validate the developed analytical system, various preparations (trivalent vaccine, monovalent split-inactivated and whole influenza viruses) have been used and characterized (HA antigen content by SRID, neuraminidase activity and total or infectious particles counting), as described in Table 1.

3.1. SPRi assay optimization

The main steps toward the development of an effective SPRi assay is the optimization of sensing and control layers, but also the study of the possible regeneration of these surfaces and their operational lifetime. The sensing layer chosen in the present study is a covalently immobilized fetuin glycoprotein bearing oligosaccharides with α-2,3- and α-2,6-linked terminal sialic acid residues [28]. Two different negative control layers were evaluated, one composed of immobilized albumin from bovine serum (BSA) and one composed of fetuin treated with bacterial neuraminidase allowing for cleavage of α-2,3, α-2,6 or α-2,8 terminal sialic acid linkage. This second control surface corresponds to a cropped fetuin structure, close to the asialofetuin structure, known for inducing no specific binding of HA [29–31]. Both sensing and control surfaces are present in a single flow cell (Fig. 1A).

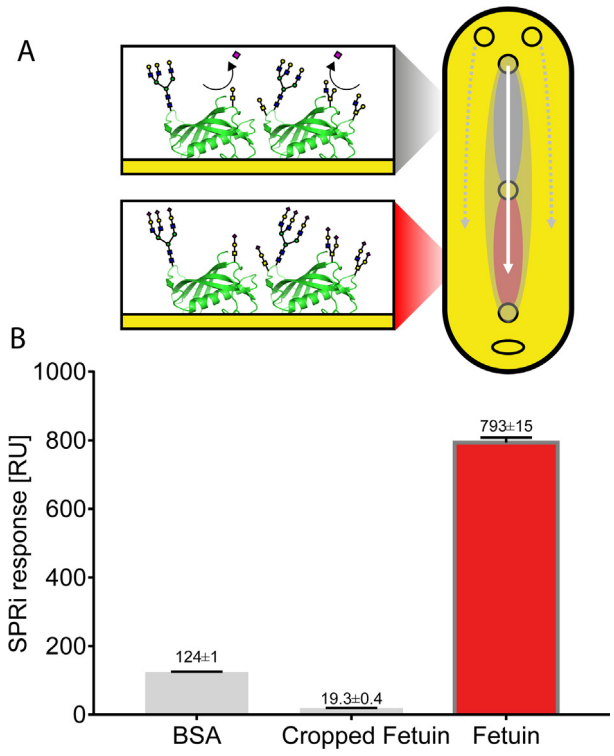


Fig. 1. Control and sensing SPRi surfaces. A: Schematic representation of the two distinct measurement zone of the SPRi biosensor flow cell: control (grey) and sensing (red) layers; white arrow: analyte flow; grey arrows: buffer flow. Glycan structures were added on fetuin (PDB 3APX) according to published data [28]. Terminal sialic acid moieties (purple diamond) are removed by bacterial neuraminidase (NA) in-situ before processing samples. B: SPRi response obtained for the control and sensing surfaces with TIV vaccine (10 μg HA/mL, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These three surfaces were prepared through the covalent grafting of each protein via amide bound formation between the gold chip surface modified with carboxylic acid groups (thioctic acid) and available primary amino groups of proteins. Fetuin grafting pH conditions were optimized to reach a maximal amount of sialic acid residues available for hemagglutinin binding. Indeed, protein grafting on the surface in flow conditions requires a contact between the surface and the protein, which is highly dependent upon the isoelectric point of the protein and then the pH of the immobilization solution. Optimum fetuin surface coverage of 1.66×10^{10} molecules/mm^2 was found using a grafting pH of 4.5. This coverage compares well with the theoretical maximum coverage of 5.43×10^{10} molecules/mm^2 calculated using protein size and comparing with immobilization SPRi signal (see *Materials and methods* for calculation).

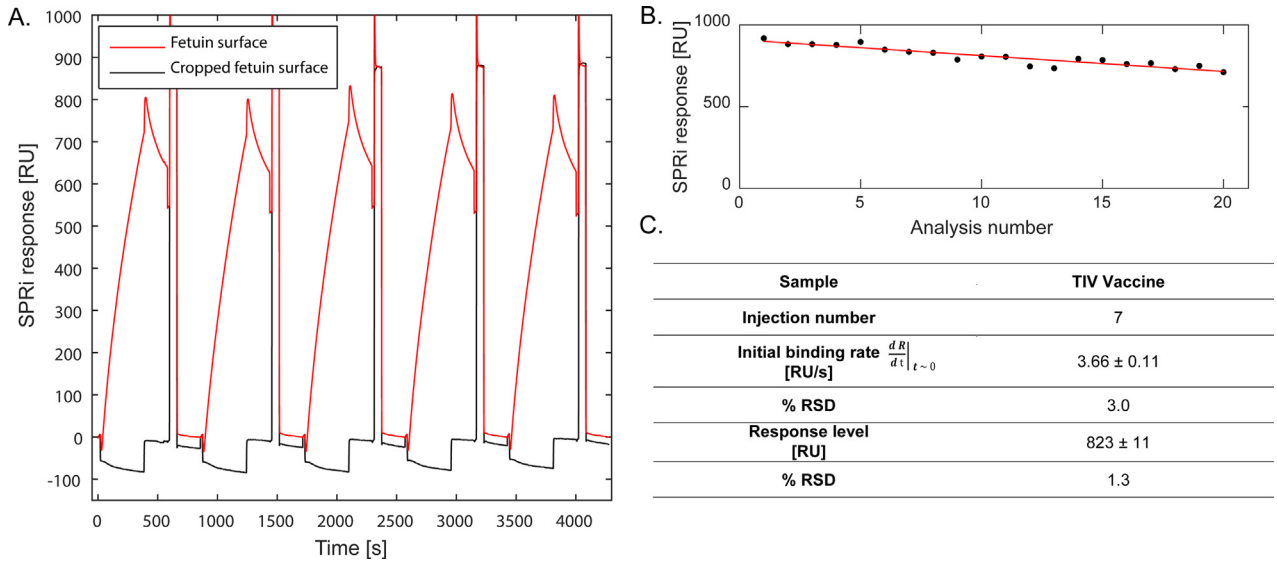
Assay specificity was first assessed using monomeric recombinant HA (A/California/04/2009). No specific interaction was observed when the protein was injected through the biosensor (data not shown). Our results were in accordance with previous studies [2,18] and confirm the HA binding to sialic acid residues requires bioactive trimeric form of antigen [30,32], thus validating our fetuin-based SPRi assay that aims to detect functional hemagglutinin. In this context, commercial Trivalent Inactivated Vaccine (TIV, Vaxigrip® 2016–2017, presented in Table 1) was used as a calibrated standard for bioactive HA antigen to determine the performances of the SPRi assay.

Fig. 1B depicted the biosensor responses when TIV vaccine (10 μg HA/mL) interacted with the different surfaces. BSA control surface was not able to hinder the non-specific interactions with the injected sample, leading to a poor specific to non-specific signal ratio of 6.4. On the contrary, cropped fetuin surface was shown to be a more reliable control surface, leading to a non-specific signal ratio of 41. Moreover, we here confirmed our fetuin-based SPRi assay is particularly suited for the evaluation of vaccine potency as the response is specifically based on hemagglutinin interactions with sialic acid-terminated glycans.

Regeneration solution composition was also optimized to achieve a complete releasing, between each sequential injection, of the glycan-bound HA. Classically proposed regeneration solutions [33] such as glycine 0.1 M, HCl 10 mM or 100 mM, NaCl 1 M and 0.25% (v/v) SDS in PBS were then evaluated. The best results were obtained using injection of 25 μL of a regeneration solution composed of 0.25% (v/v) SDS in PBS (Supplementary Information 1). Example of the signal recovery is presented in Fig. 2A where SPRi baseline was recovered between each of 5 successive injections of TIV vaccine antigen (10 μg/ HA mL).

Assay lifetime was also assessed through 20 successive cycles of TIV vaccine antigen (10 μg HA/mL) injection and consecutive regeneration. As depicted in Fig. 2B, a linear decrease of the biosensor response was observed (slope = −9.7 RU/injection; $R^2 = 0.87$). This decrease was attributed to a surface degradation during regeneration rather than to a sialic acid cleavage by influenza neuraminidase, as the neuraminidase inhibitor oseltamivir carboxylate was systematically added to each sample. A correction factor was then applied to all subsequent experiments, adjusting the measured biosensor response with the calculated decrease slope.

Finally, reproducibility of the assay was evaluated (Fig. 2C). The relative standard deviation (RSD) of the response was calculated based on 7 consecutive injections of TIV vaccine antigen (10 μg HA/mL) and regeneration. The high reproducibility of our assay was then demonstrated with RSD of 3% and 1.3% calculated based on initial binding rate and response level, respectively.



Sample	TIV Vaccine
Injection number	7
Initial binding rate $\frac{dR}{dt}\Big\|_{t\sim 0}$ [RU/s]	3.66 ± 0.11
% RSD	3.0
Response level [RU]	823 ± 11
% RSD	1.3

Fig. 2. Evaluation of sensor stability and assay reproducibility. A: Biosensor raw data obtained following 5 consecutive injections of TIV vaccine antigen (10 μg HA/mL) with consecutive regeneration. B: Operational stability of the fetuin sensing layer toward multiple regenerations using 0.25% (v/v) SDS in PBS. C: Reproducibility of the biosensor signal following several injections of TIV vaccine antigen samples (10 μg HA/mL, n = 7).

As a comparison, relative standard deviation of the classic SRID assay for influenza HA quantification is at best 6%, while the average RSD of Vaxarray sandwich immunoassay system has been estimated to 8% [2,6]. The RSD obtained are also in accordance with a previous study proposing SPR-based assay for influenza antigen quantification (2–5% for antibody-based SPR) [21]. Thus, after having validated the specificity of our regenerable sensor toward HA antigen and its excellent reproducibility, we further evaluated its sensitivity and applicability for sensing hemagglutinin present in different bioprocess-derived samples.

3.2. SPRi assay performances

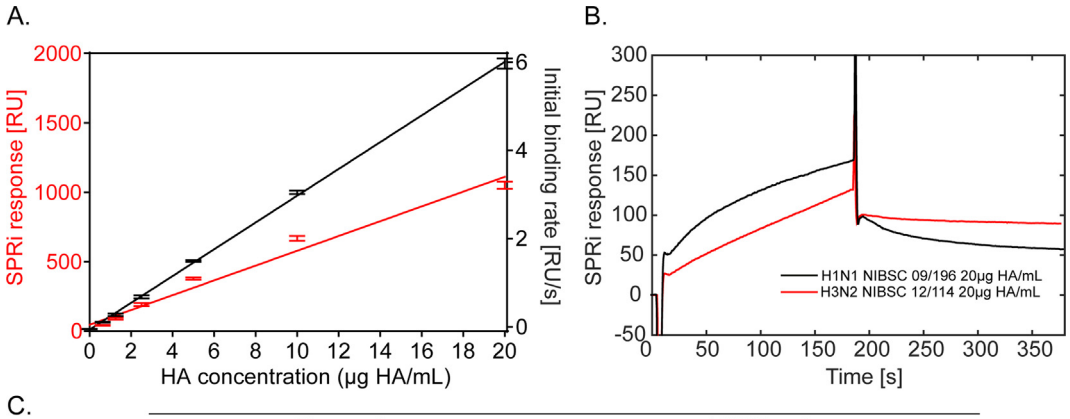
To evaluate the sensitivity of our assay toward influenza hemagglutinin antigen, SPRi signals acquired following the injection of different concentrations of TIV commercial vaccine were used to build calibration curves (Fig. 3A). Linear dynamic range of the assay together with its limit of detection and limit of quantification are presented in Fig. 3C. These parameters were determined using both the initial binding rate (RU/s) and the biosensor response (RU). Linearity of the response was shown to be optimum when the analytical parameter used to build the calibration curve was the initial binding rate. Indeed, linear regression parameters of $R^2 = 0.999$ and $R^2 = 0.98$ were calculated for initial binding rate and response, respectively. The assay was also shown to offer a large dynamic range with a linear response within three orders of magnitude, between 0.03 and 20 μg HA/mL. These results shall be compared to the dynamic range commonly obtained for SRID assay, i.e. between 3 and 36 μg HA/mL [34], demonstrating the superiority of the presently developed fetuin-based SPRi which offers 100 times higher sensitivity. Our SPRi assay also reveals to be superior to other alternative quantification methods proposed in the last decade as it presents much lower sensitivity with still a broad analytical range allowing for quantification of various types of samples (3.4–44 μg HA/mL for latex agglutination assay [8]; 1–15 μg HA/mL for antibody-based SPR assay [4]; 0.5–45 μg HA/mL for biolayer interferometry (BLI) [18]). The sole study achieving similar sensitivity and linear range than the one presented in this work used a biotinilated receptor homolog to quantify pandemic HA between 0.33 and 30 μg HA/mL [4].

The assay performances were further evaluated by comparing the response obtained for different formulations of HA produced in egg: (i) TIV vaccine commercial formulation, equally composed of antigens from H1N1, H3N2 and B strains [35], and (ii) a split-inactivated partially purified H1N1 influenza virus. This comparison enable us to evaluate the impact of both antigen formulation valence (monovalent or multivalent) and purity on the association and dissociation kinetics of the sensor surface.

Sensorgrams and corresponding association and dissociation constants calculated using 1:2 association-dissociation model (global fitting in kinetic-limited conditions) are given in Supplementary Information 2. TIV vaccine interaction with the immobilized fetuin evidenced higher dissociation constants than the freshly split H1N1 virus sample. This affinity decrease has previously been documented and characterized, and was attributed mainly to differences in quaternary structures between HA antigens and in the degree of chemical modifications between reference reagents and vaccine samples [2,6]. Thus, the present assay also has the potentiality to characterize vaccine virus production and a complementary study was performed about the interaction of H1N1 and H3N2 viruses with the present biosensor. NIBSC reference reagents (Table 1) for H1N1 and H3N2 strains were injected and the corresponding signals recorded. Results are presented in Fig. 3B. One more time, the biosensor was able to characterize interactions between immobilized fetuin and HA from two other different strains with clear variations in kinetic behaviors.

3.3. SPRi analysis of cell culture-derived influenza virus samples

The secondary objective of the study was to demonstrate the potential of SPRi fetuin-based assay to quantify virus particles production level in cell culture crude samples. It was therefore mandatory to properly distinct between specific binding and non-specific adsorption of cell culture derived impurities on sensor's surface [36]. Indeed, cell culture crude samples contain various components which might interfere with the SPRi surface, i.e. the biological product of interest (viral particles in our case), cells and debris that shall be removed during the clarification step, and a variety of either process- or product-related impurities. It was then necessary to investigate the effect of cell culture media and cell



C.

Parameters	TIV Vaccine
Slope	0.323 ± 0.002 RU/s/µg HA/mL
R^2	0.999
Limit of Detection (LOD)	**0.03 µg HA/mL**
Limit of Quantification (LOQ)	**0.10 µg HA/mL**

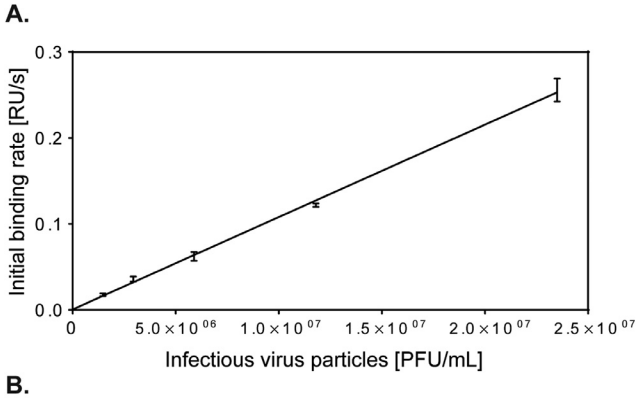
Fig. 3. Evaluation of SPRi assay performances with TIV vaccine and NIBSC reference reagents. A: Calibration curves for TIV vaccine (n = 3). B: Sensorgrams obtained for the analysis of H1N1 and H3N2 reference reagents. C: Analytical parameters determined for TIV vaccine based on initial binding rate determination, σ_{BLANK} = 0.003 RU/s (n_{BLANK} = 3).

culture-derived impurities on the response of our fetuin-functionalized surface.

A limited number of mammalian cell platform are currently in use for large-scale influenza virus production in the literature. Among them, MDCK cell line is the sole cellular platform currently in use for commercial vaccine manufacturing (Flucelvax®, Sequirus). Thus, first part of this evaluation consisted in injecting selected fresh culture media (EMEM, Ultra-MDCK, Optipro and SFM4TransFX) onto the fetuin-functionalized surface. These media were chosen for their use in reference cell-based influenza virus production described in the literature [37,38]. From this study, only SFM4TransFX demonstrated a strong non-specific response (Supplementary Information 3, Fig. S3-left) excluding it from the list of potential media useful for SPRi detection of cell culture product. Then, upon the selected media giving only negligible SPRi non-specific response (bellow 5 RUs), EMEM documented as a suitable media for the production of influenza virus using MDCK cells, was then selected for all subsequent experiments.

Then, the effect of the MDCK side-products was evaluated through the injection of clarified samples from non-infected MDCK cell cultures in EMEM medium. As can be seen in Supplementary Information 3, Fig. S3-right, even if a clear increase of the non-specific signal was observed (from 1.4 to 5 RUs), this signal was still found to be negligible and permitted to validate the use of MDCK culture in EMEM medium for potential SPRi detection of influenza virus during bioprocess.

These preliminary measurements being performed, the SPRi assay was used to evaluate virus concentration in samples from cell-based bioprocesses. H1N1 virus samples harvested at 48 h post-infection (hpi) were quantified on both SPRi sensor and reference assays, namely infectivity assay (PFU) and total particles counting (TRPS). The 48hpi sample was used to build calibration curve and to evaluate biosensor performances and linearity of the response for total influenza virus particles (Fig. 4). As detailed in Fig. 4B, the SPRi assay offered a limit of detection of 1.8 x 10^5 PFU/mL and a limit of quantification of 5.3 x 10^5 PFU/mL. The linear response was confirmed among a 2-log analytical range, allowing to reach 10^7 PFU/mL. Considering standard production kinetics obtained for cell-based influenza productions, such results allow to envision the in-process monitoring of influenza production in



B.

Parameters	H1N1 virus
Slope	2.02 ± 0.04 .10^{-8} RU/s/PFU/mL
R^2	0.998
Limit of Detection (LOD)	**1.8 x 10^5 PFU/mL**
Limit of Quantification (LOQ)	**5.3 x 10^5 PFU/mL**

Fig. 4. Evaluation of SPRi assay performances with cell culture-derived influenza virus. A: Calibration curve for H1N1 virus produced in MDCK cell culture (harvest time: 48 h.p.i.) according to infectious viral particles (n = 2). B : Analytical parameters determined for H1N1 virus based on initial binding rate determination, σ_{BLANK} = 0.001 RU/s (n_{BLANK} = 3).

cell-based bioprocess from its early production phases up to its maximal production level.

Notably, one must here take into account the discrepancy between the mechanisms involved in plaque forming units (PFU) determination and the present biosensor. Indeed, PFU assay is only impacted by the presence of infectious viral particles, whereas our fetuin-based assay quantifies both infectious and non-infectious viral particles as long as they harbor bioactive HA. In our case, from the sample harvested at 48 h post-infection, PFU and TRPS quantification allowed to calculate a VP/IVP ratio of 380 (Supplementary Information 4). It is then clear that the SPRi biosensor signal obtained shall be considered only as an indirect detection of IVP (or PFU) through the detection of the total VP concentration. Indeed, only 1/380 of the observed interactions are induced by

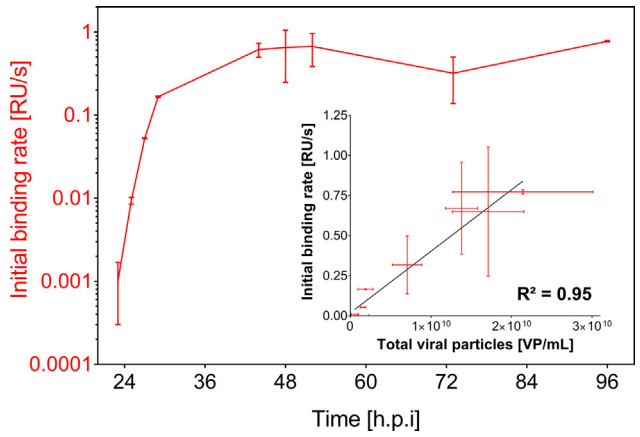


Fig. 5. Influenza virus production kinetics determined by SPRi in MDCK cell culture-based bioprocess from 23 to 96 h post-infection. Insert: correlation between SPRi biosensor response and total viral particles concentration determined by TRPS (n = 3 for SPRi analysis and n = 2 for TRPS analysis).

IVP presence and these interactions correspond to SPRi signals near the apparatus' detection limit (0.1 RU). Additionally, in terms of total viral particles the limit of detection was 6.5×10^7 VP/mL, which makes SPRi as sensitive as other (e.g. NTA) quantification techniques used for total viral particles analysis [17].

3.4. SPRi at-line monitoring of influenza virus production kinetics

VP titer is one of the main parameter of interest regarding viral vaccine production, as one ultimately wants to evaluate total antigen content in the production process. Indeed, the last set of experiments herein documented is an attempt to demonstrate the potential of the SPRi assay for its later on-line implementation for monitoring of virus particle production. H1N1-infected and mock-infected cell cultures were harvested at regular intervals from 23 to 96 h post-infection. Collected samples were further clarified and injected undiluted onto the fetuin-based biosensor ("at-line" [22]). For the sake of comparison, all samples were also quantified by reference infectivity assay (PFU) and total particles counting (TRPS). The experimental results are presented in Fig. 5, where each point corresponds to the net SPRi response between infected and control culture. As can be seen, a clear evolution of the biosensor signal was obtained with a SPRi response that levels off at 40 h post-infection. This stabilization of the total virus particle content was also observed using TRPS measurements (Supplementary Information 4). Fig. 5-insert presents the correlation between the measured SPRi signals and the TRPS measurements. A linear correlation ($R^2 = 0.95$) was found between the two techniques, proof of the usefulness of the developed technique for cell-based vaccine production monitoring.

4. Discussion

We here presented an assay for the rapid and label-free quantification of influenza virus particles and bioactive hemagglutinin in bioprocess-derived samples, i.e. egg-based and cell-based bioprocesses. The method is based on affinity capture of hemagglutinin antigen by sialic-acid terminated glycans present at the surface of the fetuin-functionalized sensor, which addresses the challenge of producing updated strain-specific sera or antibody reagents required for SRID. Sensor regeneration strategy allows for running multiple sequential analysis on the same sensor, while the use of fetuin glycoprotein provides a panel of sialylated α-2,3 and α-2,6 glycans offering specific ligands for influenza HA quantification without discriminating between influenza strains. The assay was shown to offer better analytical performances than SRID or other assays recently developed for influenza vaccine potency evaluation and requires low analyte volume (<100 μL). The high sensitivity and wide analytical range (0.03–20 μg HA/mL) provide the possibility to evaluate bioprocess samples with low HA titers. Sample time processing was 10 min while SRID turnaround time is usually 2–3 days [4].

The assay was also shown to have the potentiality to be directly applied to cell-based bioprocess monitoring. Several cell culture media showed that they induced no non-specific binding, which opens the way to work with different cell lines. Also, a minimum sample pretreatment (cell/supernatant separation through 2000 g centrifugation) was required before analysis, which might in a near future be implemented on-line using membrane and/or acoustic filtration for sample pretreatment [39]. This SPRi quantitative assay reveals a highly valuable process analytical technology (PAT) approach as it opens the path to the evaluation of influenza vaccine production consistency and robustness with the quantification of total virus particles along the production and purification processes. It is also a technology potentially allowing the evaluation of HA antigen total particles content of new cell culture-based influenza vaccine under development (e.g. Virus-like Particles vaccine). Indeed, such processes generally offer lower productivity regarding upstream process step.

Declaration of interests

The authors declared that there is no conflict of interest.

Acknowledgement and financial & competing interest disclosure

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.01.083.

References

[1] Minor P. Assaying the potency of influenza vaccines. Vaccines 2015;3:90–104. https://doi.org/10.3390/vaccines3010090.
[2] Khurana S, King LR, Manischewitz J, Coyle EM, Golding H. Novel antibody-independent receptor-binding SPR-based assay for rapid measurement of influenza vaccine potency. Vaccine 2014;32:2188–97. https://doi.org/10.1016/j.vaccine.2014.02.049.
[3] EMA/EDQM. Joint EMA/EDQM workshop on improved potency assays for inactivated influenza vaccines. meeting report 12 Dec 2011; 2012.
[4] Thompson CM, Petiot E, Lennaertz A, Henry O, Kamen AA. Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches. Virol J 2013;10:141. https://doi.org/10.1186/1743-422X-10-141.
[5] WHO Global Influenza Programme. Influenza Vaccine Response during the Start of a Pandemic. Geneva, Switzerland; 2015.
[6] Kuck LR, Saye S, Loob S, Roth-Eichhorn S, Byrne-Nash R, Rowlen KL. VaxArray assessment of influenza split vaccine potency and stability. Vaccine 2017;35:1918–25. https://doi.org/10.1016/j.vaccine.2017.02.028.
[7] Bodle J, Verity EE, Ong C, Vandenberg K, Shaw R, Barr IG, et al. Development of an enzyme-linked immunoassay for the quantitation of influenza haemagglutinin: an alternative method to single radial immunodiffusion. Influenza Other Respir Viruses 2013;7:191–200. https://doi.org/10.1111/j.1750-2659.2012.00375.x.
[8] Buffin S, Ikhelef N, Prudent J, Dubayle J, Nougarede N, Varenne MP, et al. A latex agglutination assay to quantify the amount of hemagglutinin protein in

adjuvanted low-dose influenza monovalent vaccines. J Virol Methods 2018;251:46–53. https://doi.org/10.1016/j.jviromet.2017.10.011.
[9] Pierce CL, Williams TL, Moura H, Pirkle JL, Cox NJ, Stevens J, et al. Quantification of immunoreactive viral influenza proteins by immunoaffinity capture and isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem 2011;83:4729–37. https://doi.org/10.1021/ac2006526.
[10] Wen Y, Han L, Palladino G, Ferrari A, Xie Y, Carfi A, et al. Conformationally selective biophysical assay for influenza vaccine potency determination. Vaccine 2015;33:5342–9. https://doi.org/10.1016/j.vaccine.2015.08.077.
[11] Petiot E, Kamen A. Real-time monitoring of influenza virus production kinetics in HEK293 cell cultures. Biotechnol Prog 2013;29:275–84. https://doi.org/10.1002/btpr.1601.
[12] Petiot E, Proust A, Traversier A, Durous L, Dappozze F, Gras M, et al. Influenza viruses production: evaluation of a novel avian cell line DuckCelt®-T17. Vaccine 2018;36(22):3101–11. https://doi.org/10.1016/j.vaccine.2017.03.102.
[13] Genzel Y, Dietzsch C, Rapp E, Schwarzer J, Reichl U. MDCK and Vero cells for influenza virus vaccine production: a one-to-one comparison up to lab-scale bioreactor cultivation. Appl Microbiol Biotechnol 2010;88:461–75. https://doi.org/10.1007/s00253-010-2742-9.
[14] Brown SW, Mehtali M, Sci PD a JP. The Avian EB66 ® Cell Line, Application to Vaccines, and Therapeutic Protein Production The Avian EB66 π Cell Line , Application to Vaccines , and Therapeutic Protein Production; 2010.
[15] Frensing T. Defective interfering viruses and their impact on vaccines and viral vectors. Biotechnol J 2015;10:681–9. https://doi.org/10.1002/biot.201400429.
[16] Frensing T, Heldt FS, Pflugmacher A, Behrendt I, Jordan I, Flockerzi D, et al. Continuous influenza virus production in cell culture shows a periodic accumulation of defective interfering particles. PLoS ONE 2013;8:e72288. https://doi.org/10.1371/journal.pone.0072288.
[17] Heider S, Metzner C. Quantitative real-time single particle analysis of virions. Virology 2014;462–463:199–206. https://doi.org/10.1016/j.virol.2014.06.005.
[18] Carvalho SB, Moleirinho MG, Wheatley D, Welsh J, Gantier R, Alves PM, et al. Universal label-free in-process quantification of influenza virus-like particles. Biotechnol J 2017;12:1–24. https://doi.org/10.1002/biot.201700031.
[19] Chavane N, Jacquemart R, Hoemann CD, Jolicoeur M, De Crescenzo G. At-line quantification of bioactive antibody in bioreactor by surface plasmon resonance using epitope detection. Anal Biochem 2008;378:158–65. https://doi.org/10.1016/j.ab.2008.04.019.
[20] Jacquemart R, Chavane N, Durocher Y, Hoemann C, De Crescenzo G, Jolicoeur M. At-line monitoring of bioreactor protein production by surface plasmon resonance. Biotechnol Bioeng 2008;100:184–8. https://doi.org/10.1002/bit.21725.
[21] Nilsson CE, Abbas S, Bennemo M, Larsson a, Hämäläinen MD, Frostell-Karlsson A. A novel assay for influenza virus quantification using surface plasmon resonance. Vaccine 2010;28:759–66. https://doi.org/10.1016/j.vaccine.2009.10.070.
[22] Whitford W, Julien C. Analytical technology and PAT. Bioprocess Int 2007;5:S32–41.
[23] Erickson HP. Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. Biol Proced Online 2009;11:32–51. https://doi.org/10.1007/s12575-009-9008-x.
[24] Schasfoort RBM. Chapter 3. Surface plasmon resonance instruments. handbook of surface plasmon resonance. Cambridge: Royal Society of Chemistry; 2017. p. 60–105. https://www.doi.org/10.1039/9781788010283-00060.
[25] Karlsson R. Biosensor binding data and its applicability to the determination of active concentration. Biophys Rev 2016;8:347–58. https://doi.org/10.1007/s12551-016-0219-5.
[26] Kon TC, Onu A, Berbecila L, Lupulescu E, Ghiorgisor A, Kersten GF, et al. Influenza vaccine manufacturing: Effect of inactivation, splitting and site of manufacturing. Comparison of influenza vaccine production processes. PLoS ONE 2016;11:1–19. https://doi.org/10.1371/journal.pone.0150700.
[27] Wood J, Schild G, Newman R, Seagroatt V. An improved single-radial-immunodifusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines. J Biol Stand 1977;5:237–47.
[28] Gerlach JQ, Kilcoyne M, Joshi L. Microarray evaluation of the effects of lectin and glycoprotein orientation and data filtering on glycoform discrimination. Anal Methods 2014;6:440–9. https://doi.org/10.1039/c3ay40936h.
[29] Jiang L, Eichelberger MC. Evaluation of Epic® label-free technology to quantify functional recombinant hemagglutinin. Biol Proced Online 2015;17:7. https://doi.org/10.1186/s12575-015-0019-5.
[30] Khurana S, Verma S, Verma N, Crevar CJ, Carter DM, Manischewitz J, et al. Properly folded bacterially expressed H1N1 hemagglutinin globular head and ectodomain vaccines protect ferrets against H1N1 pandemic influenza virus. PLoS ONE 2010;5:1–11. https://doi.org/10.1371/journal.pone.0011548.
[31] Takemoto DK, Skehel JJ, Wiley DC. A surface plasmon resonance assay for the binding of influenza virus hemagglutinin to its sialic acid receptor. Virology 1996;217:452–8. https://doi.org/10.1006/viro.1996.0139.
[32] Magadán JG, Khurana S, Das SR, Frank GM, Stevens J, Golding H, et al. Influenza A virus hemagglutinin trimerization completes monomer folding and antigenicity. J Virol 2013;87:9742–53. https://doi.org/10.1128/JVI.00471-13.
[33] GE Healthcare. Biacore Sensor Surface Handbook; 2005.
[34] Kuck LR, Sorensen M, Matthews E, Srivastava I, Cox MMJ, Rowlen KL. Titer on chip: new analytical tool for influenza vaccine potency determination. PLoS ONE 2014;9:1–10. https://doi.org/10.1371/journal.pone.0109616.
[35] Council of Europe. Influenza vaccine (SPLIT VIRION, INACTIVATED). Eur Pharmacopoeia 2008:867–8.
[36] Masson JF, Battaglia TM, Cramer J, Beaudoin S, Sierks M, Booksh KS. Reduction of nonspecific protein binding on surface plasmon resonance biosensors. Anal Bioanal Chem 2006;386:1951–9. https://doi.org/10.1007/s00216-006-0834-2.
[37] Lohr V, Rath a, Genzel Y, Jordan I, Sandig V, Reichl U. New avian suspension cell lines provide production of influenza virus and MVA in serum-free media: studies on growth, metabolism and virus propagation. Vaccine 2009;27:4975–82. https://doi.org/10.1016/j.vaccine.2009.05.083.
[38] Milián E, Kamen Aa. Current and emerging cell culture manufacturing technologies for influenza. Vaccines 2015 2015.
[39] Genzel Y, Vogel T, Buck J, Behrendt I, Ramirez DV, Schiedner G, et al. High cell density cultivations by alternating tangential flow (ATF) perfusion for influenza A virus production using suspension cells. Vaccine 2014;32:2770–81. https://doi.org/10.1016/j.vaccine.2014.02.016.