


RESEARCH ARTICLE

Raman-based dynamic feeding strategies using real-time glucose concentration monitoring system during adalimumab producing CHO cell cultivation

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Abstract

The use of Process Analytical Technology tools coupled with chemometrics has been shown great potential for better understanding and control of mammalian cell cultivations through real-time process monitoring. In-line Raman spectroscopy was utilized to determine the glucose concentration of the complex bioreactor culture medium ensuring real-time information for our process control system. This work demonstrates a simple and fast method to achieve a robust partial least squares calibration model under laboratory conditions in an early phase of the development utilizing shake flask and bioreactor cultures. Two types of dynamic feeding strategies were accomplished where the multi-component feed medium additions were controlled manually and automatically based on the Raman monitored glucose concentration. The impact of these dynamic feedings was also investigated and compared to the traditional bolus feeding strategy on cellular metabolism, cell growth, productivity, and binding activity of the antibody product. Both manual and automated dynamic feeding strategies were successfully applied to maintain the glucose concentration within a narrower and lower concentration range. Thus, besides glucose, the glutamate was also limited at low level leading to reduced production of inhibitory metabolites, such as lactate and ammonia. Consequently, these feeding control strategies enabled to provide beneficial cultivation environment for the cells. In both experiments, higher cell growth and prolonged viable cell cultivation were achieved which in turn led to increased antibody product concentration compared to the reference bolus feeding cultivation.

KEYWORDS

CHO cell culture, dynamic feeding strategy, limited glucose concentration, Raman spectroscopy, real-time monitoring and control

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1 | INTRODUCTION

Over the past decades, Raman spectroscopy appeared to be a very promising analytical technique with a wide variety of possible applications within the pharmaceutical field.¹⁻⁴ Due to its chemical and structural sensitivity, it enables to determine key components simultaneously even in a complex and dynamically changing biological system.⁵⁻⁷ Moreover, it can also be implemented for in-line measurement by optical biosensor providing real-time process information about the critical process parameters (CPPs) without the need of taking samples from the bioreactor. This is a key aspect in biotechnological manufacturing where the most common CPPs are determined using traditional monitoring systems based on manual sampling and off-line analysis. In general, multivariate data analysis (MVDA) techniques are necessary to employ in order to extract relevant information from large dataset generated from the Raman spectra and understand the relationship between spectroscopic measurement and the analyte of interest. In bioprocessing, principal component analysis (PCA) and partial least squares (PLS) are the most commonly used MVDA methods for building predictive regression models.⁸

Monoclonal antibodies (mAbs) show an increasing dominance within the field of biopharmaceutical approvals. Therefore, numerous studies have been investigated the feasibility of in-line Raman spectroscopy for the determination of CPPs in mammalian cell cultures. Glucose plays a very important role as an essential carbon and energy source, typically influencing the cell growth, viability, and productivity but also the protein product quality.^{9,10} For example, glycosylation pattern considered as a critical quality attribute has been shown to be sensitive to glucose concentration. However, any modification in the glycosylation pattern can affect the antibodies biological properties including bioactivity, in vivo clearance, stability, and immunogenicity.^{11,12} The first application of Raman spectroscopy was published in 2010, where Abu-Absi¹³ et al. utilized an immersion bioprobe for real-time monitoring and simultaneous prediction of multiple components (such as glucose, lactate, glutamine, glutamate, ammonium, viable cell density [VCD], and total cell density [TCD]) in industrial-scale CHO cell cultivation. Since then, further studies have confirmed the capability of Raman spectroscopy to determine the CPPs including glucose concentration with similar predictive power during fed-batch fermentation of CHO cells.^{14,15} Many other researchers have been focused to investigate the accuracy and feasibility of the Raman-based PLS regression model across different cultivation conditions: various medium, cell clones, and production scales from development to manufacturing.¹⁶⁻¹⁸ As a conclusion, the development of a robust and reliable PLS model requires a big calibration dataset generated from numerous bioreactor runs with various conditions (scale, culture time, cell density, and so forth). This is very expensive and takes also a long time. For this reason, Kozma et al.¹⁹ developed a novel shake flask model system with less time and energy investment in which the calibration data were generated from small scale and easy-to-design cultivation environment. The scalability of the developed model was tested in a 10 L and 100 L bioreactor cultivations.²⁰ The Raman spectroscopy-based shake flask model system was able to determine the glucose concentration with a good prediction error of around 4 mM.

Accordingly, the integration of in-line Raman spectroscopy as an effective Process Analytical Technology (PAT) tool opens the door to develop robust control strategies through real-time process monitoring. Traditionally, the biggest problem in fed-batch operation mode is that the nutrients are generally supplemented in bolus feeding strategy based on off-line measured concentration values using pre-determined volumes of concentrated nutrients (feed medium). This results in high fluctuations of glucose concentration in a relatively wide range, which is non-optimal for the cells due to the chance of depletion or accumulation.^{21,22} The low glucose concentration leads to starvation and declined viability, while the permanently high glucose concentration has been shown to promote the generation of oxidative species, causing undesired stress for the cells.²³⁻²⁵ Furthermore, the unbalanced nutrient supply can occur metabolic by-products accumulation at inhibitory levels, which affects negative changes in growth, productivity, and product quality. The two major metabolites are lactate and ammonia, which are mainly produced as a result of glucose and glutamine consumption, respectively.²⁶⁻²⁸

Moving away from traditional bolus feeding to an adaptive feeding strategy could be able to adjust to the dynamically changing rate of glucose consumption. Numerous studies have been aimed at maintaining the available nutrients at low levels utilizing different types of dynamic feeding algorithm.²⁹⁻³³ The purpose of these feeding strategies was to change the cell metabolism to a state where the production of inhibitory metabolites is reduced and hence to improve cell growth and product quality. The first attempt at Raman-based feeding control technology was published by Whelan et al.¹⁴ where the feed medium delivering pumps were controlled manually by switching them on and off with the aim to keep the glucose concentration at 11 mM. The same research group reported a nonlinear model predictive controller development using on-line Raman spectroscopic measurement which was applied for fixed setpoint (11 mM) glucose control.³⁴ There were other studies that confirmed the positive impact of reduced nutrients availability. For example, Berry et al.³⁵ showed that the glycation level was successfully reduced from 9 to 4% by maintaining glucose concentration at 2 g/L using a Raman-based glucose feed control system. Matthews et al.³⁶ reported about the control strategy utilizing Raman spectroscopy in which the feeding of glucose was controlled based on real-time lactate and glucose concentration measurements. The optimal amount of glucose additions allowed to minimize the lactate accumulation without the state of cells starvation and helped to increase the cultivation duration, cells viability and productivity, and the process robustness.

The aim of this research was to develop a real-time monitoring system using in-line Raman spectroscopy in order to establish an efficient feeding control strategy. Glucose was selected as a direct indicator of nutrient consumption to control the replenishment of the two multi-component feed media.³⁷ The goal was to limit the glucose at low concentration and to direct the cell metabolism to a more efficient state with reduced inhibitory by-product accumulation. The multi-component feed solutions were supplied by two different strategies—manual and automated dynamic feeding methods—as needed according to the real-time determined glucose concentration.

Our self-developed automated monitoring and control system was applied to operate the feed dosing pumps without the need of user intervention. The main concept was to maintain the glucose within the desired level of 11–12 mM and compare the effects of the Raman-based dynamic feeding and traditional bolus feeding strategies on cell metabolism, growth and on antibody product quantity and quality. To our best knowledge, this is the first reported study that applied a fully automated dynamic feeding strategy based on Raman measured glucose concentration to control the multi-component feed medium delivery in mAb producing CHO cell culture.

2 | MATERIALS AND METHODS

2.1 | Cell line, medium, and feeds

CHO DG44 cell line producing a biosimilar equivalent of adalimumab (Abbvie) was used for shake flask and bioreactor culture experiments. The cells were grown in a medium specialized for mammalian cells, which was supplemented with 4 mM glutamine. Two different feed medium, Feed A (Cell Boost™ 7a Powder, GE Healthcare) and Feed B (Cell Boost™ 7b Powder, GE Healthcare) were used to refresh the essential nutrients, from which only Feed A contained 500 mM glucose. In the fed-batch cultivation, 40 wt/wt% (2,600 mM) glucose stock solution was supplied in case to avoid starvation.

2.2 | Cell culture conditions and sample analyzing

2.2.1 | Shake flask cultivation

Shake flask cell cultures were maintained to provide a calibration dataset for PLS model building and for scaling-up the inoculum for the bioreactors. The cell cultures were incubated in shake flasks at 37°C and 5% CO₂ using SANYO Incubator and Thermo MaxQ2000 shaker agitating at 100 rpm. The cells were grown in different scales and sub-cultured every 3–4 days with a seeding density of 0.3×10^6 cells/ml.

2.2.2 | Bioreactor cultivation

Fed-batch cultivations were performed in a 2 L working volume glass bioreactor (Applikon Biotechnology). The initial seeding cell density was 0.5×10^6 cells/ml. During the bioreactor cultivations, pH (7.1), temperature (37°C), dissolved oxygen (DO, 40%), and stirring speed (120 rpm) were controlled by ez-Control system (Applikon Biotechnology). The BioXpert software was used by local area network connection to collect the cultivation parameters every 10 min.

The bioreactor was equipped with in-line probes to monitor and control critical process parameters: thermometer (AppliSens, Applikon Biotechnology), pH probe (AppliSens, Applikon Biotechnology), DO sensor (AppliSens, Applikon Biotechnology), dielectric spectrometer

(Hamilton Incyte 220), and Raman immersion bioprobe (Bio-Optic, Kaiser Optics) were used. The volume of the culture broth was monitored gravimetrically by Kern PKT24K0.1 balance. The DO was controlled using an air–oxygen gas mixture. The pH was maintained by CO₂ gas and 0.5 M sodium carbonate. Few drops of antifoam solution (Ex-cell, Sigma–Aldrich) were added every day to reduce foaming. The base, glucose, antifoam, Feed A, and Feed B were delivered separately to the bioreactor by peristaltic pumps (Watson-Marlow 120 U). Bioreactor cultivation was typically sampled two times daily; and in the reference experiment, an additional sample was taken after each bolus nutrient feeding.

2.2.3 | At-line and off-line measurements

Cell culture samples were analyzed at-line to determine the cell density and viability, the pH, and the glucose concentration. The cell density and viability were measured by Olympus CKX31 microscope using a $\times 40$ objective. The cells were stained with Trypan blue (Sigma–Aldrich) and counted in Bürker chamber (Sigma–Aldrich), two replicates were performed for each sample. The in situ pH measurements were verified using S47 SevenMulti pH meter (Mettler Toledo AG). The approximate glucose concentration was measured at-line by Accu-chek Active (Roche Diagnostics GmbH) blood glucose meter. The samples were centrifuged (Rotanta 460R, Hettich) at 25°C and 1,000 rpm (is equal to 220g) for 10 min and the cell-free supernatants were stored at -20°C until the off-line analysis. The reference concentration of glucose, lactate, glutamine, glutamate, and ammonia were quantified off-line using enzymatic Cedex Bio HT analyzer (Custom Biotech, Roche).

2.3 | Raman measurements

Cell cultures were analyzed by Kaiser Raman RXN2 Hybrid system with a stainless steel immersion bioprobe. The system was equipped with a laser providing 785 nm excitation wavelength in approximate power of 400 mW. The Raman scatter signals were transmitted via fiber optic cable to a cooled charge-coupled device camera operating at -40°C . The spectral acquisition range was between 1890 and 200 cm^{-1} with a 4 cm^{-1} resolution. The exposure time was 10 s with 75 accumulations in order to remove cosmic ray and fluorescence signal from culture components, which resulted in 15 min long spectra acquiring cycles. The spectrometer was controlled by the iC Raman™ 4.1 software (Mettler Toledo Autochem) and the spectra were exported in the format of spc files. The same settings were applied during shake flask and bioreactor experiments.

2.3.1 | Off-line spectral data collection strategy

Off-line Raman measurements were performed in shake flasks to generate a calibration dataset for PLS model development. Before Raman

spectra acquisition, each shake flasks were sampled and analyzed at-line (the same as described in Section 2.2.3). According to the initial glucose concentration, a calibration ladder was created with adjusted glucose level between 0 and 70 mM by adding calculated amounts of 40 wt/wt% (2,600 mM) glucose stock solution. After each glucose spiking, one Raman spectrum was acquired by immersing the probe manually into the actual cultivation medium. In order to ensure constant environment during spectra collection, shake flasks were maintained at 35–38°C and homogenized by magnetic stirrer working at 100–150 rpm. After the Raman measurement, a sample was taken and centrifuged (Rotanta 460R, Hettich) at 25°C and 1,000 rpm (is equal to 220g) for 10 min. The cell-free supernatants were stored at –20°C until the reference off-line glucose concentration determination by Cedex Bio HT analyzer.

2.3.2 | In-line spectral data collection strategy

In-line Raman spectroscopic monitoring was utilized from the bioreactor medium throughout the whole cultivation experiments. The Raman probe was sterilized with the bioreactor before each bioreactor cultivation. The glass bioreactor was covered with aluminum foil in order to reduce additional noise in the measurement signal originating from environment light. The bioreactors were sampled two times daily for later reference off-line measurements similar as it was described in the shake-flask experiments.

2.3.3 | Chemometric modeling (data analysis)

The PLS model development was performed with PLS toolbox 8.2 (Eigenvector Research) in Matlab R2017a environment (The MathWorks). In regression model building, Raman spectra were defined as the independent variables (X variable) and the off-line glucose concentrations were defined as the dependent variables (Y variables). The PLS model performance was evaluated considering the following statistical parameters: root mean square errors of calibration (RMSEC), cross-validation (RMSECV), and prediction (RMSEP), and square correlation coefficients (R^2) for calibration, cross-validation, and prediction.

2.4 | Feeding strategies

The bolus feeding strategy (reference) was implemented on the third cultivation day and repeated after every second day by adding predefined amounts of the two feed medium (Feed A: 4% and Feed B: 0.4% of the actual culture volume). The solutions were delivered separately by peristaltic pumps (Watson-Marlow 120 U) at a constant feeding rate.

The strategy of manual dynamic feeding was to supply the fresh nutrients based on the demand of the cell culture. The volume of the two feed medium was fixed similar as in the reference experiment

(Feed A: 4% and Feed B: 0.4% of the actual culture volume) at each feeding event. The key modification compared to the bolus feeding strategy was the integration of the in-line Raman monitoring system to determine the indicator glucose concentration in real-time. Therefore, instead of predetermined intervals (every second day) we only intervened the process if the Raman predicted glucose concentration fell below the setpoint of 11 mM. In this case, the feed solutions were delivered to the bioreactor separately by manually activating the peristaltic pumps at a constant feeding rate.

The automated dynamic feeding experiment was carried out using our self-developed real-time monitoring and control system. From the iC Raman software the acquired Raman spectra were transferred via TCP/IP network to the data analysis and the control software developed in Matlab environment. This was used for the evaluation of spectral data and to predict the glucose concentration. In this work, the integration of a microcontroller (Arduino UNO) played a crucial role to control the feed dosing peristaltic pumps automatically. The pumps were operating at a fixed feeding rate, the Feed A delivering pump was 10 times faster (1 ml/min) than the Feed B delivering pump (0.1 ml/min). The experimental setup is described in details in the *Results and Discussion* Section 3 (Figure 4).

2.5 | Off-line analysis of the antibody product

2.5.1 | Antibody concentration

The antibody concentrations were measured by OCTET K2 system (Pall ForteBio) using Dip and Read™ Protein A (ProA) biosensor in 96-well microplate (Greiner BioOne, Sigma-Aldrich). The standard curve was prepared using a series of known dilutions of HUMIRA® adalimumab standard. Based on this, the system software (Data Analysis 10.0, Pall ForteBio) calculated the binding rates and generated a standard curve, which was then used for the determination of the unknown concentrations of the cultivation samples. The assay buffer was phosphate-buffered saline (PBS) and the regeneration solution was 10% glycine (pH 1.5). The volume of the samples was 200 µl containing 10 µl 1% Tween 20 solution in order to avoid the non-specific binding. Assays were performed at 30°C with continuous agitation at 200 rpm.

2.5.2 | Kinetic characterization

The kinetic measurements were performed by OCTET K2 system (Pall ForteBio) using Anti-Penta-HIS (HIS1K) Biosensor (Dip and Read™, ForteBio) tips. The cultivation samples were purified in advance using AKTA Fast Protein Liquid Chromatography system (GE Healthcare). The volume of the samples was 200 µl. The experiment steps were the following: in Baseline 1 (60 s) the biosensor tips were equilibrated in PBS. In the loading step (200 s), the recombinant human Fc γ RIIIA/CD16a receptors (R&D Systems) diluted in PBS with 1% Tween 20 were immobilized on the surface of the biosensors. In Baseline

2 (30 s), biosensor tips were washed in PBS buffer with 1% TWEEN 20 to remove unbound molecules. In the association step (60 s), the tips were placed into the wells containing the analyte (mAb) which was followed by the dissociation (120 s), where the tips were dipped into PBS with 1% Tween 20. The regeneration solution was 0.1 M citrate buffer (pH 3.5). Assays were performed at 30°C with continuous agitation at 1,000 rpm. The Data Analysis HT 10.0 software was used to determine the kinetic parameters: binding association and dissociation rate constants (k_a and k_d , respectively) and the affinity constant (K_D). The evaluation was performed by global fitting for association and dissociation curves using mathematical equations based on 1:1 binding interactions.

3 | RESULTS AND DISCUSSION

3.1 | Calibration model building

PLS calibration model was developed to determine the glucose concentration by Raman spectroscopy in real-time during bioreactor cultivations. The Raman spectra for calibration dataset generation were collected from shake flask and bioreactor cultivations. The shake flask environment enabled to plan the chemical and physical parameters of the culture (especially glucose concentration with a spiking method) in a simpler way as it is less time- and workload-intensive compared to bioreactor process. Therefore, the shake-flask cultures were performed with a wide range of cultivation parameters in order to simulate the biochemical variability of the bioreactor medium. The VCD was maintained between 7 and 14.5×10^6 cells/ml and the cell viability was maintained between 10 and 100%. Moreover, some of the shake flasks were fed using the two feed medium (Feed A 4% and Feed B 0.4% of the actual cultivation medium volume).

The calibration dataset included a total of 106 individual observations from nine shake flask cultures (56 points) and three independent bolus feeding fed-batch fermentations (50 points). Several preprocessing methods were investigated and compared their effect (data not shown) in order to emphasize relevant analytical information and to eliminate noise. The best preprocessing method for raw Raman spectra was resulted by the combination of baseline correction (Automatic Whittaker Filter) ($\lambda = 10,000$; $p = .0001$), standard normal variate, and mean centering. The reference glucose concentrations were mean-centered. After the pretreatment process, PLS regression was used to build the calibration model from Raman spectra combined with their reference concentration data. Internal validation was used for model qualification with the same 106 data points. Venetian blinds cross-validation was applied with six splits. The optimal number of latent variables was selected for five considering the minimum value of RMSEC in order to develop a stable model but to minimize the overfitting. As shown in Figure 1, the model training data covered a glucose concentration range of 0–70 mM. It can be observed that the sample points within a low range (0–15 mM) were gathered only from shake flask cultures. This was due to the fact that the glucose concentration was high over the course of bioreactor runs and could not

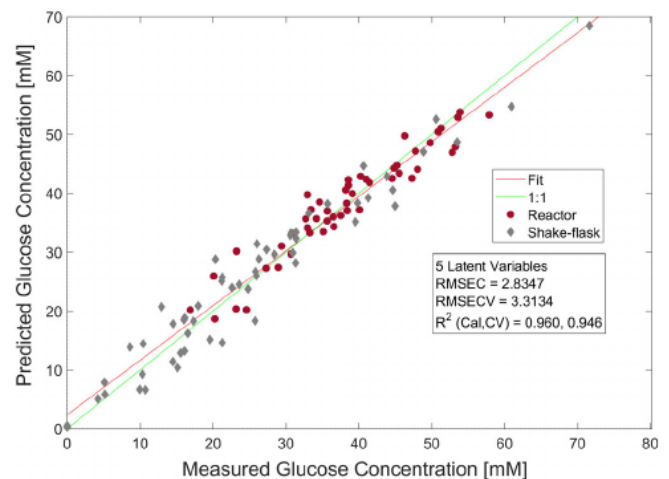


FIGURE 1 Scatter plot of off-line measured versus Raman predicted glucose values by the partial least squares calibration model

decrease below 15 mM as a consequence of frequent fresh nutrient replenishment by bolus feeding strategy.

The PLS model has a good predictive power evaluated by an R^2 for calibration of 0.96, a RMSEC of 2.83, an R^2 for cross-validation of 0.95, and a RMSECV of 3.31 mM. Therefore, an independent bolus feeding bioreactor experiment that was not used for the model development, acted as the validation run in order to verify the robustness and accuracy of the model. For this purpose, a real-time data analysis program developed in Matlab was used to evaluate the Raman spectra acquired from the bioreactor and to predict the glucose concentration. The PLS model was able to determine the glucose concentration in real-time through the duration of the bioreactor run with a RMSEP value of 5.2 mM. This result is comparable to the reported errors of many other groups^{14–16} taking into consideration that the model was built by combining the data from nine shake flask cultures and three bioreactor runs. In addition, at the end of the cultivation, the presence of significant amounts of bubbles and cell mass can also interfere with Raman measurement.¹⁴ The prediction power of the model could be improved by adding new reference data gathered from bioreactor cultivations with lower glucose level and higher VCD. However, it is sufficiently accurate for the development of real-time monitoring and control system.

3.2 | Dynamic feeding strategies based on real-time glucose concentration

In this work, two types of dynamic feeding algorithms were employed: manual and automated. The aim of these feeding strategies was to maintain the nutrient concentrations within a constant and narrow range in order to provide a more ideal environment for the cells. We decided to control the volume of the two multi-component feed medium (Feed A and Feed B) instead of controlling only one nutrient component solution (e.g., glucose or lactate). Glucose was selected for the indicator of cell metabolism because of its key role as a primary

carbon and energy source. In this case, when the culture was fed based on the glucose concentration then all other components were replenished proportionally according to the stoichiometric composition of the feed medium. The benefit of this strategy is that it can prevent the depletion or overfeeding of nutrients, so the starvation of the cells or the accumulation of toxic by-products can be avoided. In order to provide a reference experiment, a bioreactor cultivation was performed where the nutrients were refreshed by traditional bolus feeding. The feedings were performed on cultivation Day 3, 5, 7, and 9 by adding predefined volumes of the two feed media. As a result of frequent and large volume of feed replenishments, the cells were

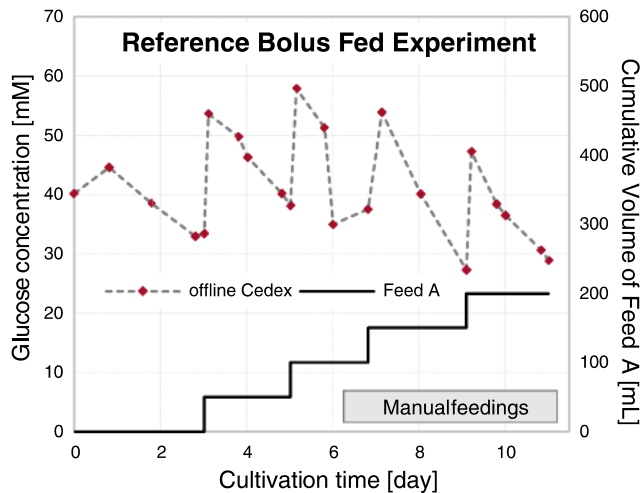


FIGURE 2 Off-line glucose concentration monitoring in the bolus fed experiment. The nutrients were replenished four times (on Day 3, 5, 7, and 9). The glucose concentration was high (above 30 mM) throughout the bioreactor cultivation

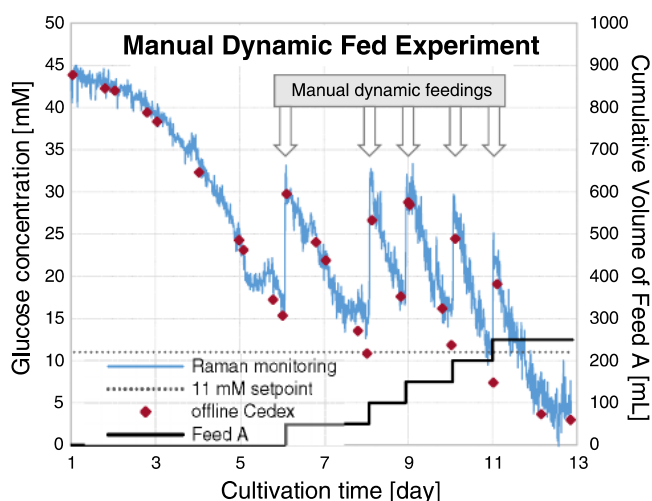


FIGURE 3 Raman-based glucose concentration monitoring in the manual dynamic fed experiment. The nutrients were replenished four times (on Day 6, 8, 9, 10 and 11). The glucose concentration was maintained within a lower and narrower concentration range (10–30 mM)

subjected to a much higher nutrient concentration than those was required for energy production. Figure 2 shows the concentration of the off-line measured indicator glucose throughout the bioreactor run. According to this, the glucose level was changing within a wide concentration range above 30 mM. Several research has been proved that high and variable glucose concentration could negatively influence cell growth, but also the antibody product quality.^{23,25} In the manual dynamic feeding experiment, the glucose concentration was monitored in real-time by Raman spectroscopy with the aim to investigate and understand the nutrient uptake rate of the cells. The glucose concentration decreased below the setpoint of 11 mM five times. According to this, the nutrients were replenished by the feed solutions on cultivation Day 6, 8, 9, 10 and 11. It can be observed that the first feed addition was 3 days later than the first bolus feeding was performed in the reference experiment. This resulted in that the manual dynamic feedings based on Raman-derived glucose concentration allowed to maintain the glucose level at a narrower range and to avoid the over accumulation (Figure 3). Although, the glucose concentration still fluctuated between 10 and 30 mM, which was caused by the predetermined volume of the feed additions. The use of manual dynamic feedings ensured better conditions for the cells compared to the bolus feeding. Nevertheless, this manual control strategy can still not enable a robust and rapid response to the dynamically changing nutrient uptake rates.

To improve the process consistency and robustness an automated control system was developed, which is represented in Figure 4. The principle of the control strategy was the following: if the Raman-measured glucose level dropped below the setpoint of 11 mM, the evaluation software calculated the volume of Feed A solution (in the unit of ml based on the predicted glucose concentration and the actual volume of the culture) which was needed to raise the concentration to 12 mM after dosing. The feed dosing pumps were automatically operated by the microcontroller at fixed feeding rate for the time that was required to supply the precalculated amount of the solutions to the bioreactor.

The Raman-based process control was initiated on cultivation Day 10, when the glucose concentration decreased to 11 mM and after this, the cultivation was maintained for five more days without nutrient exhaustion (Figure 5). Until Day 10, the bolus feedings should have been repeated four times leading to excess glucose accumulation for the cells. Unfortunately, the system suffered from an automation problem, because the communication was lost between the Raman spectra acquisition and the analysis software. This issue caused the interruption of the feed additions between cultivation hours 315 and 330, but this half-day omission did not lead to glucose exhaustion. The automated control system allowed to respond to the cells glucose consumption very quickly and enabled to maintain the concentration close to the desired level. More frequent additions but always smaller quantities of feed solutions were required to supply for the cells (according to the black curve in Figure 5, which represents the cumulative volume of Feed A solution fed by the pump). As a result, the continuous automated dynamic feeding was performed successfully in a CHO cell cultivation.

FIGURE 4 The experimental setup of real-time monitoring and automated control system

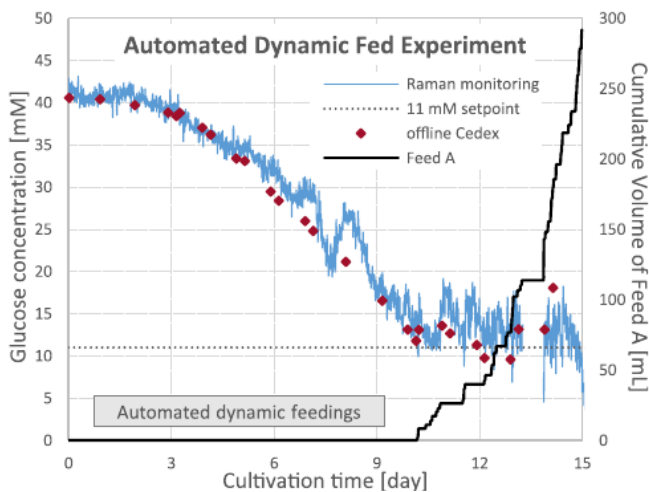
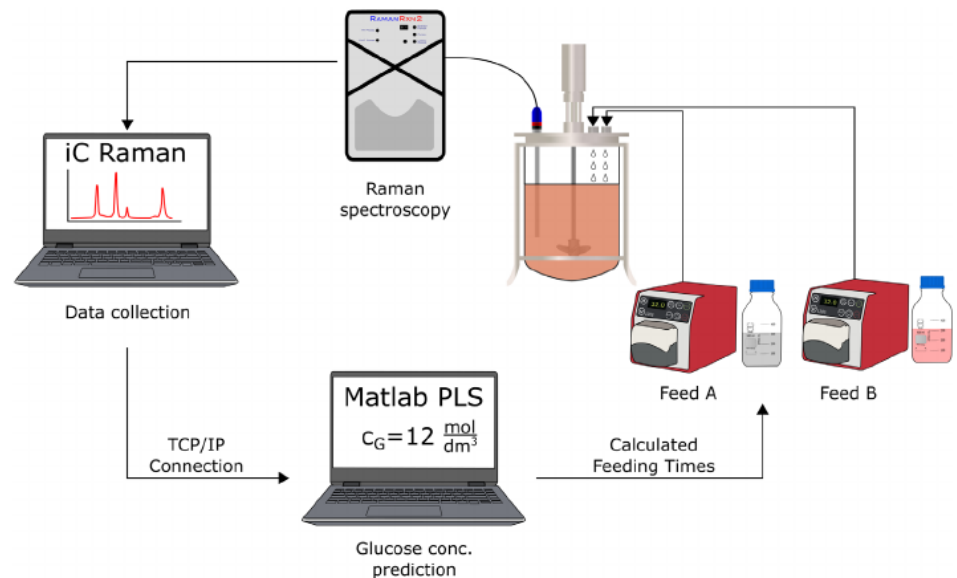


FIGURE 5 Raman-based glucose concentration monitoring in the automated dynamic fed experiment. The nutrients were replenished more frequently and in smaller quantities. This resulted in that the glucose level was limited within the desired concentration range (10–15 mM)

3.3 | Effect of dynamic feeding strategies

3.3.1 | To the metabolism

In order to see the impact of dynamic feeding strategies, the results were evaluated on the cells metabolism and compared to the reference bolus fed experiment. In the metabolism of CHO cells, glycolysis and glutaminolysis are two key pathways.³⁸ Through glycolysis, the cells consume glucose and generate lactate, while through glutaminolysis, the cells metabolize glutamine by deamidation to glutamate which is then converted to α -ketoglutarate releasing two moles of ammonia as the by-product.^{9,39} The goal of our dynamic feeding strategies was to alter the metabolism in a more efficient way

to ensure beneficial cultivation environment by moderating the inhibitor by-products (lactate and ammonia) accumulation.

Figure 6 represents the off-line measured concentrations of the five key metabolites including glucose, glutamine, glutamate, lactate, and ammonia. As can be seen in Figure 6a, during the dynamic fed experiments the glucose concentration was successfully maintained within a lower range compared to the reference experiment. The implementation of the automated control system enabled to target the desired glucose level with only moderate fluctuations around 10–15 mM.

The dynamic feeding methods (both manual and automated) had a significant impact on the lactate metabolite profile (Figure 6b). In the case of bolus feeding, the cells lactate production was high throughout the whole cultivation process. In the presence of high glucose concentration, the cells could not change the metabolism from lactate production toward lactate consumption. In contrast, when the available glucose was controlled at low level, the lactate concentration peaked on cultivation Day 6 in the manual controlled and on cultivation Day 7 in the automatic controlled cultures. Comparing the results to the glucose values (Figure 6a), it can be seen that in the manual dynamic feeding experiment the glucose was consumed faster than in the automated dynamic feeding experiment as a consequence of higher viable cell density (see Figure 7a). Thus the glucose concentration decreased to the desired level of 11 mM on Day 6, while the formation of lactate ended one day later. In contrast, in the automated dynamic fed cultivation the glucose concentration was well above the setpoint of 11 mM (between 20 and 25 mM) when the shift happened in the lactate accumulation. However, in both cases the lactate production was followed by a decline, suggesting that not only the limitation of glucose was responsible for shifting the cellular metabolism into a more efficient phase, where lactate was consumed as an alternative carbon and energy source.

The dynamic feedings were especially beneficial to the other nutrient components. In our strategy, glutamine was replaced by

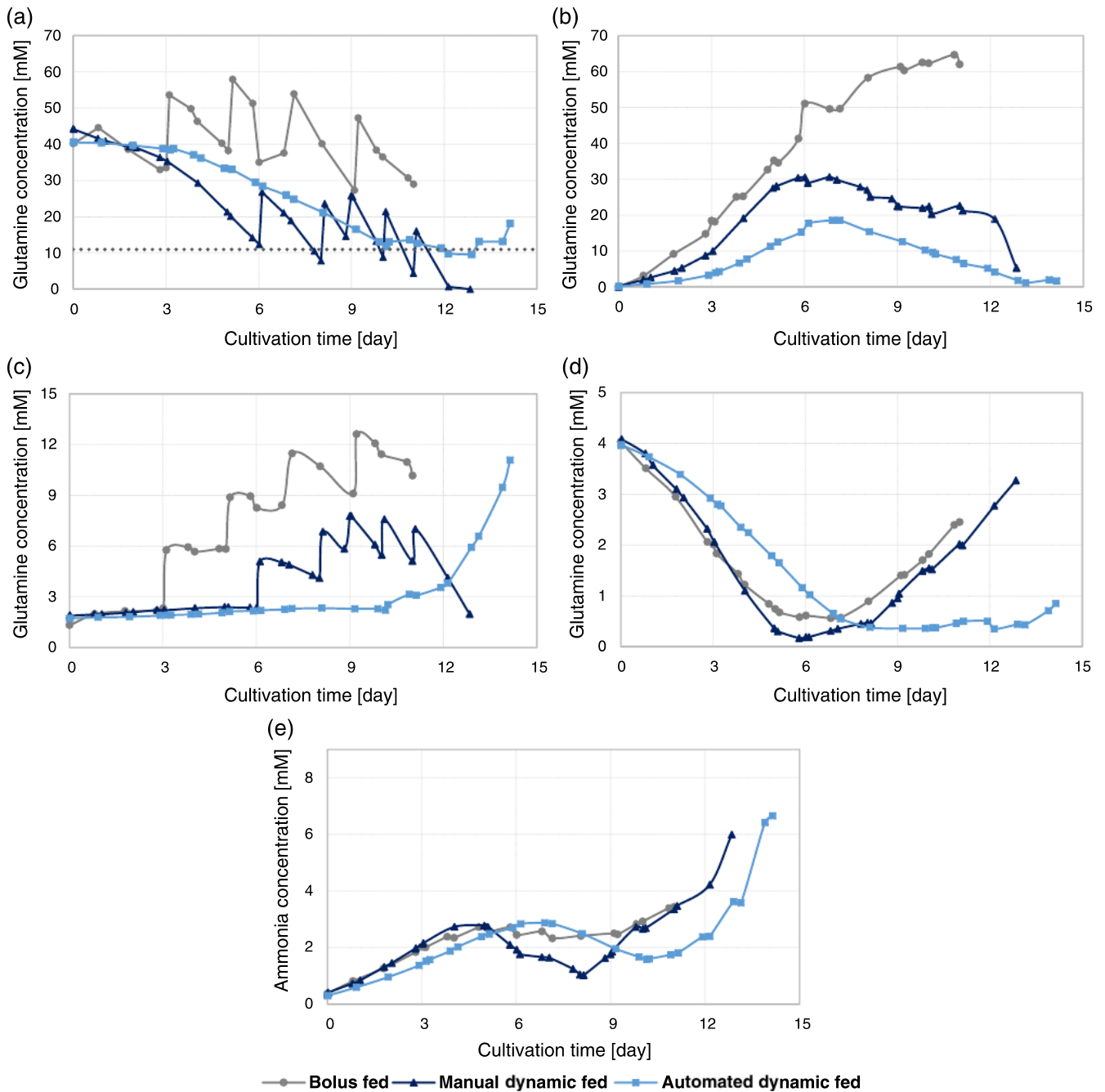


FIGURE 6 Off-line measured metabolite concentration profiles of glucose (a), lactate (b), glutamate (c), glutamine (d), and ammonia (e) in the bolus feeding, manual, and automated dynamic feeding cultivations

glutamate as it can be metabolized in a more efficient way since it has only one amino group and produces lower ammonia accumulation. According to this, the applied feed solutions contained only glutamate and the basal medium was supplemented with 4 mM glutamine to support cell growth. Figure 6c shows the glutamine profile of the three bioreactor runs. As can be seen, during the first half of the cultivations the available glutamine was depleted by the cells on Day 6 and Day 7 in manual and in automated dynamic fed cultivations, respectively. The two major pathways (glutaminolysis and glycolysis) are interconnected in the metabolism of mammalian cells.^{40,41}

Accordingly, the low glutamine level has also played an important role in preventing lactate accumulation. If we compare the results, it can be seen that the depletion of glutamine was consistent with the peak of lactate accumulation in both dynamic fed cultivations. After the depletion of the initial glutamine, the nitrogen source was replenished by glutamate from the fresh feed medium additions. The pattern of glutamate fluctuations is correlated to the profile of feed medium replenishments (Figure 6d). Thus in the manual dynamic fed experiment, glutamate was generally maintained at a higher concentration, while in the automated dynamic fed experiment it was maintained at

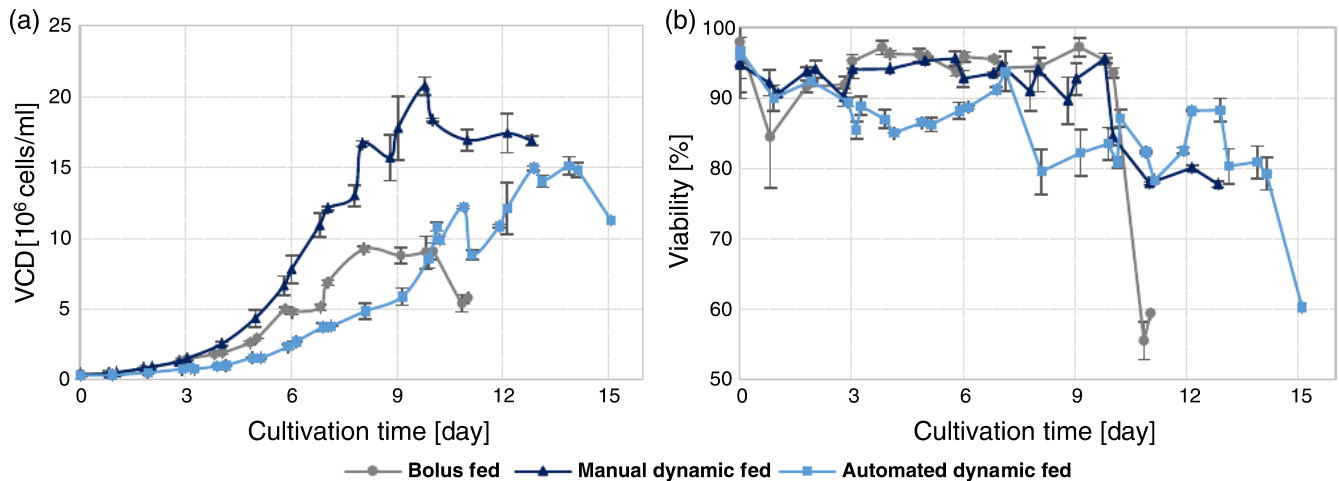


FIGURE 7 Growth profile (a) and viability (b) in the bolus feeding, manual, and automated dynamic feeding cultivations. VCD, viable cell density

a low level (until Day 12). As a consequence of the applied dynamic feeding strategies, both glucose and glutamate were maintained at reduced levels compared to the bolus fed cultivation.

Figure 6e shows the ammonia profile, which is the main by-product of amino acids (e.g., glutamine and glutamate) metabolism. The initial phase of all the three cultivations was characterized by glutamine consumption and ammonia production. Subsequently, the ammonia concentration was stagnated (bolus feeding) or decreased (manual and automated dynamic feeding). At the end of the bioreactor runs, ammonia was continued to accumulate due to the increased available nitrogen source but did not reach the inhibitory level in either case.

3.3.2 | To the cell growth and viability

The manual and automated dynamic feeding strategies showed significant improvement in cell growth compared to the reference bolus fed experiment. The VCDs during the three cultivations are shown in Figure 7a. The two dynamic fed cultures showed significant improvements in VCD compared to the traditional bolus fed culture. The results confirmed that the formation of toxic lactate inhibits cell growth after a certain point.^{26,29} Thus, the lowest maximum VCD was achieved in the reference cultivation (9.21×10^6 cells/ml), where the accumulation of lactate could not be prevented in the presence of high glucose concentration caused by the bolus feed additions. In contrast, when the available glucose was limited through dynamic feeding strategies, greater cell growth could be observed. The highest maximum VCD was achieved in the manual dynamic fed experiment with 2.14×10^7 cells/ml, which was twice as high as in the reference experiment. The growing phase was started later in the automated dynamic feeding experiment, which can be attributed to the consequence of the bioprocesses variability (e.g., variation in the cell density of the inoculum). Therefore, these experiments should be repeated in the

future in order to execute a comprehensive examination and clear conclusions. However, the results that the maximum VCD was obtained at 1.51×10^7 cells/ml which signified a 64% increase compared to the bolus feeding cultivation. The implementation of dynamic feedings extended the duration of the cultivation. The manual dynamic feeding experiment was maintained for 2 days longer and the automated dynamic feeding experiment for 4 days longer than the reference bioreactor run. The viability of the cells was between 75 and 100% in each fed-batch cultures (Figure 7b). It can be observed that the period of feed interruption (between 315 and 330 hr) in the automated feeding cultivation caused only a negligible decrease in culture growth and viability.

3.3.3 | To the concentration of the antibody product

The determination of the antibody product concentration (titer) was performed by Octet K2 instrument based on Bio-Layer Interferometry (BLI) technique that measures the changes in interference pattern between waves of light. The results are shown in Figure 8. The harvest titer was 0.49 g/L in the reference bolus feeding cultivation. In contrast, 1.28 and 0.72 g/L antibody concentrations were achieved in the manual and automated dynamic fed cultivation, respectively. Based on the results, the dynamic feeding strategies could substantially increase the amount of the produced antibody due to the favorable cultivation environment and to the increased cell growth.

3.3.4 | To the kinetics of the antibody product

The binding affinities of the antibodies produced in the three different bioreactor cultivations (bolus, manual dynamic and, automated

dynamic fed experiments) were determined by kinetic assay. The method was developed using the Octet K2 system, the same as for the quantification of the antibodies. This analytical technique is similar to the commonly used Enzyme-Linked Immunosorbent Assay with the difference that it utilizes label-free detection based on BLI, thus enables to monitor the binding interactions in real-time.

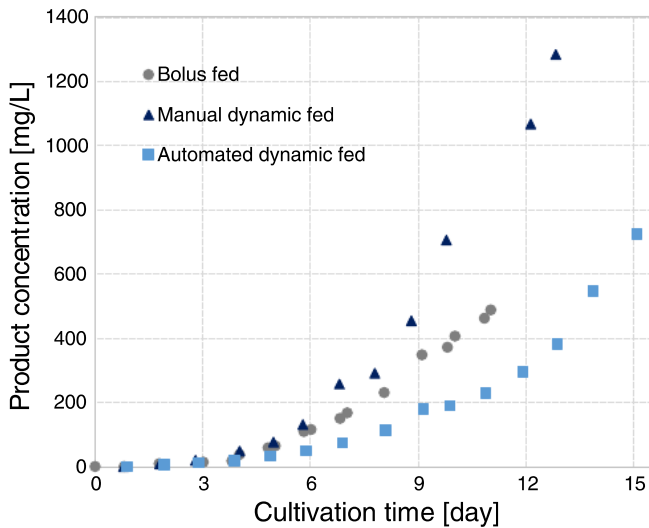


FIGURE 8 Product concentration throughout the bioreactor cultivations using bolus, manual, and automated dynamic feeding strategies. mAb, monoclonal antibody

Eight concentration settings (5; 2.5; 1.25; 0.625; 0.313; 0.156; 0.078, 0.039 μM) of the antibody were measured for each sample to generate binding graphs in real-time. Figure 9 represents the binding sensogram of adalimumab produced in the reference experiment (bolus fed). The kinetic measurement includes the following steps: baseline step (0–30 s), association (30–90 s), and dissociation step (90–200 s). During the evaluation, the dissociation step was limited to 110 s and the fitting was truncated to 5 s. The blue curves are corresponding to the dilution series of the analyte. The red curves are fitted globally by the software based on the 1:1 binding model. It can be seen that the model fitting was more optimal at lower concentration range. The binding sensograms showed that all the three adalimumab products performed a similar binding pattern (data not shown).

The individual association (k_a), dissociation (k_d), equilibrium binding constants (K_D) (calculated as k_d/k_a ratio), their standard values (SE), and the model fitting parameters (R^2 and χ^2) are summarized in Table 1. ADA1, ADA2, and ADA3 are the antibodies produced in the bolus fed, manual dynamic fed, and automated dynamic fed experiment, respectively. The fitted model can be considered reliable and accurate because the SE do not exceed $\pm 10\%$, the total $R^2 > 0.8$ and the total $\chi^2 < 3$. The calculated kinetic parameters were very similar for all adalimumab products. The association rate for ADA3 was $4.00 \times 10^5 \text{ M/s}$, which was greater than the association rates for ADA1 and ADA2 with a value of 1.50×10^5 and $3.78 \times 10^4 \text{ M/s}$, respectively. This means that the fastest antibody-receptor complex formation was achieved by the antibody product of automated

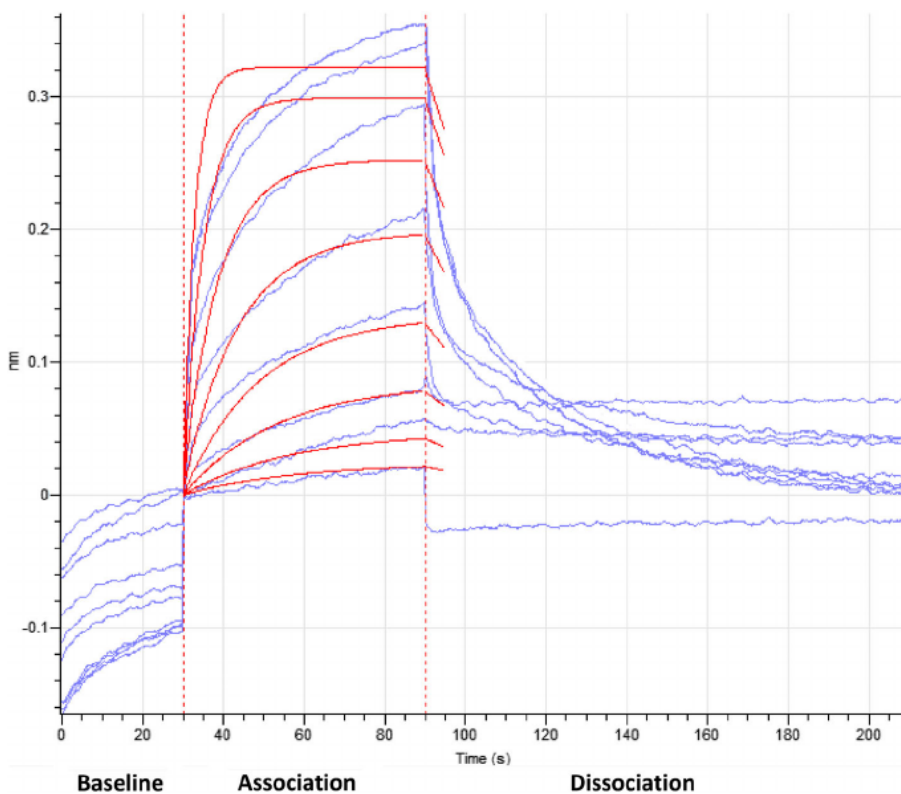


FIGURE 9 Example binding sensogram of adalimumab (produced in bolus fed cultivation) interacting with Fc γ RIIIA/CD16a receptors. The measurement steps are: baseline step (0–30 s), association (30–90 s), and dissociation step (90–200 s) The blue curves are corresponding to the dilution series of the analyte and red lines represent the fitted curves

TABLE 1 Results of the kinetic analysis obtained by global fitting of binding curves to the 1:1 interaction model

Sample	K_D [M]	K_D SE	k_a (1/Ms)	k_a SE	k_{dis} (1 s ⁻¹)	k_{dis} SE	R ²	χ^2
ADA1	5.35E-07	2.12E-08	1.50E+05	3.83E+03	8.02E-02	2.43E-03	0.9231	2.6803
ADA2	8.70E-07	2.32E-08	3.78E+04	4.87E+02	3.29E-02	7.69E-04	0.9695	1.3699
ADA3	4.85E-07	1.76E-08	4.00E+05	1.05E+03	1.94E-01	4.89E-03	0.9253	1.8820

Note: ADA1 (reference experiment); ADA2 (manual dynamic feeding experiment); ADA3 (automated dynamic feeding experiment).

dynamic fed cultivation. However, the greatest k_d value ($1.94 \times 10^{-1} \text{ s}^{-1}$) belonged for MAB3 too which means that the dissociation of its complex was the fastest either. Comparing the K_D values, it can be seen that there was no remarkable difference between the antibody products, each obtained an adequate binding affinity for the Fc γ RIIIA/CD16a receptor.

4 | CONCLUSION

In this study, in-line Raman spectroscopy-based monitoring and control system was developed to maintain the concentration of glucose and other nutrients at a fixed level during adalimumab producing CHO cell cultivation. MVDA technique was used for the evaluation of Raman spectra to determine the glucose concentration of the culture medium. The PLS calibration model was built from shake flask and bioreactor cultures as they enabled a faster and cost-effective way to control the biochemical and physical variability of the medium surrounding the cells. The model proved to be reliable and accurate for real-time monitoring of glucose concentration during an independent fed-batch bioreactor cultivation. Two dynamic feeding algorithms (manual and automated) were performed to control the available glucose by feed medium additions at a low setpoint (around 11 mM). The experimental data demonstrate that the dynamic feeding control strategy is an effective and reliable method to keep the nutrients (e.g., glucose and glutamate) within a narrow and low concentration range. Furthermore, the implementation of our self-developed, fully automated control system was able to respond to the cells dynamic nutrient consumption and proved to be an appropriate alternative for controlling the glucose concentration at the desired level only with moderate fluctuations. As a result, the cells metabolism was successfully pushed to consume lactate as a primary carbon source with moderate toxic by-products accumulation. This ultimately led to a favorable cultivation environment for the cells. The VCD increased in the two dynamic fed cultivations compared to the bolus fed experiment. Moreover, prolonged viability could be accomplished and thus resulted in increased harvest titer in the dynamic fed experiments compared to the reference experiment. The kinetic assay showed only minor differences in the binding affinities. Accordingly, the different types of feeding strategy did not affect significantly the kinetic properties of the antibody products. The presented Raman-based real-time monitoring and control system could be a potential opportunity in the future to promote the transition from traditional, industrially used bolus feeding to a more efficient dynamic feeding strategy and hence to improve the cell growth, productivity, and product quality.

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REFERENCES

1. Vankeirsbilck T, Vercauteren A, Baeyens W, et al. Applications of Raman spectroscopy in pharmaceutical analysis. *Trends Analyt Chem.* 2002;21(12):869-877.
2. Fini G. Application of Raman spectroscopy pharmacy. *J Raman Spectrosc.* 2004;35(5):335-337.
3. Kudelski A. Analytical applications of Raman spectroscopy. *Talanta.* 2008;76(1):1-8.
4. Nagy B, Farkas A, Borbás E, Vass P, Nagy ZK, Marosi G. Raman spectroscopy for process analytical technologies of pharmaceutical secondary manufacturing. *AAPS PharmSciTech.* 2019;20(1):1-16.
5. Abu-Absi NR, Martel RP, Lanza AM, Clements SJ, Borys MC, Li ZJ. Application of spectroscopic methods for monitoring of bioprocesses and the implications for the manufacture of biologics. *Pharm Bioprocess.* 2014;2(3):267-284.
6. Hirsch E, Pataki H, Farkas A, et al. Raman-based feedback control of the enzymatic hydrolysis of lactose. *Org Process Res Dev.* 2016;20(10):1721-1727.
7. Buckley K, Ryder AG. Applications of Raman spectroscopy in biopharmaceutical manufacturing: a short review. *Appl Spectrosc.* 2017;71(6):1085-1116.
8. Lourenço ND, Lopes JA, Almeida CF, Sarraguça MC, Pinheiro HM. Bioreactor monitoring with spectroscopy and chemometrics: a review. *Anal Bioanal Chem.* 2012;404(4):1211-1237.
9. Fan Y, Jimenez Del Val I, Müller C, et al. Amino acid and glucose metabolism in fed-batch CHO cell culture affects antibody production and glycosylation. *Biotechnol Bioeng.* 2015;112(3):521-535.
10. Altamirano C, Paredes C, Cairó JJ, Gòdia F. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. *Biotechnol Prog.* 2000;16(1):69-75.
11. Zheng K, Bantog C, Bayer R. The impact of glycosylation on monoclonal antibody conformation and stability. *MAbs.* 2011;3(6):37-41.
12. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annu Rev Immunol.* 2007;25(1):21-50.

13. Abu-Absi NR, Kenty BM, Cuellar ME, et al. Real time monitoring of multiple parameters in mammalian cell culture bioreactors using an in-line Raman spectroscopy probe. *Biotechnol Bioeng.* 2011;108(5):1215-1221.
14. Whelan J, Craven S, Glennon B. In situ Raman spectroscopy for simultaneous monitoring of multiple process parameters in mammalian cell culture bioreactors. *Biotechnol Prog.* 2012;28(5):1355-1362.
15. Moretto J, Smelko JP, Cuellar M, et al. Process Raman spectroscopy for in-line CHO cell culture monitoring. *Am Pharm Rev.* 2011;14(3):18-25.
16. Berry B, Moretto J, Matthews T, Smelko J, Wiltberger K. Cross-scale predictive modeling of CHO cell culture growth and metabolites using Raman spectroscopy and multivariate analysis. *Biotechnol Prog.* 2015;31(2):566-577.
17. Mehdizadeh H, Lauri D, Karry KM, Moshgbar M, Procopio-Melino R, Drapeau D. Generic Raman-based calibration models enabling real-time monitoring of cell culture bioreactors. *Biotechnol Prog.* 2015;31(4):1004-1013.
18. Webster TA, Hadley BC, Hilliard W, Jaques C, Mason C. Development of generic raman models for a GS-KOTM CHO platform process. *Biotechnol Prog.* 2018;34(3):730-737.
19. Kozma B, Párta L, Zalai D, Gergely S, Salgó A. A model system and chemometrics to develop near infrared spectroscopic monitoring for Chinese hamster ovary cell cultivations. *J Near Infrared Spectrosc.* 2014;22(6):401-410.
20. Kozma B, Hirsch E, Gergely S, Párta L, Pataki H, Salgó A. On-line prediction of the glucose concentration of CHO cell cultivations by NIR and Raman spectroscopy: comparative scalability test with a shake flask model system. *J Pharm Biomed Anal.* 2017;145:346-355.
21. Xie L, Wang DIC. Fed-batch cultivation of animal cells using different medium design concepts and feeding strategies. *Biotechnol Bioeng.* 1994;95(2):270-284.
22. Xie L, Wang DIC. Applications of improved stoichiometric model in medium design and fed-batch cultivation of animal cells in bioreactor. *Cytotechnology.* 1994;15(1-3):17-29.
23. Hu WS, Dodge TC, Frame KKHV. Effect of glucose on the cultivation of mammalian cells. *Dev Biol Stand.* 1987;66:279-290.
24. Lu S, Sun X, Zhang Y. Insight into metabolism of CHO cells at low glucose concentration on the basis of the determination of intracellular metabolites. *Process Biochem.* 2005;40(5):1917-1921.
25. Ha H, Bahl Lee H. Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose. *Kidney Int Suppl.* 2000;58(77):19-25.
26. Ozturk SS, Riley MR, Palsson BO. Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. *Biotechnol Bioeng.* 1992;39(4):418-431.
27. Hayter PM, Curling EMA, Baines AJ, et al. Chinese hamster ovary cell growth and interferon production kinetics in stirred batch culture. *Appl Microbiol Biotechnol.* 1991;34(5):559-564.
28. Yang M, Butler M. Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms. *Biotechnol Prog.* 2002;18(1):129-138.
29. Glacken MW, Fleischaker RJ, Sinskey AJ. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. *Biotechnol Bioeng.* 1986;28(9):1376-1389.
30. Zhou W, Rehm J, Hut W. High viable cell concentration fed-batch cultures of Hybridoma cells through on-line nutrient feeding. *Biotechnol Bioeng.* 1995;46(6):579-587.
31. Wlaschin KF, Hu WS. Fedbatch culture and dynamic nutrient feeding. *Adv Biochem Eng Biotechnol.* 2006;101:43-74.
32. Gagnon M, Hiller G, Luan YT, Kittredge A, Defelice J, Drapeau D. High-end pH-controlled delivery of glucose effectively suppresses lactate accumulation in CHO fed-batch cultures. *Biotechnol Bioeng.* 2011;108(6):1328-1337.
33. Goldrick S, Lee K, Spencer C, et al. On-line control of glucose concentration in high-yielding mammalian cell cultures enabled through oxygen transfer rate measurements. *Biotechnol J.* 2018;13(4):1-33.
34. Craven S, Whelan J, Glennon B. Glucose concentration control of a fed-batch mammalian cell bioprocess using a nonlinear model predictive controller. *J Process Control.* 2014;24(4):344-357.
35. Berry BN, Dobrowsky TM, Timson RC, Kshirsagar R, Ryll T, Wiltberger K. Quick generation of Raman spectroscopy based in-process glucose control to influence biopharmaceutical protein product quality during mammalian cell culture. *Biotechnol Prog.* 2016;32(1):224-234.
36. Matthews TE, Berry BN, Smelko J, Moretto J, Moore B, Wiltberger K. Closed loop control of lactate concentration in mammalian cell culture by Raman spectroscopy leads to improved cell density, viability, and biopharmaceutical protein production. *Biotechnol Bioeng.* 2016;113(11):2416-2424.
37. Lu F, Toh PC, Burnett I, et al. Automated dynamic fed-batch process and media optimization for high productivity cell culture process development. *Biotechnol Bioeng.* 2013;110(1):191-205.
38. Quek L-E, Dietmair S, Krömer JO, Nielsen LK. Metabolic flux analysis in mammalian cell culture. *Metab Eng.* 2010;12(2):161-171.
39. Robitaille J, Chen J, Jolicoeur M. A single dynamic metabolic model can describe mAb producing CHO cell batch and fed-batch cultures on different culture media. *PLoS One.* 2015;10(9):e0136815.
40. Zielke HR, Sumbilla CM, Sevdalian DA, Hawkins RL, Ozand PT. Lactate: a major product of glutamine metabolism by. *J Cell Physiol.* 1980;104:433-441.
41. Alvi K. Cell culture technology for pharmaceutical and cell-based therapies. *Journal of Natural Products.* 2007;70(4):712-713.

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