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Real-Time VCC Monitoring and Forecasting in HEK-Cell-Based rAAV Vector Production Using Capacitance Spectroscopy

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ABSTRACT

Recombinant adeno-associated virus (rAAV) vector production is a complex process in which the robust cultivation of human embryonic kidney cells (HEK293) plays a critical role in generating high-quality viral vectors. Tracking the viable cell concentration (VCC) during upstream production is essential for process monitoring and for implementing actions that ensure optimal process management. The advent of inline capacitance probes has introduced a crucial process analytical technology (PAT) tool for real-time VCC measurement. Here, we present the development and application of a method for real-time monitoring of VCC in HEK293-based rAAV vector production. In a first step, BioPAT Viamass probes were used to record capacitance data of individual 10 L rAAV-8 batches within a frequency range of 50 kHz–20 MHz. Based on the capacitance data, a linear single-frequency model and an orthogonal partial least square (OPLS) multifrequency model for VCC prediction were developed. Subsequently, these models were deployed inline, and predictions were exposed into BioPAT MFCS bioprocess control software, enabling real-time VCC monitoring in subsequent rAAV-8 production batches. In addition, the continuous VCC signal was used as input for an exponential cell growth model that was deployed inline to provide accurate real-time forecasting of the transfection time point. To the best of our knowledge, this is the first example of inline deployment of VCC and Time-Till-Transfection predictive models to the bioprocess control system for real-time monitoring and forecasting of these parameters in HEK-cell-based transient rAAV vector production.

1 | Introduction

Process analytical technology (PAT) is a concept of designing, monitoring, and controlling manufacturing processes through timely measurements of critical process parameters that affect process efficiency and critical quality attributes of the product. The Food and Drug Administration's PAT initiative stresses the importance of implementing efficient inline or online monitoring and control strategies as a crucial prerequisite to achieve improved and robust production processes [1].

In the emerging field of gene therapy, recombinant adeno-associated virus (rAAV) vectors serve as key tools for the precise and safe delivery of genetic material to cells. The production of rAAV vectors is a complex process involving human embryonic kidney cells (HEK293) [2, 3], expressing the adenoviral helper genes E1A/B, which improve rAAV titer yields [4]. However, real-time monitoring and control of rAAV production remain a challenge, as there are not many advanced inline sensors providing signals that reliably represent specific biological cell culture states. Amongst others, the ability to accurately monitor

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Summary
• Monitoring of VCC during mammalian cell culture processes is crucial for achieving robust operations, enhancing process understanding, conducting accurate assessments, and implementing actions that ensure optimal process management.
• In transient rAAV production, VCC monitoring is essential as this parameter is used to time critical process events, such as the transfection step. Thereby, it has pronounced influence on the repeatability, efficiency, and quality of the rAAV production process.
• Historically, VCC determination in mammalian cell culture has been achieved through offline assays requiring manual sampling, ultimately limiting real-time process insights and the ability of timely interactions.
• Therefore, capacitance-based approaches have lately garnered increased interest as these can provide real-time, inline VCC measurement data. In this study, we present the development and application of capacitance-based models for accurate real-time, inline VCC monitoring in HEK-cell-based rAAV vector production.
• In addition, VCC forecasting is showcased for the prediction of the optimal transfection time. This approach represents a robust solution for VCC monitoring and forecasting in HEK-cell-based rAAV vector production.

viable cell concentration (VCC) during cell cultivation is essential to achieve expected process outcomes. Traditional methods for assessing VCC in HEK293 cell cultures rely on offline analytics, such as trypan-blue-based optical measurements. These methods do not provide real-time data and rely on culture sampling, additionally increasing the risk of process contamination. Therefore, access to real-time, inline data is preferable for process monitoring, as it ensures timely process interventions and enables precise process control strategies. These include optimal timing of transfection with plasmid DNA, which is critical for maximizing rAAV yield in transient expression systems, and for accurate determination of the optimal harvesting time.

Capacitance measurement has emerged as a promising, non-invasive technique for cell culture monitoring. It can provide continuous signals depending on the polarizability of living cells in suspension and can be directly correlated with the VCC by uni- or multivariate modeling approaches [5]. Further, several additional parameters, like critical frequency or Cole–Cole alpha, can be obtained from the capacitance spectroscopy data. These can be related to cell size and its distribution in the population of cells and hence bring additional information about the culture [6]. An increasing number of publications demonstrate how capacitance measurement can provide deeper insights into viral production processes. It has for example supported the identification of distinct phases of the process that correlate directly with the kinetics of lentivirus (LV) production [7], facilitated the observation of cell attachment to microcarriers, pinpointing the ideal time for measles virus harvesting [8], and demonstrated the potential for scalable monitoring of Vero cell proliferation on microcarriers [9]. In human HEK293 and Sf9 insect cell systems, characteristic capacitance parameters (intracellular conductivity and membrane capacitance) strongly aligned with the phases of viral replication [10]. In batch cultures of Sf9 cells, capacitance spectroscopy provided insights into cell size alterations after infection [11] and enabled precise prediction of the cell infection time [12]. Further, uni- and multivariate modeling of capacitance data was tested for prediction of viable cell density and cell viability during adherent Vero-based live-virus vaccine production [13]. Capacitance measurements at high frequencies (15 MHz) were also reported to be correlated with virus titer [14].

Most of the mentioned applications of capacitance measurements in the viral production space focused on inline or online recording of single- or multi-frequency capacitance signals and their postprocess analysis with univariate approaches, and eventually analysis of parameters derived from the Cole–Cole equation. Only two studies discussed how multivariate models could be applied to predict important cell parameters in viral production like VCC/density, viability, and average cell diameter in rAAV production in Sf9 cells [12] and in LV production by Vero cells grown on microcarriers [13]. In both cases, the models were however never applied for real-time inline prediction.

Although there are examples of CHO-based processes with multivariate models being deployed inline to provide capacitance-based real-time VCC prediction to the bioprocess control unit [15], the application of such approach in HEK-cell-based transient rAAV vector production has not yet been reported. The integration of this technology could enhance real-time process monitoring and forecasting, enable immediate feedback on cell culture conditions, and facilitate timely process interventions.

2 | Materials and Methods

2.1 | rAAV-8 Production and Reference Analytics

Expi293F Inducible cells were thawed and subcultivated in FreeStyle 293 Expression Medium (both Thermo Fisher Scientific). To initiate rAAV-8 vector expression, a target of 0.3×10^6 viable cells/mL with a viability of ≥95% was inoculated into a 10 L Univessel Glass bioreactor (Sartorius Stedim Biotech GmbH, Germany) and cultivated in batch mode. Main bioreactor process parameters were set to 37.0°C temperature, 40% DO, 201 rpm stirrer speed, 0.01 vvm total gas flow rate, pH ≤7.25 (using CO_2), and were controlled by a Biostat control unit (Sartorius Stedim Biotech GmbH). After approximately 48 h, cells were transfected with an optimized plasmid ratio of pTransfer and pDP8.ape (Plasmidfactory) and an optimized total pDNA-to-viable cell ratio. Before cell culture addition, FectoVIR-AAV (Sartorius Polyplus) was used for the complexation of pDNA. The transfection complex was generated in 5% (v/v) of culture medium and incubated for 30 min at room temperature and at rest. Approximately 72 h after transfection, cell cultures were terminated by lysis and harvested from the bioreactor. A total of six independent rAAV-8 production batches were run at the described conditions.

Cell attributes (VCC, viability, and average cell diameter) were analyzed offline once or twice per day using a Cedex HiRes Analyzer (Roche Diagnostics GmbH).

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2.2 | Capacitance Frequency Scanning

Inline capacitance spectroscopy data was recorded using the BioPAT Viamass system equipped with an BioPAT Viamass 12 mm Annular Probe for 10 L (both Sartorius Stedim Biotech GmbH). Capacitance data was obtained at 25 discrete frequencies between 50 and 20,000 kHz using the FUTURA SCADA software (Aber Instruments Ltd).

2.3 | Calibration Modeling

For calibration modeling, the capacitance spectroscopy data of four rAAV production batches were used. A single-frequency (SF) model based on the capacitance at 580 kHz and a (multi-frequency) orthogonal partial least square (OPLS) model were built using SIMCA 18 software (Sartorius Stedim Data Analytics AB). For the OPLS model, the mean-centered capacitance data were selected as X-block variables while the offline VCC reference measurements, scaled to unit variance, were used as Y-block variable. For the generated OPLS model, one predictive and one orthogonal component were used.

The root mean square error of cross-validation (RMSECV) was used to evaluate the predictive ability of uni- and multivariate models based on the calibration data according to the following equation, where y_i and $\hat{y}_i$ describe the measured and predicted VCC value for observation i, respectively, while n describes the total number of observations:

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n} (\hat{y}_i - y_i)^2}{n}}$$

Specifically, leave-one-group-out (LOGO) cross-validation with each group corresponding to a single batch was utilized. Additionally, the predictive performance of both models was evaluated on two external test sets (Batches #5 and #6) using the root mean square error of prediction (RMSEP), which was calculated using the same equation.

2.4 | Inline Deployment of an OPLS Model and Integration Into a SCADA System

To facilitate the inline implementation of the OPLS model, a wrapping component for OPC UA (provided by Unified Automation GmbH) was employed. This component enabled the native OPC DA server of the FUTURA SCADA software to interact with OPC UA clients. A specialized middleware component was created using Node-RED 1.3.4 (OpenJS Foundation) to deploy the model. This component includes an OPC UA client that routinely collects capacitance spectroscopy data from the FUTURA SCADA software. The data collected by this client is then sent to a function component that integrates the model equation (Equation S1), previously exported from SIMCA 18. The predictions of the model were made accessible through an OPC UA server component. This server, along with the inline VCC prediction, was accessed by the SCADA system BioPAT MFCS version 4.9 (Sartorius Stedim Biotech GmbH) which served as a client for the purpose of process monitoring [15].

2.5 | Time-Till-Transfection Forecaster

The Time-Till-Transfection (TTT) Forecaster was designed to predict when the transfection event should be initiated based on the current cell growth curve of the cell culture and a user-specified transfection VCC target. Practically, this was achieved by fitting an exponential growth curve to the VCC predictions of the OPLS model and extrapolating into the future to estimate when the culture would reach the given VCC target. This time-point was transformed into a timespan based on the current process time, indicating the time left until transfection needed to be initiated. Specifically, a sliding regression window of 15 h was utilized when fitting the exponential growth curve to ensure that only the most relevant (meaning the most recent) culture data is utilized for forecasting. To evaluate the forecasting results retrospectively, the actual timepoint for the target VCC value of 1.5 million cells/mL was obtained from an exponential growth curve fitted to the offline Cedex HiRes references (Figure S2).

2.6 | Inline Deployment of the TTT Forecaster and Integration Into a SCADA System

The TTT Forecaster Python script has been implemented using Node-RED 1.3.4. In this setup, the outcomes of the OPLS model are stored in a CSV file, which is continuously updated with new VCC prediction values. This CSV file is then utilized as the input for the Python script previously described. The script automatically executes 1 h after inoculation and subsequently runs every 36 s following its initial execution until the end of the batch process. Script outcome is then fed into an OPC UA server component allowing the integration and visualization with BioPAT MFCS version 4.9.

3 | Results and Discussion

Four rAAV-8 production Batches (#1–4) were used to generate uni- and multivariate models for real-time VCC monitoring. A flow chart of the applied procedure, from calibration data generation to model building and finally inline VCC monitoring and forecasting, is depicted in Figure 1. The SF and OPLS models were integrated into a BioPAT MFCS SCADA system and used for real-time VCC monitoring and TTT Forecasting in two additional rAAV-8 batch cultivations (Batches #5 and #6).

Developed OPLS and SF models demonstrated very good model fit, as evidenced by their high cross-validated coefficients of determination (R2CV) of 0.96 and 0.95, respectively (Figure 2A). Both models showed similar RMSECVs at 0.16 million cells/mL indicating similar predictive accuracy.

To assess these model accuracies relative to the reference method, we calculated the standard deviation of the reference VCC measurement (Cedex HiRes Analyzer). This involved analyzing VCCs from six independent measurements being taken per sample by the analyzer. The standard deviation values were averaged across all samples ($N = 53$), resulting in a mean standard deviation of 0.11 ± 0.05 million cells/mL, which closely aligns with the obtained RMSECVs of the models.

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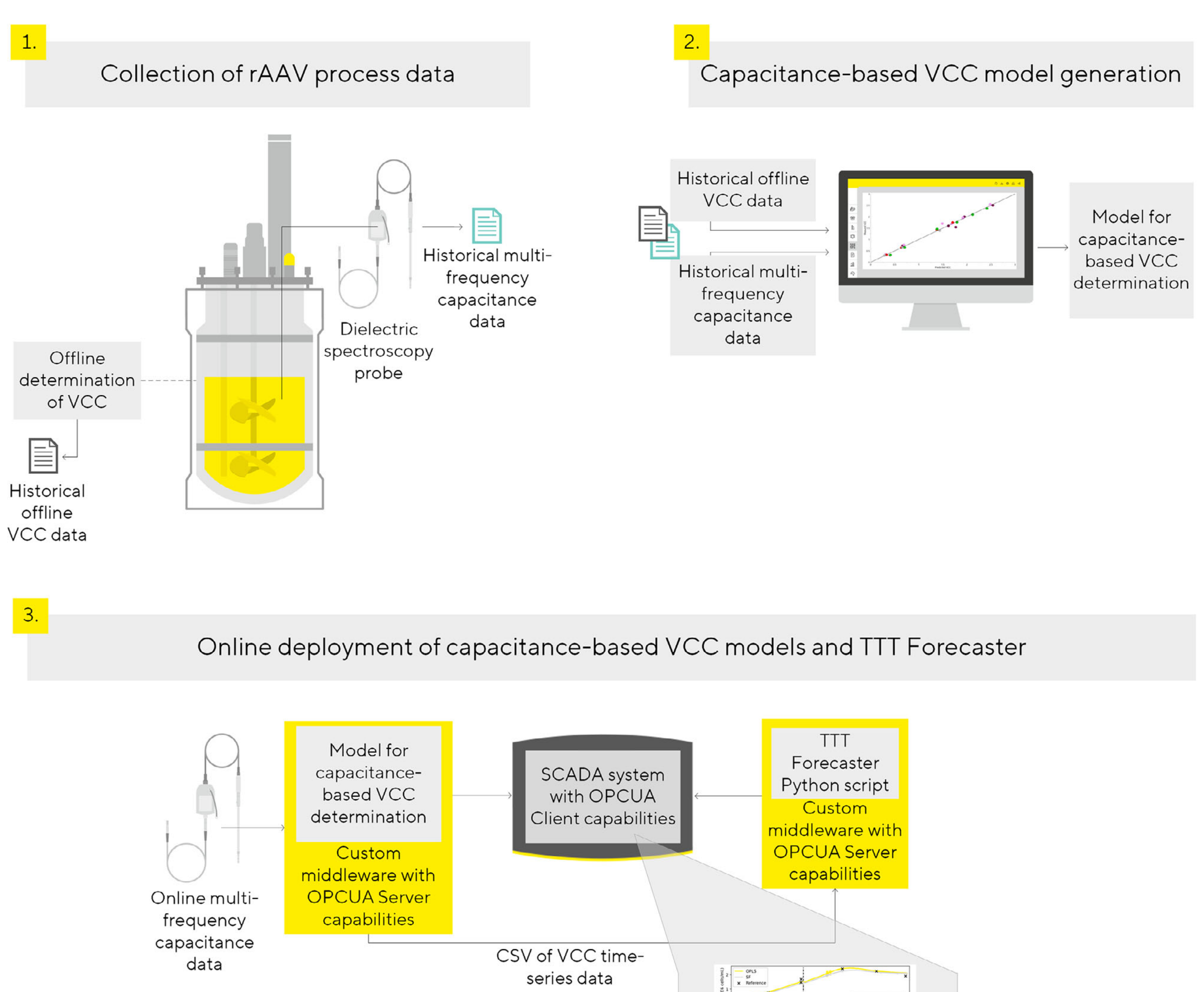

FIGURE 1 | Flowchart depicting the approach for inline deployment of a capacitance-based VCC model and the integration into an SCADA system, as adapted from [15]. First, historical single- and multifrequency capacitance data as well as historical offline VCC measurement data were collected in several rAAV batch runs. Second, SF and OPLS models that can predict the VCC based on capacitance data were generated. Third, a custom IT infrastructure was set up for an inline execution of the models and SCADA integration, as well as the integration of the online VCC data into a TTT Forecaster. OPLS, orthogonal partial least squares; rAAV, recombinant adeno-associated virus; SCADA, supervisory control and data acquisition; SF, single-frequency; TTT, time-till-transfection; VCC, viable cell concentration.

Significant differences between single- and multifrequency capacitance-based VCC models are frequently observed in certain mammalian cell culture processes. This is because the capacitance signal correlates with the viable cell volume, which is dependent not only on the VCC but also on the diameter of the cells, which can substantially change during the process, particularly during the cultures' death phase [16]. SF models are limited in their ability to accurately capture this effect, which is why multifrequency approaches have gathered increased interest lately [5]. The high similarity between our SF and OPLS models can be attributed to the minimal change in average cell diameter of less than 2 µm throughout the applied rAAV-8 vector production process (Figure S1A), which suggests that for this process SF-based VCC prediction is sufficiently accurate. The presented results are in line with those published earlier for CHO cultivations [16] and show that the SF approach is accurate enough for VCC monitoring in processes where cell size does not change significantly throughout the process.

In the final step, the OPLS and SF models were deployed inline to test their predictive ability in two additional cultivations,

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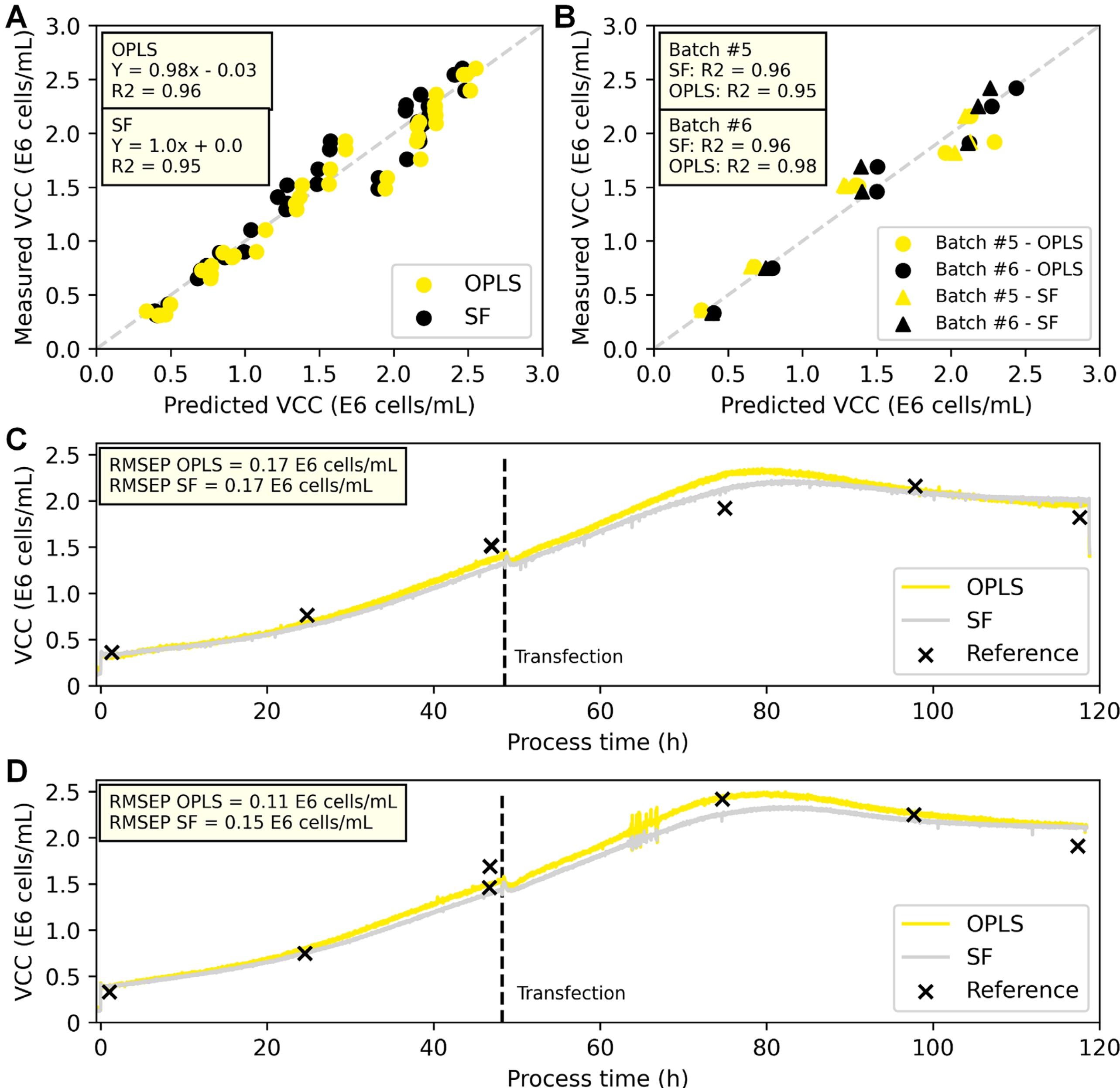

FIGURE 2 | (A) Observed versus predicted (cross-validation) plot of SF and OPLS models; (B) Observed versus predicted plot of SF and inline OPLS model predictions for Batches #5 and #6; SF and OPLS predictions with offline references over process time for (C) Batch #5 and (D) Batch #6. OPLS, orthogonal partial least squares; SF, single-frequency; TTT, time-till-transfection; VCC, viable cell concentration.

supplying real-time VCC prediction data to the BioPAT MFCS SCADA system. For evaluation, the models' predictions were compared to the offline references (Figures 2B–D). The OPLS model demonstrated very good prediction performance throughout all cultivation phases, represented by RMSEPs of 0.17 and 0.11 million cells/mL for Batches #5 and #6, respectively. The SF model provided the same accuracy for Batch #5 and a slightly larger RMSEP of 0.15 million cells/mL for Batch #6. Considering the strong agreement between the inherent measurement uncertainty of the reference method and the models' RMSEPs, we find that the developed models are able to account for the batch-to-batch variability of our process (Figures S1A–C) and as such are suitable for monitoring VCC in the rAAV vector production process.

Real-time VCC monitoring is especially beneficial for viral production processes that incorporate a transfection step at a predetermined target VCC. Such processes often necessitate the timely preparation of reagents manually, which must be completed within a constrained time window before the transfection event, indicating the profound importance of accurate VCC forecasting.

Herein, we propose a novel, stacked model architecture consisting of a capacitance-based model for real-time VCC monitoring combined with an exponential growth model, called the TTT Forecaster. The TTT Forecaster is designed to predict in real-time the time left until a transfection event should be initiated based on the growth kinetics of the cell culture and a user-specified

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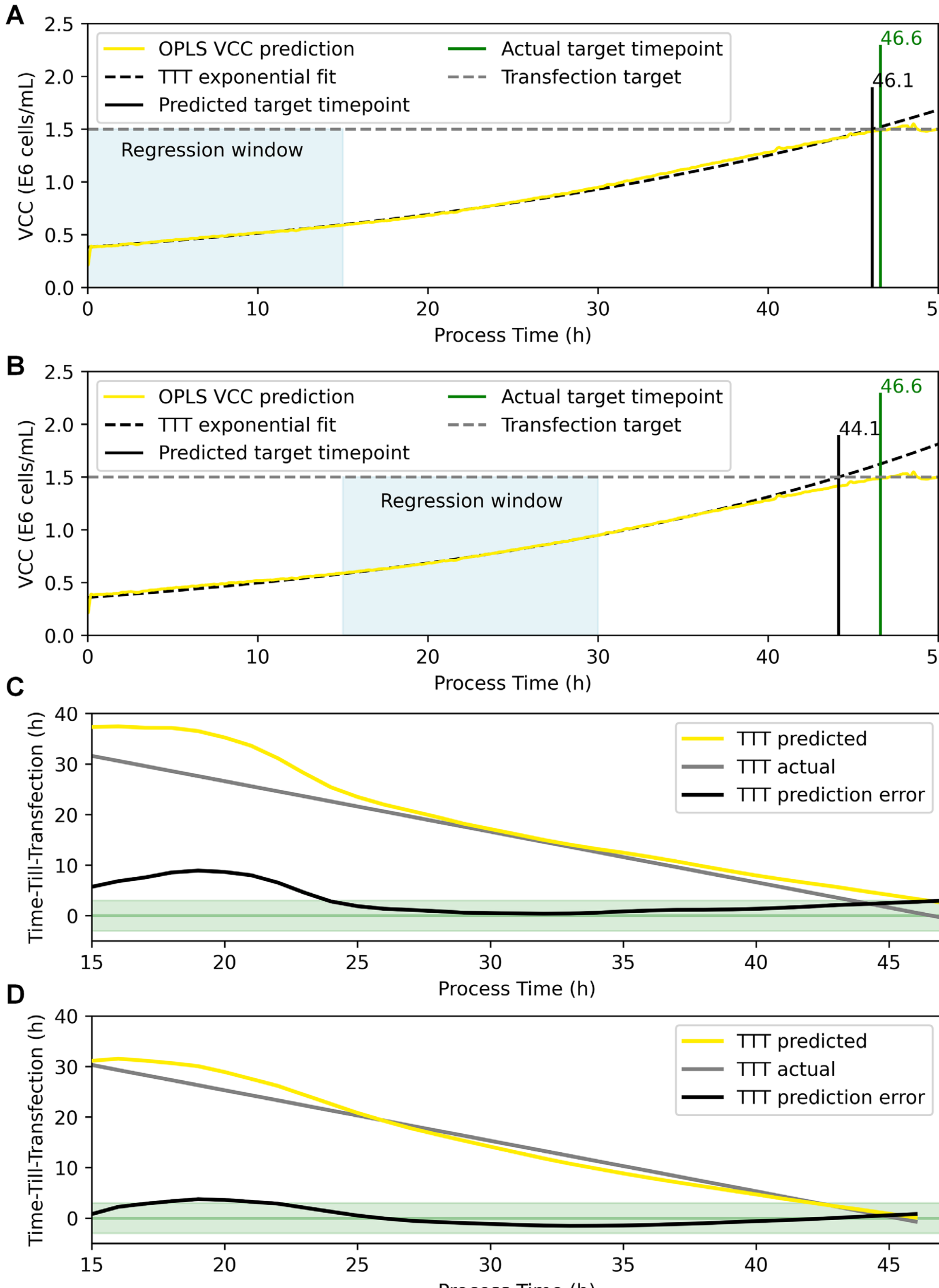

FIGURE 3 | Exponential fit and forecasting results of the TTT Forecaster for Batch #5 utilizing OPLS-based VCC predictions with exemplary regression window between (A) 0–15 h and (B) 15–30 h. Actual and predicted TTT with prediction error (predicted TTT–actual TTT) for (C) Batch #5 and (D) Batch #6. The green-shaded area marks an error range of ±3 h; the actual target timepoint was obtained from an exponential growth curve fitted to the offline Cedex HiRes Analyzer references (Figure S2). OPLS, orthogonal partial least squares; TTT, Time-Till-Transfection; VCC, viable cell concentration.

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transfection VCC target. The method presented in [12] utilized a time-weighted linear model based on raw SF capacitance measurements for forecasting. In contrast, the TTT Forecaster utilizes an exponential model based on VCC, which makes the approach wider and more compatible with any type of sensor that provides continuous VCC data. In this study, the VCC data is derived from real-time predictions of the previously discussed OPLS model, which is trained on multi-frequency capacitance spectroscopy data.

The TTT Forecaster was deployed inline, providing real-time predictions to the bioprocess control system during batch cultivations #5 and #6. The transfection VCC target was specified at 1.5 million cells/mL. Forecasting results and prediction errors for both batches are presented in Figure 3. In the beginning, the TTT Forecaster was overestimating the time left until transfection due to relatively low cell growth rates, likely during the cultures' lag phases upon bioreactor inoculation (data not shown). Over time, the influence of the lag phase on the forecast was reduced as a direct consequence of the moving regression window that was employed. This led to a stabilization of the forecast projections starting from 24 h of process time and lasting until the transfection target was reached. During this time frame, the TTT prediction error stayed within ±3 h of the error margin for both cultivations (green shaded area in Figure 3C, D). Assuming a growth rate of 0.03 million cells/ (mL h) and a (maximum) TTT prediction error of ±3 h (after stabilization), the deviation from the VCC transfection target of 1.5 million cells/mL would correspond to ±0.09 million cells/mL.

These results show that the TTT Forecaster can provide an accurate prediction of the transfection time point already one full day in advance of the transfection event, which is comparable to the results published for Sf9 cell culture rAAV production [12]. This highlights the ability of the TTT Forecaster to support repeatable and consistent transfection at specific culture states (e.g., VCC targets), enhancing the reproducibility of rAAV vector production processes and ultimately increasing product yield and quality. Notably, the TTT Forecaster's architecture is designed to be independent of any specific VCC source, ensuring compatibility with any (continuous) VCC signal, including those beyond capacitance-based methods.

4 | Conclusion

Monitoring of VCC during mammalian cell culture processes is crucial for achieving robust operations, enhancing process understanding, conducting accurate assessments, and implementing actions that ensure optimal process management. As described before, and confirmed in this study, inline capacitance spectroscopy is an effective PAT tool for real-time VCC monitoring in HEK293-based rAAV vector production. In extension, the real-time VCC data can serve as a basis for predicting the transfection time point, a crucial event influencing process yield and repeatability. We have demonstrated accurate prediction of the transfection time as early as 24 h after inoculation and 22 h before the event itself, which provides an opportunity for optimization of lab resources and automated control of transfection in the future.

Whereas monitoring of VCC in the bioprocess control unit based on the SF models is currently an accepted approach, to the best of our knowledge, we have presented the first example of an inline deployment of a multivariate VCC predictive model for monitoring and TTT forecasting in HEK-cell-based transient rAAV production, as demonstrated in our rAAV-8 model upstream process. The presented approach shows the potential to be applied for other rAAV serotype production batches such as, for example, rAAV-9. Whereas for the studied process SF capacitance measurement would be sufficient for accurate VCC monitoring, this might not necessarily be the case for other rAAV production processes where cell diameter changes significantly. As such, here presented approach for inline deployment of multivariate models, combined with the TTT Forecaster, for optimized transfection timing, enables the advancement of the virus production field toward more stable and efficient processes.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

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