Implementation of Real-time Inline Acidification in Continuous Viral Inactivation

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Abstract:

Existing low pH viral inactivation methods ~~to enable~~for continuous downstream processing of biologics typically rely on predictive models to estimate the necessary pH adjustments. However, these methods are of limited use during the process development stage due to the dynamic nature of capture chromatography, where batch variations can alter the eluted protein titre. This study introduces an Inline Viral Inactivation System (IVIS) that utilises real-time adaptive control and inline sensor readings to precisely regulate the pH manipulation for inline acidification and continuous viral inactivation. The IVIS, which includes a coiled flow inversion reactor (CFIR), is integrated with a multi-column capture chromatography system to demonstrate a fully continuous process from protein capture chromatography to inline pH manipulation. The ~~IVIS~~ system achieved precise inline pH manipulation within ± 0.15 and a narrow residence time distribution of 13.5 minutes ~~and~~ with a relative width of 0.7. The introduction of real-time inline pH manipulation with the IVIS signifies a notable advancement in managing critical process parameters (CPPs) and ensuring consistent product quality ~~in~~ across varied production environments for continuous downstream bioprocessing.

Keywords: Continuous Downstream Processing, Coiled Flow Inverse Reactor, Process Control, Process Development, pH Manipulation, Inline Viral Inactivation, Critical Process Parameters (CPPs)

Abbreviations

CFIR: Coiled flow inversion reactor

CPPs: Critical process parameters

CV: Column volume

HPLC: High performance liquid chromatography

IgG1: Immunoglobulin G1

IVIS: Inline Viral Inactivation System

OD: outer diameter

pDNA: plasmid DNA

RTD: Residence time distribution

SEC: Size exclusion chromatography

UV: Ultraviolet

1. Introduction

The bioprocessing industry is currently moving towards continuous downstream processing to ~~realise its benefits~~capitalize its advantages over batch-mode processing, such as smaller footprints, reduced cycle times, higher equipment utilization rates and better scalability.[1–3] In downstream biologics processing, viral inactivation is a crucial step, where protein elutes from capture chromatography are exposed to ~~The duration of viral inactivation in downstream processing of biologics subject protein eluates by capture chromatography in~~ a lower pH environment for a period dictated ~~is determined~~ by

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viral log kinetics and product stability. This process is essential in ensuring the safety and quality of products. ~~and is typically conducted in two stages: a pH manipulation stage and an incubation stage.~~[4]

~~Currently, three main approaches are used to inactivate viruses in continuous bioprocessing: (i) alternating mixer tanks, (ii) columns packed with resin, and (iii) plug flow reactors. Alternating mixer tanks coordinate between multiple batch-mode processes to facilitate semi-continuous production of eluate, pH manipulation, and incubation, as seen in the Cadence virus inactivation system (Cytiva, USA). However, this method suffers from a large footprint and a lower equipment utilization rate, because the time required for incubation in the surge tank imposes limitations on the operation of upstream and downstream units. For example, if one incubation cycle of virus inactivation takes approximately one hour, the Protein A elution cycle time cannot be less than one hour, as the system would not be ready to process material for the next cycle. The use of resin-packed columns for inactivating viruses suffers from the questionable long-term stability of the resin at low pH.[5,6] Finally, plug flow reactors may allow low RTDs for low pH inactivation,[7–10], but cannot be integrated with continuous pH manipulation. These issues highlight the lack of a system that integrates inline pH adjustment with low pH incubation at low residence times and at a fully continuous modality.~~

pH is a critical process parameter (CPP) in viral clearance that affects virus clearance efficiency and product quality. A real-time continuous pH manipulation system is ~~needed~~ essential to enable fully continuous viral inactivation.[11] Current state-of-the-art uses model-based approaches where the protein titre from the capture chromatography process is first analysed to construct a predictive model. Based on the model, ~~T~~the amount of acid required for viral inactivation is ~~then~~ calculated ~~with the predictive model~~ and then delivered to the product stream ~~for viral inactivation~~. This model can be derived from predetermined pH titration curves,[12] or with an invariant method coupled with Bayesian estimation.[13,14] These approaches assume that the protein titre behaves very similar to the predictive models and do not account for the dynamic nature of capture chromatography where batch variations often occur. As a result, they are less responsive and accurate in processing lines with higher variability, especially during the process development stage before manufacturing, where process parameters and scale change frequently. Such changes may cause the protein titre to stray from the forecasts of predictive models based on earlier parameters, necessitating extra time and data collection for its effective application.

Currently, three main approaches are used for low pH incubation in continuous bioprocessing: (i) alternating mixer tanks, (ii) columns packed with resin, and (iii) plug flow reactors. Alternating mixer tanks, such as the Cadence virus inactivation system (Cytiva, USA), coordinate between multiple batch-mode processes to facilitate semi-continuous pH manipulation, and incubation. However, this method suffers from a large footprint and a lower equipment utilization rate, as the time required for incubation in the surge tank imposes limitations on the operation of upstream and downstream units. For example, if one incubation cycle of virus inactivation takes approximately one hour, the Protein A elution cycle time cannot be less than that, as the system would not be ready to process material for the next cycle. Resin-packed columns, though an option for viral inactivation, faces challenges due to the questionable long-term stability of the resin at low pH.[5,6] Plug flow reactors, while capable of achieving low residence time distribution (RTD) for low pH inactivation,[7–10], have not been integrated with continuous pH manipulation.

This study introduces an inline viral inactivation system (IVIS), which ~~continuously manipulates pH in~~enables real time pH manipulation using an inline acidification system and dual-mode adaptive control, ~~as well as~~while maintaining continuous low pH incubation through~~continuously incubates using~~ a coiled flow inversion reactor (CFIR). Unlike conventional model-based approaches, the dual-mode adaptive control leverages inline sensor readings and real-time ~~measurements~~ data to reduce the need for intense computation or ~~comprehensive~~ complex modelling. ~~It is designed to~~This design enhances robustness and accuracy ~~for~~ in pH ~~manipulation control over model-based approaches for~~especially during the process development stage. Additionally, the IVIS system allows automated, cross-platform control that is integrated with both single and multi-column configurations of AKTA Chromatography systems, enabling a fully interconnected and continuous process from protein capture chromatography to viral inactivation.

2. Materials and methods

2.1. Materials and reagents

The CHO K1 cell line was used to produce recombinant immunoglobulin G1 (IgG1) in-house. Cells and cell debris were removed via centrifugation, followed by depth filtration through filter cassettes with

PDK5 membrane (Pall Life Sciences, USA). The filtered cell culture harvest was used for the validation study.

All chemicals including sodium phosphate, sodium chloride, sodium acetate, acetic acid and sodium hydroxide were purchased from Merck. For IgG purification, 4 mL Protein A chromatography columns were packed in-house with MabSelect PrismA™ using Tricorn 10 columns (Cytiva, USA). The column was first equilibrated with 5 column volumes (CV) of 100 mM sodium phosphate and 150 mM sodium chloride at pH 7.2. 10 CV of wash buffer containing 10 mM sodium phosphate at pH 7.2 was then applied followed by 6 CV of elution buffer containing 100 mM sodium acetate at pH 3.4. 3 CV of 1M sodium chloride was used to regenerate the column. The IVIS used 4M acetic acid (pH 2.0) and distilled water.

2.2. Equipment

2.2.1. Chromatography systems

Two configurations of chromatography systems were used: a single-column verification set-up to investigate the real-time acidification performance of the IVIS, and a multi-column validation set-up to demonstrate the fully continuous process from capture chromatography to inline acidification.

The single-column configuration ~~uses~~ used an AKTA Explorer 100 (Cytiva, USA) which is a Fast Protein Liquid Chromatography system and a single chromatography column. The multi-column configuration used an AKTA pcc 75 (Cytiva, USA) and ~~employs~~ employed four chromatography columns to continuously direct protein eluates to the IVIS.

2.2.2. IVIS

~~The IVIS is located downstream of the chromatography system to receive protein eluates via a tubing with an inner diameter (ID) of 1.6 mm (Masterflex, L/S 14, Tygon®, Germany). It~~ The IVIS ~~is~~ was developed in-house and composed of an inline acidification system which employs~~, employing~~ a dual-mode adaptive control for pH manipulation, and a CFIR for continuous incubation. ~~Details of the system are provided in the Results section. It used three~~Three pH sensors (C-900, AKTA, Cytiva, USA) and one inline mixer (M-925, AKTA, Cytiva, USA) ~~were used~~ at the recommended usage configurations. Details of the system are provided in the Results section. The IVIS was ~~is~~ located downstream of the chromatography system to receive protein eluates via a tubing with an inner diameter (ID) of 1.6 mm (Masterflex, L/S 14, Tygon®, Germany). The pH probes were calibrated with standard solutions at pH 4.01 and pH 7.00 (Mettler-Toledo) before the experiments.

2.2.3. Operation of chromatography systems and the IVIS

The methods on the UNICORN software of AKTA system were synchronised with the control of the IVIS. ~~During the elution phase, fluid would be channelled towards the IVIS. With a flow sensor in place, the IVIS would only begin operation when flow was detected~~.

The single-column configuration used an AKTA Explorer 100 (Cytiva, USA) and a single chromatography column for Protein A purification of monoclonal antibody IgG1 from filtered cell culture harvest. During the elution phase, the protein solution was dissociated from the column and channelled into the IVIS. The flow sensor of IVIS detected the incoming flow and consequently triggered the operation of IVIS. During all other phases (e.g., equilibration or sample application), the solutions from the column outlet were directed to the waste line. To understand the pH distribution and the RTD of the IVIS, a pulse test was conducted by loading 2.5 mg of filtered cell culture harvest per mL of resin. During the robustness test with actual sample volume, 30 mg per mL of resin were used in sample loading phase.

The multi-column configuration used an AKTA pcc 75 (Cytiva, USA) to continuously elute proteins by staggering the elution phases of four chromatography columns. The AKTA pcc allowed the loading and non-loading phases to occur simultaneously. During the loading phase, 30 mg per mL of resin were used in the first and second columns that were connected in series. Concurrently, a third column underwent the non-loading phase within which elution occurred. After all the columns fully completed the loading and non-loading phases, a column switch occurred in which the columns progressed along the sequence of loading and non-loading phases - the second column underwent the non-loading phase while the first and fourth column underwent the loading phase. Given that the non-loading phases were shorter than loading phases, the column in the non-loading phases would complete elution first and

become idle, resulting in periodic elution from the column output. One cycle was completed after all four columns experienced elution once, producing four elutions. Applying fixed time operation, the switch time was set to 100 minutes with one elution per switch. The AKTA chromatography system was set for a constant protein elution rate of 1.0 ml/min and elution duration of 24 minutes. The idle time between elutions can be reduced, but this requires synchronisation of the AKTA pcc and the IVIS.

2.3. Sample collection and analysis

For the single-column configuration, one elution was performed and the output solutions from the IVIS were collected in a fractionator to analyse the acidification performance of the IVIS, the pH profile, RTD of the CFIR and protein recovery. For multi-column configurations, one cycle of up to at least four elutions was conducted with in-line sensors to gather pH data and UV absorbance, then collected for offline analysis.

The filtered sample (100 µL) was injected to size exclusion-high performance liquid chromatography (SEC-HPLC) using an UltiMate™ 3000 UHPLC system (ThermoFisher, US) installed with a TSKgel G3000SWxl column (Tosoh Bioscience, Japan). The mobile phase was 0.2M arginine, 50mM MES, 5mM EDTA and 0.05% sodium azide (w/w) at pH6.5 introduced. UV absorbance at 280 nm was monitored and the relative purity of monomer was determined.

2.4. Measurement of RTD

The RTD of the CFIR was determined by measuring the concentration of a tracer with respect to time. The sample (10 ml) was loaded onto the AKTA Explorer to elute small amounts of Protein A which acted as the tracer. The concentration was measured by determining UV absorbance at 280 nm. Two UV sensors were used - the inline UV sensor of the AKTA Explorer located just upstream of the IVIS, and an offline UV reader using an Infinite M Plex plate reader (Tecan, Switzerland) which ~~measures~~ measured the samples obtained from the fractionator.

3. Results

3.1. Development of the IVIS

The IVIS comprises an inline acidification system and dual-mode adaptive control for regulating the injection of acid into the flow pathway based on inline pH readings, and a CFIR which enables continuous incubation at the target pH with low RTD. Figure 1 illustrates the configuration of the IVIS and its integration with AKTA chromatography and fractionator.

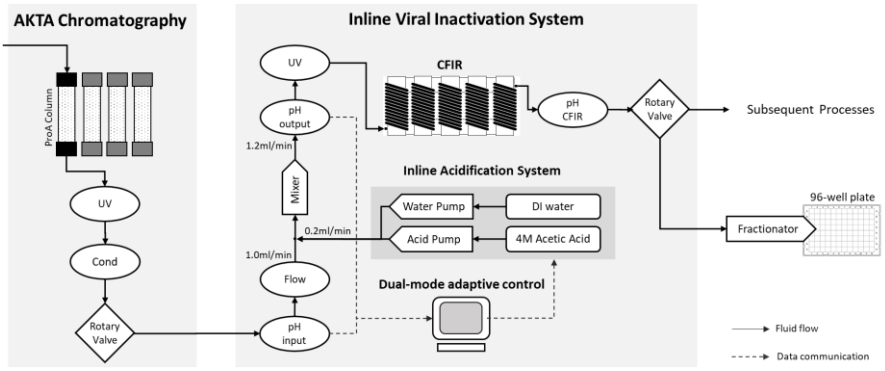

Figure 1. Diagram of the IVIS integrated with AKTA Chromatography and fractionator. The elution sample from AKTA Chromatography flows into the IVIS, which comprises an inline acidification system and dual-mode adaptive control for real-time inline pH manipulation, and a CFIR for low-pH incubation, before proceeding to continuous downstream processing or the fractionator.

3.1.1. Inline acidification system

As the protein eluate from the chromatography system enters the IVIS at a constant flow rate (Q_{in}), the inline acidification system injects acid and water into a single point along the fluid pathway and an inline mixer mixes the reagents and protein eluate immediately. An acid fluid pathway with 4M acetic

acid and a water pathway with DI water are each driven by a peristaltic pump (Longer, UK) via 0.51 mm ID tubing (BPT Pump tubing, PharMed). The total injection flow rate of the acid and water (Q_{acid} + Q_{water}) is kept constant at 0.2 ml/min, while the ratio of acid to water is regulated based on the strength of acid needed to equilibrate the protein elution to the target pH for viral inactivation. This constant total injection flow rate is needed to drive the acidified protein elution through the CFIR at a constant output flow rate (Q_{out}) for uniform incubation and continuous processes downstream of the CFIR.

The inline acidification system uses two inline pH sensors to measure the real-time pH level. The first inline pH sensor (*pH input*) is located at the fluid pathway leading into the IVIS and measures the pH of the protein eluates from the chromatography system. The second one (*pH output*) is located immediately downstream of the inline mixer and measures the pH of the acidified protein solution (Figure 1).

3.1.2. Dual-mode adaptive control

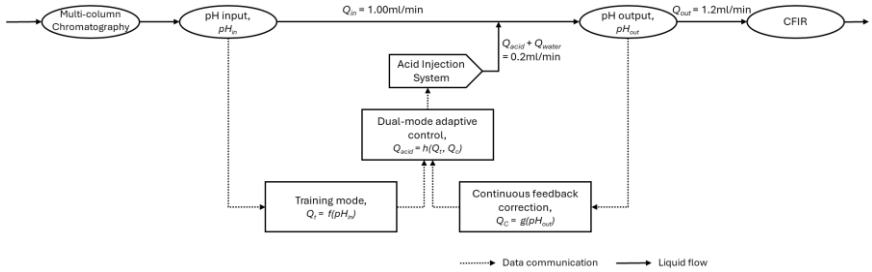

Figure 2. Logic diagram showing the relationship of acid injection flow rate and the dual-mode adaptive control. Each mode of the dual-mode adaptive control (training mode and continuous feedback correction) are functions of the real-time pH sensor readouts.

Precise control of acidification is necessary to ensure that the target pH is met for viral inactivation. Over-acidification can denature proteins while under-acidification may not completely inactivate viruses. The IVIS leverages on readings from inline pH sensors (Cytiva, USA) and flow sensors (Sensirion, Switzerland) ~~to implement~~and implements the dual-mode adaptive control for precise continuous pH manipulation. The dual-mode adaptive control utilises two control modes consecutively. The first is a simple model created in the training mode to determine the tentative action - the baseline flow rate of acid injection *(Q_t)* needed to equilibrate the sample pH close to the target pH. The second control mode is a directing action where continuous feedback correction determines the correction flow rate of acid *(Q_c)* injected according to the real-time pH measurements (Figure 2). The dual-mode adaptive control calculates the final flow rate of acid *(Q_{acid})* that the inline acidification system delivers. The training mode determines the injection flow rate needed across the three stages of elution, which are firstly dispensing wash buffer at pH 6.8-7.2, secondly transitioning between wash and elution buffer from pH 6.8 to pH 3.8, and finally dispensing elution buffer at pH 3.4-3.8 (Figure 2). This training mode ~~was~~ is executed once with buffer blanks without samples prior to the elution sequence of the capture chromatography. A simple model is created which maps the injection flow rate of acid and water respective to the pH inputs during each of the three stages of elution. Proportional control is used to determine the required injection flow rate with the process variable being the pH output of the inline acidification system and the controller output being the acid injection rate. The set point of pH for viral inactivation is ~~set at~~ pH 3. The cycle time is set at 15 seconds which accounts for the response time of the pH probes and the time for liquid flow from the point of injection of acid and water through the inline mixer and into the inline pH output sensor. K_i and K_d are set to 0 to reduce the reliance on tuning and reduce the possible occurrence of overshoot or oscillatory response. The Cohen-Coon tuning method is used because of its suitability for non-linear systems and short rise time.[15,16]

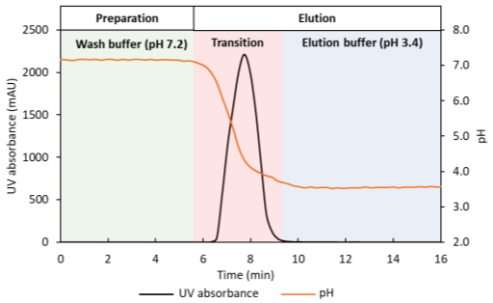

Figure 33. pH and UV absorbance profile of the reagents dispensed from the chromatograph columns across one round of elution. These reagents enter the IVIS, and the pH reading is used as the pH input for the dual-mode adaptive control. For preparation phase (green, left region), the wash buffer was loaded into and dispensed from the column at pH 6.8 to 7.2. Once elution phase began, the elution buffer at pH 3.4 was loaded and dispensed instead, with the pH transiting from 6.8 to pH 3.8 (pink, middle region). Eventually, the pH equilibrates at 3.8 to 3.4 (blue, right region).

During protein elution, the rate and concentration of protein elution are not uniform as seen with the UV absorbance in the protein titre profile (Figure 3). The protein titre profile varies with changing parameters such as the buffers used, methods employed by the chromatography system, and amount of samples loaded, which leads to a deviation from the simple model. To account for this deviation, a continuous feedback correction is implemented on top of the simple model to provide the second layer of the dual-mode adaptive process control. This acts as a self-regulating mechanism that continuously measures and restores the pH output to its target value whenever the deviations occur [17,18]. Another proportional control is employed, but the cycle time and K_p are halved from that used in the training mode. As the continuous feedback correction adjusts the acid injection flow rate to bring the pH output closer to the set point, the new injection flow rate is recorded to further refine the simple model for future elution cycles.

3.1.3. Coiled Flow Inverse Reactor

A narrow RTD ensures all the fluid elements are subjected to the same conditions uniformly. It must be monitored, especially in fluid flow through the tubing where the flow tends to increase due to axial dispersion as fluid elements under laminar flow travel faster at the centre of the tube than when closer to the walls. The CFIR lowers the RTD by incorporating curves at 90° angles in the flow path which create flow inversions. These features enhance the radial mixing within the tubing and reduce axial dispersion [19]. The CFIR has demonstrated high efficiency in virus inactivation when employed in both batch and continuous processing.[9,10,20]

The CFIR of the IVIS was fabricated following previous studies.[10,21] The design entails coiling 1.6 mm ID tubing (Masterflex, L/S 14, Tygon®) around a cylindrical core of 24 mm outer diameter (OD) for five helical turns to form a primary helix. It then extends to another cylindrical core that is orientated perpendicularly to the previous one. This configuration is repeated to create a secondary helix of cylindrical coil until the length of tubing contains sufficient volume for incubation.

The cylindrical cores were 3D printed using Ultimaker S3 (UltiMaker, Netherlands) with grooves and inter-core alignment guides to secure both the primary and secondary helices in a repeated, identical fashion. 50 cylindrical cores were used to construct the IVIS with a capacity of 43.2 ml.

3.1.4. Cross-platform communication between the IVIS with chromatography system

The control software of the IVIS allows cross-platform communication and coordination, providing a graphic user interface and handling data presentation and logging. It is written in Python and can be accessed with a computer. It is paired with Arduino micro-controller for controlling the peristaltic pumps, inline pH sensors, UV and flow sensors of the IVIS and can receive readouts from AKTA.

The IVIS relies on sensors such as inline flow, pH, conductivity and UV sensors at its interfaces with neighbouring equipment to determine the sequence of events. Communication between the IVIS and

AKTA system has been established to allow initiation and termination of signals, unified data logging, flow-path selection, and automatic execution and termination of operations.

3.1.5. Operation

~~Production start~~ The process begins with a training phase, where ~~that dispenses~~ blank buffers from the chromatograph system are dispensed into the IVIS. The inline flow sensor in the IVIS detects the influx into the IVIS and triggers the initiation of the training mode as described above. Upon completion of the training mode, the chromatograph system directs the output to the waste line. When the IVIS flow sensor~~The IVIS~~ detects ~~the absence of~~no further influx ~~by the flow sensor~~, ~~ends~~ the training phase ends and the system prepares for the elution(s) ~~- the chromatography column(s) are washed, loaded with samples, and rinsed of the excess unbound sample~~. ~~Elution then begins with the~~ The chromatography system ~~first primingthen primes~~ the columns with blank buffers ~~once more~~again~~, which is~~ and dispenses ~~themed~~ into the IVIS. Upon detecting the influx of the blank wash buffer,~~The flow sensor of~~ the IVIS ~~detects the influx of blank wash buffer and~~ initiates the inline acid injection. This involves priming the tubing with acid, flushing the pH, UV sensors and CFIR, removing air and tracers in the tubing (if any), and zeroing the UV sensor. Once these priming steps are finished, protein elution begins.~~Protein elution begins shortly after the priming steps are complete.~~

Acid and water are injected according to the pH input readings and the simple model obtained from the training mode while the continuous feedback correction continuously adjusts the acid-water ratio according to the pH output readings.

In the multi-column configuration, the column undergoing the non-loading phase completes elution and enters an idle phase while the other columns undergoing the loading phase complete the remaining tasks. Once all the columns have completed their tasks, a column switch occurs and another elution phase begins. The column switch is programmed at one elution peak every 100 min.

After the inline acidification system, the protein solution proceeds into the CFIR at a flow rate of 1.2 ml/min. In the multi-column configuration, the idle time is 76 minutes, during which no solution is supplied to IVIS. The IVIS detects the end of the elution cycle and injects DI water at 1.2 ml/min to drive the pH-adjusted eluate through the CFIR to achieve the targeted incubation time. The duration of water injection can be shortened if the idle time is reduced by programming the multi-column chromatography system to continuously supply ProA eluates. A rotary valve (Cytiva, USA) is used downstream of the CFIR to channel the protein-containing solutions into either a storage bottle (in the multi-column configuration) or a fractionator (in the single-column configuration), and the blank solutions into a waste carboy.

3.2. Evaluation of pH manipulation of the inline acidification system

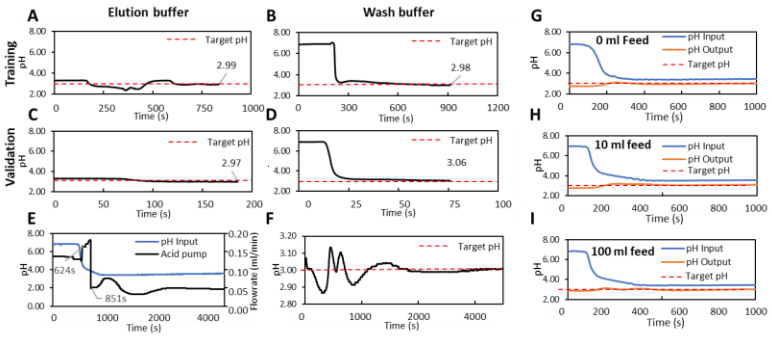

Figure 44. Inline pH control evaluation across various scenarios in single column verifications. Training of the inline acidification system (A, B) and the respective verifications (C, D) with elution (100mM Sodium Acetate pH 3.4) and wash buffers (10mM Sodium Phosphate pH 7.2). (E, F) The adjustment of flow rates for acid pump according to the simple model as the pH input progress to the different elution stages at 624s and 851s. Following that, gradual changes to the flow rate to correct for deviations in pH output as seen in (F). (G, H, I) The pH input and pH output of runs loaded with different feed volumes onto the protein A column (0 ml, 10 ml, 100 ml, respectively) of a single column verification.

The training mode of the IVIS involved the training of blank wash and blank elution buffers. Figures 4A and 4B depict the training data used to equilibrate the elution buffer (100mM sodium acetate, pH 3.4) and the wash buffer (10mM sodium phosphate, pH 7.2) to the target pH of 3, respectively. The training process ended when stable inline pH measurements at pH 3 ± 0.05 were achieved for 60 seconds. Verification rounds were conducted with the established flow rates (Figures 4C and 4D), which confirmed an error margin of less than pH 0.06. The elution buffer achieved (and remained stable at) the target pH in 80 seconds, with the wash buffer doing the same in 70 seconds.

Figures 4E and 4F illustrate the dual-mode adaptive control in action. The sharp changes in flow rate of acid at time points 624 seconds and 851 seconds correspond to the change in flow rates obtained in the training mode at the different stages of elution, while the gradual change corresponds to the continuous feedback correction as the IVIS continuously regulates the flow rate as the pH output readings deviate from the target of pH 3. Figures 4G to 4I showcase the system's adaptability by demonstrating how it maintains consistent pH output throughout the acidification process even under varying sample loads.

3.3. IVIS demonstration with single column setup of Protein A chromatography

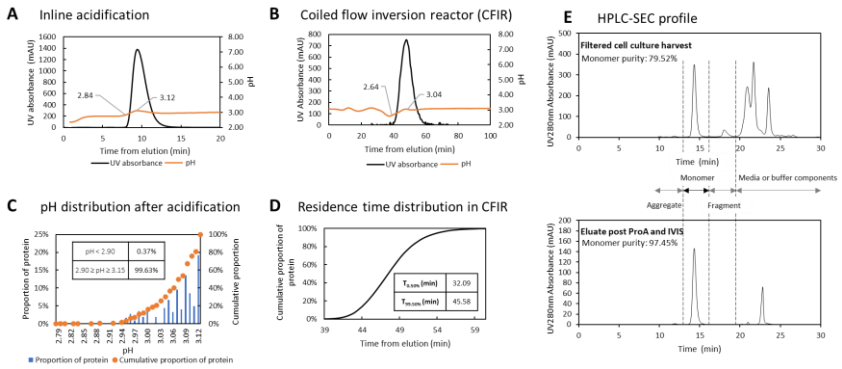

Figure 55. Demonstration run of the IVIS with a single column setup of Protein A chromatography with pulse test. Measurement of pH and UV absorbance of eluted protein after (A) inline acidification and (B) Coiled flow inversion reactor (CFIR). The pointed numbers indicate the lowest and highest pH value. (C) pH distribution in proportion to protein proportion. (D) Residence time distribution. (E) HPLC-SEC profiles of samples before and after purification and the IVIS.

To assess the efficiency of inline acidification, the IVIS system was firstly integrated with a single-column AKTA chromatography setup for a pulse test. The intended objectives in this case study were to i) adjust the pH of eluate to pH 3, and ii) incubate the acidified protein solution for a residence time of 30 to 60 minutes via CFIR. The pH and UV absorbance of the protein solutions were measured both before and after CFIR to appraise pH control and residence time within the reactor (Figures 5A and 5B).

After inline acidification, the pH of the protein solution was reduced to 3.0 which was sustained throughout the entire elution process (Figure 5A). pH output measurements were taken at time points coinciding with UV measurement of the protein, providing a more accurate representation of the eluted sample's pH (see Supplementary information S1 for calculation of pH distribution). The process set 99.63% of protein to pH between pH 2.90 to 3.12, indicating the successful implementation of real-time inline acidification of protein elutes from capture chromatography (Figure 5C).

Subsequently, the acidified sample underwent incubation in the CFIR at constant flow rate of 1.2 ml/min with an expected residence time of 30 min. The minimal residence time (first 0.5% of the population) was determined to be 32.09 min while the maximal residence time (first 99.5% of the population) was 45.58 min (Figure 5D). This proved that more than 99 % of product achieved a low pH inactivation of more than 30 minutes to ensure sufficient viral inactivation of all proteins while limiting the hold to less than 60 minutes to minimise protein degradation. The difference between $T_{99.5\%}$ and $T_{0.5\%}$ was 13.5 minutes. The relative width ($F_{0.5\%}$ / $F_{99.5\%}$) of the RTD was 0.7, which was consistent with the results of previous studies.[10] The detailed calculation for RTD is provided in Supplementary

information S2 (Table S3). The HPLC-SEC analysis shows that the purification of the protein increased from 79.52 % to 97.45 % (Figure 5E).

3.4. IVIS demonstration with multiple column setup of Protein A chromatography

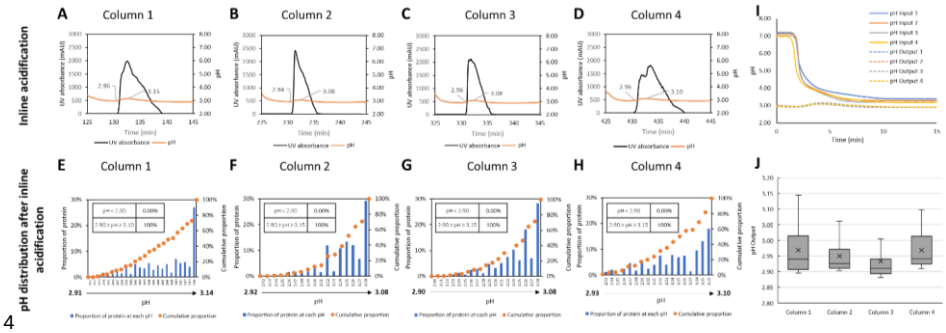

Figure 66. Integration of the IVIS with multi-column chromatography of Protein A. Measurement of pH output and UV absorbance of eluted protein after inline acidification from (A) Column 1, (B) Column 2, (C) Column 3 and (D) Column 4. pH distribution in proportion to protein concentration from (E) Column 1, (F) Column 2, (G) Column 3 and (H) Column 4. (I) Overlay of pH profiles across the 4 columns, showing deviations at the pH input while maintaining similar pH output profiles. (J) Box plot illustrating the error range of pH outputs, spanning from 2.9 to 3.15.

The AKTA pcc 75 with a four-column configuration was used for periodic elution of protein product, which was directed to the IVIS for continuous virus inactivation. Each column was set to 100 minutes, including a 24-minute elution phase in which 24 mL of product was eluted. The intended objectives of this case study were to i) validate the consistency in pH manipulation by the IVIS for multiple columns (Columns 1 and 4 were aged columns while Column 2 and Column 3 were freshly packed columns), and ii) demonstrate the integration and efficacy of the IVIS with continuous capture chromatography. pH output and UV absorbance of the protein solutions were measured.

Figures 6A to 6D illustrate the real-time pH profiles and UV absorbance of the elute after inline acidification system during the elution of the four columns, respectively. There are clear variations in the protein concentrations of the elutes across the different columns. As Column 1 and 4 were aged columns, the elution peaks are wider than the fresh columns (Columns 2 and 3). The protein concentrations downstream of the inline acidification system, measured via UV absorbance for each column, are shown together with the pH output distribution in Figure 6E to Figure 6H, respectively. Despite variation in the elution profile across four columns, 100 % of the protein remained at pH levels between 2.90 and 3.15, demonstrating the excellent pH control of the protein elute (with maximum errors in pH at +0.15, +0.08, +0.08 and +0.10 for each respective column). Figure 6I overlays the pH profiles of protein elutes downstream of the inline acidification system for all four columns, highlighting the consistent pH output profiles despite input variations in pH and protein concentration. Additionally, Figure 6J is a box plot of the pH output errors, showing consistent maintenance of the pH within the controlled range of 2.9 to 3.1. This effectively illustrates the IVIS's capability in controlling pH precisely during continuous capture chromatography operations. The IVIS is also robust enough to handle potential deviations in the Protein A elution profile caused by the columns aging with extended use.

4. Discussion

A real-time IVIS has been developed and implemented for continuous inline viral inactivation of protein elutes after capture chromatography. It comprises an inline acidification system for inline real-time pH manipulation, and a CFIR system for incubating acidified protein elutes. The inline acidification system utilises dual-mode adaptive control to regulate pH manipulation based on real-time inline sensor readings to correct for unexpected fluctuations and batch variations, which is especially relevant in process development. Validation studies showed that the inline acidification system could acidify precisely to within ± pH 0.15 at target pH 3 for continuous protein elution of Protein A, with the CFIR having a narrow RTD of 13.5 minutes and a relative RTD width of 0.7, which compare favourably to current continuous downstream processing techniques that utilise model-based strategies. On top of that, the IVIS eliminates the usage of mixer tanks to mitigate the drawbacks of batch-mode operations, while enabling process intensification with easy to implement simple models and cross-platform

integration.[13,14] Compared to the conventional PID controls which require careful tuning to ensure effective control, the dual mode adaptive control of the IVIS simplifies the tuning methods for both training and continuous feedback control modes. This reduction in tuning reliance makes IVIS more suitable for rapid implementation in process development. As process development proceeds and more parameters are fixed, other tuning methods such as Chien-Hrones-Reswich method can be implemented to reduce the settling time, especially in the continuous feedback control.

Conventional model-based approaches typically have high data and computational requirements for accurately predicting individual protein titre profiles, especially if there are frequent variations in protein elute behaviours. In contrast, the IVIS requires minimal data for the training process. The simple model generated after one instance of training process is sufficient to enable inline acidification with an error of only 0.15. Furthermore, the continuous feedback correction during subsequent elution phases further refines the trained model, reducing the error to 0.08 and 0.10 in subsequent runs.

The robustness of the control system within the IVIS is a critical focus of our study, particularly in its ability to adapt to variations in fluid stream properties, such as protein concentration and buffer composition. The continuous feedback correction plays a vital role in maintaining stable pH control despite these fluctuations, as demonstrated in Figures 4 and 6 where different feed volumes were loaded onto the columns, and continuous elution performed across different columns. Additionally, different batches of buffers were prepared throughout the verification and validation studies, which is a common source of batch variation. The IVIS demonstrated its effectiveness in managing this with similar pH output profiles across all batches. These features highlight the system's capability to provide precise pH control under variable conditions, making it highly suitable for process development where such variations are common. Cross-platform integration enables automation and longer cycles with larger scales of processing, especially with unit operations that are currently standalone. The integration of the IVIS with the multi-column chromatography demonstrates the feasibility of a fully continuous protein purification to the viral inactivation process. Although there were time gaps between elution cycles, improvements can be made by increasing the number of columns or adjusting elution strategies. Peristaltic pumps offer good accuracy and precision for injecting small amounts of acid in inline viral inactivation systems, though precision may vary based on factors like pump model and tubing size. Notably, the IVIS can be easily used for the neutralization process to support fully continuous protein purification from viral inactivation to the neutralization process, further reducing human intervention and increasing equipment utilization rates in downstream processing. The inline acidification system of the IVIS can be potentially adapted to other unit operations for downstream processing of biologics. Similar inline fluid manipulation techniques can be used to introduce different reagents continuously and achieve feedback control based on real-time inline sensor readings. This can be foreseeably applied to ion exchange chromatography or in reactions involving hydrophobic interactions, where inline conductivity sensors can be used.

The design of the IVIS facilitates scaling up with its relatively simple design and equipment used. Several critical factors must be addressed to ensure robustness and efficiency at larger volumes. Scaling up or increasing the capacity of the IVIS typically involves increasing the duration and/or increasing the flow rates. The former entails increasing the length of tubing primarily at the CFIR, where incubation takes up the longest time for the viral inactivation process. An important design consideration for the CFIR is maintaining a high Reynold's number by modifying the diameter of the tubing, number of helical coils and flow rate. However, increasing the flow rate must be balanced with the length of tubing; higher flow rates require longer tubing for sufficient incubation time which comes with higher pressure losses and larger footprints. Meanwhile, increasing the flow rate requires equipment ~~with the appropriate precision. More advanced pumping systems like piston pumps and mixers specific for the chosen flow rates are needed to ensure consistent accuracy and to prevent localised pH de~~vira~~viations. Additional attention must be paid to the delay and precision of sensors. Multiple injection points can be considered.

In addition to biologics, other modalities that require precise regulation of CPPs such as pH, reagent concentration, and incubation duration can also potentially benefit from the real-time monitoring and control techniques of the IVIS. For instance, in the continuous manufacturing of mRNA from *E. coli*, real-time monitoring and control can be effectively applied during the cell lysis and the in-vitro transcription (IVT) processes.[22] During cell lysis, NaOH and SDS are commonly added to disrupt the cells and release plasmid DNA (pDNA). It is crucial to maintain NaOH concentration below 0.15M or pH below 12 and to incubate within the optimal lysis time to prevent irreversible degradation of pDNA. During IVT, the enzymatic reaction to synthesise mRNA will decrease pH and affect the reaction

efficiency. This makes a close-loop control system similar to that of the dual-mode adaptive control of the IVIS in adjusting the pH in real-time and maintaining it at optimal reaction conditions very useful.

5. Conclusion

We successfully demonstrated continuous and real-time inline acidification for continuous virus inactivation with an Inline Viral Inactivation System (IVIS). The integration of the IVIS with capture chromatography and the validation of a fully continuous protein purification process led to precise acidification within ±0.15 pH, affirming the robustness of the system. An advanced control model (dual-mode adaptive control) was employed to ensure swift and accurate pH manipulation in the face of rapid changes in pH profiles and variations from chromatography elutes. The IVIS holds significant promise in advancing fully continuous downstream processing, as well as controlling CPPs in production processes.

6. Author contributions:

Jia Sheng Zach Lee: Conceptualization (equal); formal analysis (lead); investigation (equal); methodology (equal); project administration (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). Tan Dai Nguyen: Conceptualization (equal); formal analysis (lead); investigation (equal); methodology (equal); project administration (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). Zi Ying Zheng: Conceptualization (equal); formal analysis (lead); investigation (equal); methodology (equal); project administration (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). Wei Zhang: Project administration (supporting); resources (supporting); supervision (supporting); writing—review and editing (supporting). Dan Liu: Conceptualization (equal); project administration (equal); supervision (lead); writing—review and editing (supporting).

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8. Conflict of Interest:

9. References:

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