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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, single-radial immunodiffusion (SRID) assay remains the only validated assay for the evaluation of influenza vaccine potency. We hereby report an imaging surface plasmon resonance (SPRi) biosensor for the quantification of both split vaccine antigen and influenza virus particles from egg- and cell-based production samples, respectively. The biosensor, based on the fetuin-hemagglutinin interaction, 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-time lower than the SRID's one. Finally, viral particles production through cell culture-based bioprocess was also successfully monitored using the developed assay and a clear correlation was found between the biosensor response and the sample virus particle content.

INTRODUCTION

Current gold standard influenza vaccine production is based on egg inoculation technology which is every year endangered by the threat of a new pandemic outbreak. Alternative production methods based on standardized cell culture processes have been successfully proposed and it is believed to be the future of the influenza vaccine production since it should be standardized, monitored and controlled, like other bio-production processes. Nevertheless, when it comes to optimization and monitoring of vaccine production processes, one of the most interesting parameters to follow is the virus particles content, directly in the bioreactor. Unfortunately, accessible methods are not useful for on-line monitoring, while WHO and regulatory agencies are strongly supporting the development of improved influenza vaccine potency assays^{1,2}. Presently, influenza vaccine lots release relies on the standard single-radial immunodiffusion (SRID) assay¹. SRID requires strain-specific reference sera and antigen reagents to determine the amount of influenza hemagglutinin (HA) antigen. The annual preparation of these reagents takes up to 6 months and constitutes critical bottleneck for the release of vaccine lots³⁻⁵.

In order to push forward the development and optimization of cell culture-based virus production, rapid, specific, sensitive and in-process quantification tools need to be developed. This will enable to precisely follow the production level and optimize the bioprocess operating conditions.

A large set of analytical technologies has been evaluated to replace or complement SRID in early phases of process developments. Various immunoassays including ELISA, latex bead agglutination or slot-blot were developed with either monovalent antibodies or reference sera to target influenza antigens⁶⁻⁸. Alternative chromatography-based strategies, like RP-HPLC and LC-MS, were also proposed to directly quantify HA content in vaccine samples^{9,10}. Regarding in-process quantification of influenza viruses, physical characterization of the viral particles through Dynamic Light Scattering (DLS), Tuneable

Resistive Pulse Sensing (TRPS), Flow Virus counter and FFF-MALS were proposed¹¹. Although 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 on the absence of information regarding HA bioactivity. We are proposing herein the use of imaging Surface Plasmon Resonance (SPRi), which represents an interesting alternative being able of real time detection of specific biomolecular interactions with hemagglutinin, with a potential to be on-line integrated to the production process via incorporated fluidic system.

SPR is commonly used to detect biomolecular interactions and has also been extensively used to determine affinity constants of several different 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^{12,13}, very few studies were performed on the use of SPR-based assay for quantitative evaluation of influenza hemagglutinin antigens or virus particles^{4,12}. 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 content in influenza vaccine. Based on immobilized fetuin, a generic sialic acid-containing ligand of influenza hemagglutinin antigens, our quantitative assay harbored specificity and reproducibility with both influenza split vaccine and virus particles produced from egg- and cell-based systems. Moreover, particular attention has been given to the regeneration capability of the biosensor surface, leading to further on-line real-time monitoring and development for cell-based bioprocesses.

MATERIEL AND METHODS

Reagents

Fetuin from fetal bovine serum, bovine serum albumin (BSA), 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 from Roche (Bale, Switzerland). Trivalent inactivated vaccine were 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 12/214) 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>).

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 micro-fluidic 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 leads to changes in local refractive. 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 (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 response rates ($[RU \cdot s^{-1}]$) were calculated based on the slope of the referenced sensorgrams for the first 30 seconds 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 calibration (LOQ) were subsequently determined based on the following equations:

$$LOD = 3.3 \frac{\sigma}{slope}; LOQ = 10 \frac{\sigma}{slope}$$

where σ was the standard deviation of 3 consecutive injections of negative sample (0 μg HA/mL). Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA).

Proteins' immobilization

For immobilization purpose, proteins were used in a concentration of 200 $\mu\text{g}/\text{mL}$ 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 $\mu\text{L}/\text{min}$ on the surface of a SPRi sensor for 4 min. Then, fetuin immobilization solution was injected and applied for 4 minutes. In parallel, a separate surface of the same sensor (see **Figure 1-B**) 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 minutes.

Protein surface density

According to the average density of proteins (i.e. $\rho_{\text{prot}} \approx 1.35 \times 10^{-21} \text{ g} \cdot \text{nm}^{-3}$) the minimum volume of a globular protein can be calculated based on the following formula¹⁴:

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

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

The mean SPRi signal obtained during fetuin and BSA immobilization were 1340 RU and 868 RU, respectively. The measured values of reflectivity variation can be converted into protein surface density using the classical correspondence¹⁵ where 1 SPR unit corresponds approximately 1 pg/mm^2 . Surface densities of 1.66×10^{10} molecule/ mm^2 and 7.9×10^9 molecule/ mm^2 were calculated for fetuin and BSA, respectively.

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 $\mu\text{L}/\text{min}$ was used during all experiments while temperature in the measurement chamber was maintained at 15°C. Sample injection flow rate was fixed to 5 $\mu\text{L}/\text{min}$ for 6 minutes, leading to the injection of 30 μL of each sample. Regeneration of the biosensor surface was performed through the injection, at a flow rate of 25 $\mu\text{L}/\text{min}$, of 25 μL of PBS containing 0.25% (v/v) of sodium dodecyl sulfate.

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 modified sensor.

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 hours post infection, concentrated by ultracentrifugation (UC) and purified through 25-60% sucrose gradient UC. Visible virus band was harvested and layered onto PBS buffer before a final UC. Resuspended pellets were fragmented with 0.5% Triton-X100 detergent for 1 hour at

37°C before being 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 72h 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 months¹⁶.

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-175 T-flasks in serum-free Ultra-MDCK medium supplemented with L-glutamine and penicillin/streptomycin until reaching 70-80% confluency (approximately 115 000 cell/cm²). 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. 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. Cell cultures were harvested and clarified by 10 minutes low-speed centrifugation at 2000g before being used for SPRi detection. Control cell cultures were obtained using identical protocol except for virus infection.

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

Titration of infectious viral particles (IVP) by plaque forming unit assay

Infectious titer (IVP/ml) of influenza virus particles were quantified using the tissue culture infectious dose at 50% (TCID₅₀) and plaque forming unit (PFU) assays. For plaque 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 hour 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% CO₂ before visual examination of cytopathic effect.

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 mem-

branes with a tunable nanopore of 150 nm (NP150). Prior analyses, samples were diluted at 1/5 in PBS buffer.

Quantification of Hemagglutinin (HA) antigen content and activity

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/7/2009¹⁷. Standard and samples were incubated with a final concentration of 1% Zwittergent 3-14 for 30 minutes at room temperature, diluted at 1:1; 3:4; 1:2; 1:4 and loaded in triplicate into wells. Gels were incubated for 20 hours at 22°C to enable 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.

RESULTS AND DISCUSSION

The present study aims at developing and validating the use of a SPRi-based assay for the characterization of influenza virus production (egg or cell-based process). In order to fully validate the developed analytical system, various samples have been collected or prepared and characterized. A description of these samples is presented in Erreur ! Source du renvoi introuvable. together with their characterization methods and corresponding characteristics. Three complementary characterizations were performed on these samples: HA content (SRID), neuraminidase activity and total particles counting. Within the present study, commercial TIV vaccine (equally composed of antigens from H1N1, H3N2 and B strains) was used as a standard while split-inactivated and whole virus were used as bioprocess derived products issued from egg and cell-based production, respectively.

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.

Table 1 : Detailed description of the different influenza samples used in the present study. * TIV: Trivalent Inactivated Vaccine. †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 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) ‡	-
Neuraminidase assay (nmol 4-Me/h/ml)	900	3000	-	80
Total Particles (particles.mL ⁻¹)	1.15 x 10 ¹¹	1.58 x 10 ¹²	-	1.71 x 10 ¹⁰

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 sialic acid -2,3, α -2,6 or α -2,8 terminal link cleavage. This second control surface corresponds to a cropped fetuin structure, close to the asialofetuin structure, known for inducing no specific binding of HA¹⁸⁻²⁰. Both sensing and control surfaces are present in a single flow injection cell (**Figure 1-A**).

These three surfaces were prepared through the covalent grafting of each protein via amide bond formation between the gold chip surface modified with carboxylic acid groups (thioctic acid) and available primary amino groups of the proteins. Fetuin grafting pH conditions were optimized to reach a maximal amount of fetuin and NANA residues available for hemagglutinin binding. Indeed, protein grafting on 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. An optimum fetuin surface coverage of 1.66×10^{10} molecule/mm² was found using a grafting pH of 4.5. This coverage compares well with the theoretical maximum coverage of 5.43×10^{10} molecule/mm² calculated using protein size and comparing with immobilization SPRi signal (see *Methods* for calculation).

Specificity of the assay was first assessed using monomeric recombinant HA (H1N1 A/California/04/2009). No specific interaction was observed when the protein was injected through the biosensor (data not show). Our results were in accordance with previous studies^{4,21} and confirm the binding to sialic acid residues only of the bioactive trimeric form of HA antigen^{19,22}, thus validating our fetuin-based SPR 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 of bioactive HA antigen to determine the performance of the SPRi biosensor assay.

Figure 1-B depicted the biosensor responses when TIV vaccine (10 μ g HA/mL) was brought into contact with the different surfaces. As expected, BSA control surface is 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 functionalized surface was shown to be a more reliable control surface, leading to a non-specific signal ratio of 41. Moreover, the observed interactions between sensing surface and influenza purified vaccine antigens was proven to specifically due to HA interactions with fetuin glycans.

Regeneration solution composition was also optimized to achieve a complete releasing, between each sequential injection, of the bound HA. Classically proposed regeneration solutions²³ such as glycine 0.1M pH 2, HCl 10mM or 100mM, NaCl 1M and 0.25% (v/v) SDS in PBS were then evaluated. The best results were obtained using injection of 25 μ L of a regeneration solution composed of 0.25% (v/v) SDS in PBS (**Supporting Information 1**). Example of the signal recovery is presented in **Figure 2-A** where SPRi baseline was recovered between each of 5 successive injections of TIV vaccine antigen (10 μ g/HA mL).

Assay lifetime was also assessed through 20 successive cycles of TIV vaccine antigen (10 μ g HA/mL) injection and consecutive regeneration. As depicted in **Figure 2-B**, a linear decrease of the biosensor response was observed (slope = -9.7 RU/injection; R² = 0.8685). This decrease was attributed to a surface degradation during regeneration rather than to a sialic acid hydrolysis by the virus 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 using the calculated decrease slope.

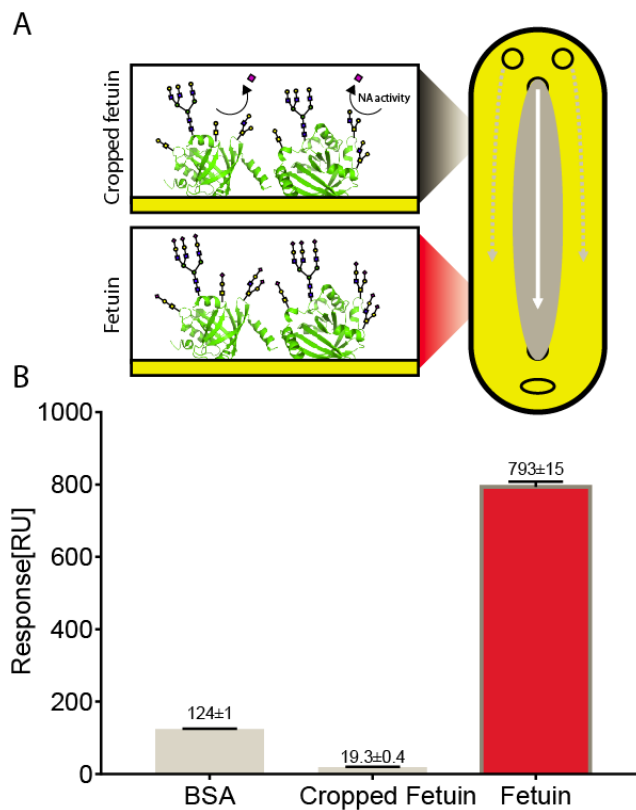
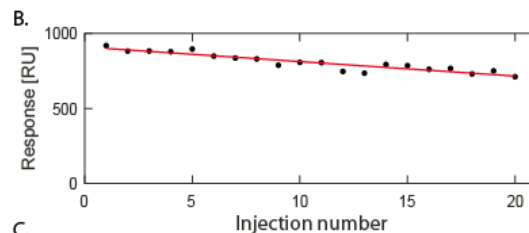
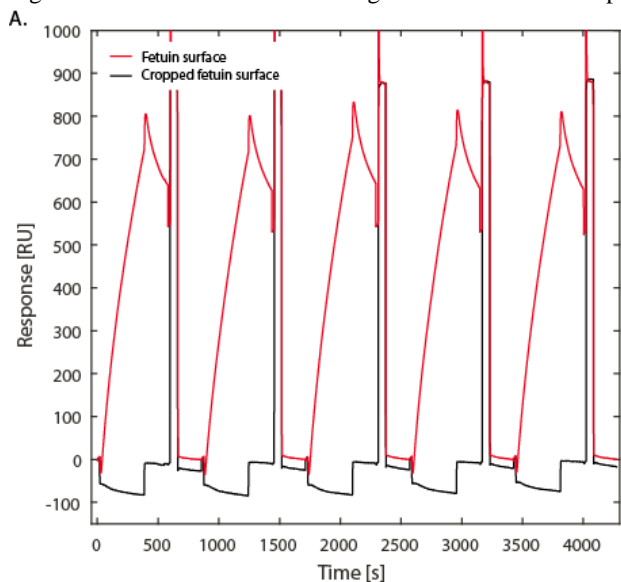


Figure 1 Control and sensing SPRi surfaces. A: Schematic representation of the two distinct measurement zone of the SPRi biosensor flow cell: control (grey) and sensing (red) layers; white arrow: analyte flow; grey arrows: buffer flow. Glycan structures on fetuin (PDB 3APX) according to published data²⁴. Terminal sialic acid moieties (purple diamond) are removed by bacterial neuraminidase (NA) in-situ before processing samples. B: SPRi responses obtained for control and sensing surfaces with injected TIV vaccine (10 μ g HA/mL).

Finally, reproducibility of the assay was evaluated (**Figure 2-C**). The relative standard deviation (RSD) of the response was calculated on the basis of 7 consecutive injections of TIV vaccine antigen (10 μ g 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. 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%^{4,6}. Thus, after having validated the specificity of our regenerable sensor toward HA antigen and its excellent repro-



C. Reproducibility of the biosensor signal following several injections of TIV vaccine antigen (10 µg HA/mL).

Sample	TIV Vaccine
Injection number	7
Initial binding rate dR/dt t=0 (RU/µg/HA/mL)	3.66 ± 0.11
% RSD	3.0
Response level (RU/µg/HA/mL)	823 ± 11
% RSD	1.3

Figure 2 Biosensor reproducibility, stability and regeneration. 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 (10 µg HA/mL).

SPRi biosensor performances

In order to evaluate the sensitivity of our biosensor for hemagglutinin-containing samples, sequential injections of vaccine dilutions were performed, the SPRi signal quantified and the corresponding calibration curve built (Figure 3-A). Linear dynamic range of the assay together with its limit of detection and limit of quantification are presented in Figure 3-C. These parameters were determined using both the initial binding rate of the biosensor (RU/s) and the biosensor response (RU). Linearity of the biosensor 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 $R^2=0.9998$ and $R^2=0.9778$ were calculated for initial binding rate and response, respectively. The assay was also shown to offer a large dynamic range with a linear response over 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 validated assay, i.e. between 3 and 36 µg HA/ml²⁵, demonstrating the superiority of the presently developed fetuin-based SPRi which offers a 100-time higher sensitivity than conventional SRID potency assay and to quantify samples with lower HA antigen content. These results open the path to evaluation of influenza vaccine production consistency and robustness in-process with the quantification of HA antigens along the production and purification. It is also a new technology potentially allowing the evaluation of HA antigen content of new cell-based or VLP-based influenza vaccine under development. Indeed, such processes generally display lower HA concentrations within the production phases, especially in the upstream production steps.

We further evaluated the biosensor performances by comparing the response obtained in the presence of two bioactive forms of HA produced in egg: i) TIV vaccine, equally composed of antigens from H1N1, H3N2 and B strains²⁶, and ii)

ducibility, we further evaluated its sensitivity and applicability for sensing hemagglutinin obtained from different influenza strains.

Split-inactivated H1N1 influenza virus. Sensorgrams and corresponding association and dissociation constants calculated using the classical 1:1 association-dissociation model (global fitting in kinetic-limited conditions) are given in Supporting Information 2. As can be seen, TIV vaccine interaction with the immobilized fetuin evidenced a much higher dissociation constant than the freshly split H1N1 virus sample. This affinity decrease was documented and characterized previously by Kuck and co-workers and Khurana et al.^{4,6}, 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. Thus, the present biosensor also has the potentiality to deeply 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 for H1N1 and H3N2 strains were injected in the biosensor and the corresponding signals recorded. Results are presented in Figure 3-B. One more time, the biosensor was able to characterize interactions between immobilized fetuin and HA from two different strains with clear variations in kinetic behaviors.

SPRi detection of cell-based bioprocess samples

One of the aims of the present study being the demonstration of the biosensor's capacity to follow virus production from various bioprocesses, detection within samples obtained from cell-based process was evaluated. First, various culture media in use for cell-based processes described in the literature^{27,28} (EMEM, Ultra-MDCK, Optipro and SFM4TransFX) were studied according to non-specific signal triggering. Figure 4 presents the obtained signals in the presence of the different culture media.

These results demonstrate that among the four tested media, three of them, EMEM and Ultra-MDCK used for adherent MDCK cell culture and Optipro used for Duck-T17® avian

cell line give limited non-specific background. The last media, i.e. SFM4TransFX, used for HEK293 cell culture demon-

strates high non-specific response.

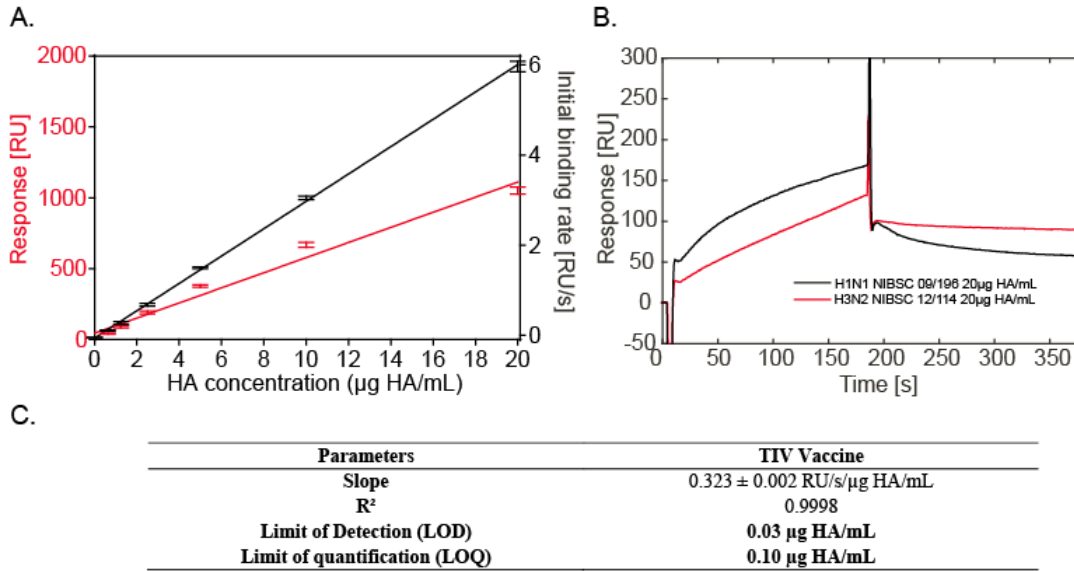


Figure 3 Detection of HA in TIV vaccine. A: Calibration curves for TIV vaccine. B: Sensorgrams obtained following the injection of H1N1 and H3N2 reagents in the biosensor. C: Analytical parameters determined for TIV vaccine using initial binding rate, $\sigma=0.003$ RU/s (n=4).

These preliminary media measurements being performed, the influenza virus SPRi biosensor was used to characterize bioactive virus from cell-based process. As a matter of fact, when it comes to non-specific signal minimization, serum free Ultra-MDCK is the best option and the evaluation of SPRi virus quantification in cell-based production processes was pursued with this medium. Then, in order to account for the non-specific signal generated by process-derived side products (cell debris and extracellular vesicles mainly), samples from non-infected cell culture (20×10^6 cell/T-175 flask at t=0) were harvested after 48h and 96h of culture, clarified through 2000g centrifugation and injected in the SPRi biosensor. The obtained signals are depicted in **Figure 4**. As can be seen, when using MDCK as culture medium, a non-negligible increase of the non-specific signal (between 4- and 7-time) in the presence of the cells and their side-products was observed. These background signals, generated by cell culture side products, will be subtracted to all subsequent SPRi measurements as to obtain net signals from cell-based bioprocess virus production.

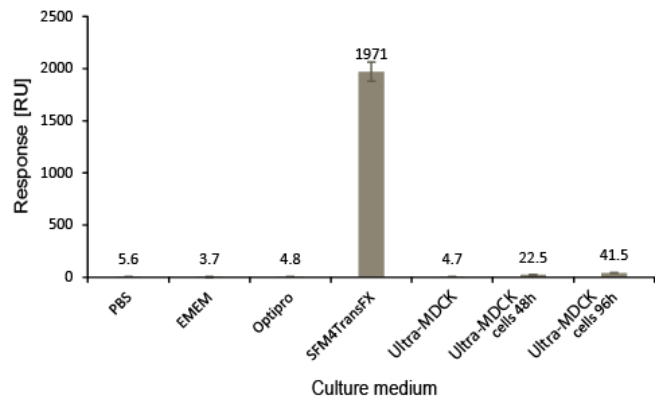


Figure 4 SPRi biosensor responses in the presence of different cell culture media together with SPRi biosensor response of non-infected cell culture harvested after 48h and 96h.

These preliminary measurements being performed, the influenza virus SPRi biosensor was used to characterize bioactive virus from cell-based process. In this case, the sample composition the closest to the production route was used, i.e. 2000g clarified cell culture medium. Calibration curve was built toward infectious viral particles since HA concentrations in harvested cell-based culture samples were too low to be determined using SRID method. Results are presented in **Figure 5**. As presented above, a particular data treatment was applied to these experiments by subtracting sensorgrams from control samples (non-infected culture) to the actual cell-based virus production sample sensorgrams. This treatment was necessary to overcome the matrix effect obtained in the presence of cell culture. As presented in **Figure 5-table**, the SPRi biosensor allowed to reach a limit of detection of 1.8×10^5 pfu. ml^{-1} and a limit of quantification of 5.3×10^5 pfu. ml^{-1} . The

linear response was confirmed among a 2 log analytical range, allowing to reach a 10^7 pfu.ml⁻¹ content. Such results allow to envision the monitoring of influenza production in cell-based bioprocess from its early production phases up to its maximal production rates.

These results have to be analyzed taking into account the discrepancy between the mechanisms involved in plaque forming unit (pfu) calculation and the present biosensor. Indeed, pfu assay is impacted only by the presence of infectious viral particles, whereas our fetuin based biosensor quantifies both infectious and non-infectious particles as long as they exhibit active HA, but also cell membrane embedded HA released during cell lysis process.

While infectious viral particles harbor a complete viral genome and are properly conformed for infection, replication and propagation in cell culture, most of the particles generated are defective [11]. Such defective particles were described in numerous previous works as defective interfering particles (DIPs), i.e. particles structurally similar to viruses but partially lacking viral genome [28]. Such DIPs are commonly determined as total viral particles (VP) in production processes monitoring and could be numbered by physical quantification techniques. The ratio between influenza total viral particles (VP) and infectious viral particles (IVP) is commonly evaluated between 10-1000 for influenza A virus, and appeared to vary during cell culture [11, 29, 30]. In the case of the sample used for **Figure 5** calibration and harvested 48H after infection, TRPS quantification of total particles allowed to calculate a VP/IVP ratio of 380 (*Supporting Information 3*). 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 IVP presence and these interactions correspond to SPRi signals near the apparatus' detection limit (0.1 RU).

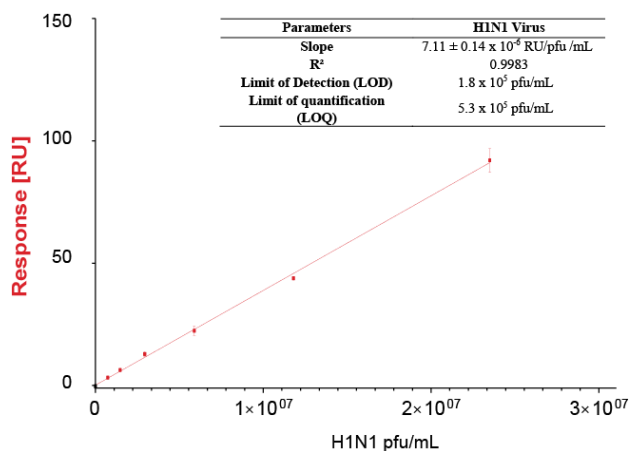


Figure 5 Calibration curve for H1N1 virus from cell-based bioprocess. Insert: Analytical parameters determined for H1N1 virus using SPRi response (n=3).

Nevertheless, when it comes to vaccine production bioprocess, VP titer is the primary parameter of interest, 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 capacity of the SPRi biosensor for one-line monitoring of virus particle production. Thus, H1N1-infected cell culture and control cell culture were

harvested at regular intervals from 23 to 96 hours post-infection, clarified and injected without any further dilution in the fetuin-based biosensor. For sake of comparison, all cell culture supernatants were also quantified with reference infectivity assays (pfu) and particles counting (TRPS). The experimental results are presented in **Figure 6** where each point corresponds to the net signal between infected and control culture. As can be seen, a clear evolution of the biosensor signal was obtained with a SPRi signal leveling off 40 hours after culture infection. This stabilization of the total virus particle content was also observed using TRPS measurements (*Supporting Information 3*). **Figure 6-insert** presents the correlation between the measured SPRi signals and the TRPS measurements. A linear correlation ($R^2=0.9528$) was found between the two techniques, proof of the usefulness of the developed technique for monitoring vaccine production using cell-based bioprocess.

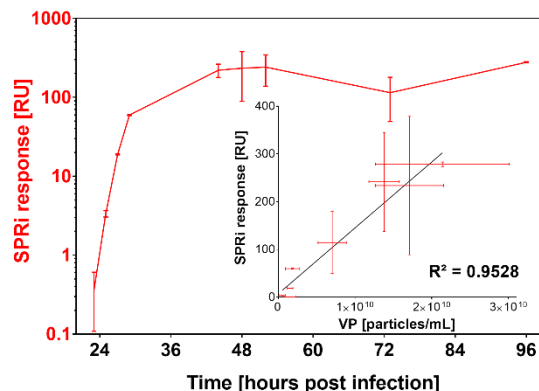


Figure 6 SPRi biosensor signal variations obtained from influenza virus production in MDCK cell-based bioprocess. Insert: correlation between SPRi biosensor response and total viral particles determined using TRPS.

CONCLUSION

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. 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

evaluation and requires low volume (<100µL) of reagent. The high sensitivity and wide analytical range (0.03 and 20 µg HA/ml) provide the possibility to evaluate bioprocess samples with low titer. Sample time processing was 10 minutes and is then fully compatible with on-line bioprocess monitoring while SRID turnaround time is usually 2-3 days [1]. The assay was also shown to have the potentiality to be directly applied to cell-based bioprocess monitoring. A minimum sample pre-treatment (cell/supernatant separation through 2000g centrifugation) was required before analysis, which might in a near future be implemented on-line using membrane and/or acoustic filtration for sample pre-treatment [31].

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting Information 1 – Sensor regeneration optimization (vaccine 10 µg HA/mL)

Supporting Information 2 – Comparative evaluation of kinetic constants obtained for reference standards

Supporting Information 3 – Total particles and infectious particles determination in cell-based bioprocess.

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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TABLE OF CONTENT

