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RESEARCH ARTICLE

Continuous optical in-line glucose monitoring and control in CHO cultures contributes to enhanced metabolic efficiency while maintaining darbepoetin alfa product quality

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Abstract

Great efforts are directed towards improving productivity, consistency and quality of biopharmaceutical processes and products. One particular area is the development of new sensors for continuous monitoring of critical bioprocess parameters by using online or in-line monitoring systems. Recently, we developed a glucose biosensor applicable in single-use, in-line and long-term glucose monitoring in mammalian cell bioreactors. Now, we integrated this sensor in an automated glucose monitoring and feeding system capable of maintaining stable glucose levels, even at very low concentrations. We compared this fed-batch feedback system at both low (< 1 mM) and high (40 mM) glucose levels with traditional batch culture methods, focusing on glycosylation and glycation of the recombinant protein darbepoetin alfa (DPO) produced by a CHO cell line. We evaluated cell growth, metabolite and product concentration under different glucose feeding strategies and show that continuous feeding, even at low glucose levels, has no harmful effects on DPO quantity and quality. We conclude that our system is capable of tight glucose level control throughout extended bioprocesses and has the potential to improve performance where constant maintenance of glucose levels is critical.

KEYWORDS
bioprocess monitoring, biosensors, cell culture, CHO cells, recombinant proteins

Abbreviations: AB, 2-aminobenzamide; AGE, advanced glycation end products; ALA, alanine; ASN, asparagine; CZE, capillary zone electrophoresis; DDI, distilled de-ionized; DO, dissolved oxygen; DPO, darbepoetin alfa; DTT, dithiothreitol; EPO, human erythropoetin; FDA, food and drug administration; FLD, fluorescence detector; G418, geneticin; GLC, glucose; GLN, glutamine; GU, glucose units; HG, high glucose; HILIC, hydrophilic interaction liquid chromatography; HIS, histidine; HPLC, high performance liquid chromatography; LAC, lactate; LEU, leucine; LG, low glucose; MET, methionine; PAT, process analytical technology; PHE, phenylalanine; POF, polymeric optical fiber; PYR, pyruvate; SAX, strong anion exchange chromatography; SDS, sodium dodecyl sulfate; SER, serine; SFR, shake flask reader; TCA, tricarboxylic acid cycle; TRP, tryptophan; VCD, viable cell density

1 | INTRODUCTION

In 2004, the Process Analytical Technology (PAT) initiative by the Food and Drug Administration (FDA) stimulated a paradigm shift in the bioprocess environment. The active shaping of product quality using real-time process supervision and control has complemented the quality testing after production –"quality cannot be tested into products, it should be built-in".[1] Since then, devices for real-time measurements have been in the focus of any bioprocess development, and particularly so in animal cell bioprocesses. CHO based processes dominate this field and account for 80% of animal cell derived biopharmaceuticals.[2] Ideally, process parameters are monitored in-line without sampling and breaching the sterile barrier of a cultivation vessel.[3] Physical parameters like pH, temperature, oxygen, and carbon dioxide partial pressure (pO_2, pCO_2) are measured with in-line sensors, resulting in a constant stream of (monitoring) data, which often enables automated control of the respective parameter. However, for the monitoring of equally important metabolic or cell-related parameters, in-line systems are still sparse. Several technologies have been developed and are extensively used in R&D, for example UV-, MIR-, and NIR-spectroscopy.[4] In particular, Raman-based in-line spectroscopy is a very promising tool for real-time in-process measurements. Furthermore, formerly off- or at-line operated methods can be combined with cultivation vessels via automated samplers, for example automated metabolite analyzers. Spectroscopy-based methods, in general, have the aforementioned advantage of being non-destructive and non-invasive. The possibility for the acquisition of multiple parameters with one measuring probe is another advantage, however complex chemometric data evaluation is necessary.[5] Nevertheless, very few of all those technologies have yet made it into a GMP regulated production environment as, for example, dielectric spectroscopy.[6]

In the case of glucose, it is still standard practice to withdraw samples from the bioreactor for offline time-delayed measurements, which are not suitable for real-time glucose control. Several sensors have been developed for continuous glucose measurements.[7] However, with very few exceptions,[8] these sensors have not outreached research levels.

We previously developed an optical glucose biosensor capable of in-line glucose measurements[9] and its manufacture was described in detail.[10] The ease of handling and disposable feature of this sensor enables integration into various cultivation systems for single-use glucose monitoring. In this study, we used the biosensor to set up an automated glucose feeding system for CHO cell culture. Sufficient nutrient levels during fermentation are not only crucial for cell growth and prolonged viability but for proper product glycosylation.[11–13] However, high consumption rates of glucose and glutamine typically lead to an overflow metabolism causing excess accumulation of metabolic waste products like lactate and ammonia, which in turn hamper cell growth and protein production.[14,15] Maintaining constant but low level of nutrients has been shown to render metabolism more efficient.[15] Also, the amount of advanced glycation end products (AGEs), which are unwanted by-products arising from the reaction of reducing sugars with a free amino group of a protein, can be minimized by lower glucose levels.[16,17] On the other hand, low glucose levels bear the risk of nutrient depletion, which can reduce the availability of intracellular nucleotide sugars that are precursors for the glycosylation process.[18] This eventually leads to reduced glycan site occupancy and complexity.[11] Protein glycosylation, in particular N-glycosylation, the predominant type of glycosylation, is critical for many important characteristics including solubility,[19] thermal stability,[20] antigenicity,[21] in vivo clearance,[22] secretion,[23] and bioactivity[24,25] of a protein. Therefore, variability in the glycan profile of biotherapeutics is a critical measure of protein quality and functionality.

In this study, we applied our recently developed glucose biosensor to generate an advanced glucose feeding system for mammalian cell cultures. Further, the effect of low and high glucose feed-back control on cell growth, viability as well as metabolism, protein production, glycosylation, and glycation were investigated for the protein darbepoetin alpha (DPO). We further validated our glycan analytics by conducting additional experiments at a lower temperature (31°C), which has been described in literature to cause a decrease in sialylation.[26]

DPO is a highly glycosylated protein with over 20 isoforms.[27] However, only six to eight isoforms remain in the final pharmaceutical product, because of their desired high content of sialic acidic groups, which is an important quality criterion for DPO. Therefore, the goal was to use continuous glucose monitoring and supply to assess different cultivation conditions without compromising product quality.

2 | MATERIALS AND METHODS

2.1 | Continuous glucose feeding system

Shake flasks (125 mL) with pH and oxygen sensors (PreSens GmbH, Regensburg, Germany) were equipped with a sampling port and an adjacent clave connector as a sterile barrier, feed port, air inlet/outlet filter and a glucose biosensor (Figure 1). The production and sterilization process of the oxygen-dependent glucose biosensor was performed as described before.[9] An automated glucose feeding system for shake flasks was established using the developed biosensor (Figure 1). The biosensor signal was transmitted via polymeric optical fibers (POFs) and processed by a four-channel oxygen meter (OXY-4 mini; PreSens GmbH). A pre-integrated reference oxygen sensor at the bottom of the flask allowed measurement of dissolved oxygen (DO) using a Shake Flask Reader (SFR) from PreSens GmbH. After an initial one-point calibration of the sensor, a concentrated glucose solution was supplied to the cell culture broth automatically depending on the measured glucose level. For feedback control experiments at 40 mM, a 0.5 M glucose feed solution (in PBS) was used and for feed-back control experiments at 0.8 mM, a 0.1 M solution was supplied. Our custom-made pump was controlled using LabVIEW (National Instruments, Austin, Texas, USA) for automated glucose addition. Glucose measurements were set to be performed every two to three min.

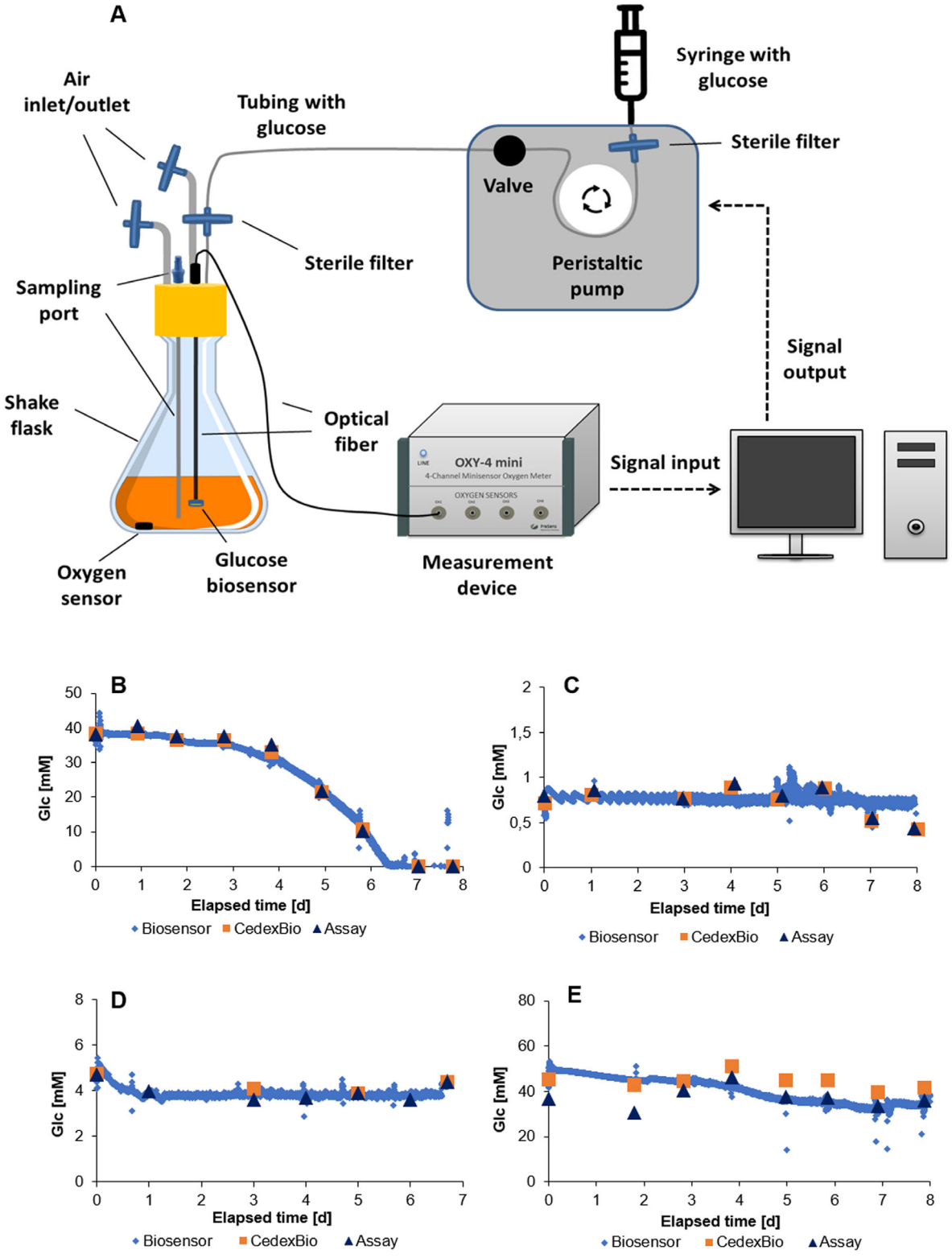

FIGURE 1 Automated glucose feed-back control in a shake flask. (A) A shake flask, equipped with an oxygen sensor, sampling port, feed port, air inlet/outlet filter, and a glucose biosensor is depicted. Signals from the glucose biosensor are transmitted via fiber optics to a measurement device (OXY-4 mini). The signal is processed with a LabVIEW-based program, which allows for the automated adjustment of glucose to defined levels by the regulation of a peristaltic pump and a valve. Glucose is pumped from a syringe through feeding tubes and two sterile filters into the shake flask. Glucose feed-back is exemplified with only one flask here. The actual set-up involved splitting of the glucose tubing downstream of the pump into four tubes with four valves. Four flasks can be monitored and controlled in parallel. In (B), the sensor signal of a batch experiment with 40 mM of glucose initially is shown. Fed-batch experiments with continuous glucose feeding were performed at 0.8 mM (C), 4 mM (D), and 40 mM (E) of glucose. Sensor signals were confirmed by two different periodical offline measurements (Cedex Bio (Roche); hexokinase kit (Sigma, Taufkirchen, Germany)). One-point calibrations on day 0 were performed without re-calibration at a later stage

2.2 | Cell cultivation

A DPO producing, suspension adapted CHO-K1 cell line was kindly provided by the Steinbeis Transferzentrum for Applied Biological Chemistry (Mannheim, Germany). For continuous glucose feeding experiments in 125 mL shake flasks, the cell inoculum was centrifuged and resuspended in fresh chemically defined medium (PowerCHO 2, Biozym, Hess. Oldendorf, Germany). The medium was supplemented with glucose, 4 mM glutamine and 0.4 mg mL^{-1} G418. The initial cell concentration was 2.5×10^5 cells per mL and the total volume 50 mL. Experiments were carried out with three different glucose feeding strategies; a batch experiment with 40 mM of glucose initially, and two constant control fed-batch regimes at low (0.8 mM) or high (40 mM) glucose concentrations. For each condition, triplicates or quadruplicates were carried out in parallel. 40 mM were chosen because it reflects the technical limit of our glucose sensor and also a high glucose concentration in commercially available cell culture media; 0.8 mM were chosen because lower glucose levels were reported to potentially negatively affect glycosylation.[12,28] The experiments with different glucose feeding were independently repeated with a new cell culture medium lot and a second batch of cryopreserved cells and were designated as second set of experiments. Cells were cultured at 37°C, 5% CO_2, 80% humidity and DO controlled at 80% air saturation on an orbital shaker set to 130 RPM (Lab-Therm LT-X Kühner, Basel, Switzerland). The pH value in flask experiments was maintained between 6.8 and 7.2 by manual addition of $NaHCO_3$. Additional experiments under hypothermic conditions were performed with a high cell density inoculum, $3–6 \times 10^6$ cells per mL, and cultivated at 31°C.

2.3 | Offline analytical methods

Periodically, samples were withdrawn for offline measurements. VCD and viability were measured with a ViCell analyzer (Beckman Coulter Inc., Brea, USA). Glucose, lactate, glutamine, ammonia, and pyruvate were measured with a Cedex-Bio metabolic analyzer (Roche Diagnostic GmbH, Mannheim, Germany). Glucose was also measured with a hexokinase kit (Sigma, Taufkirchen, Germany). In continuous glucose feeding experiments, the VCD was corrected for dilution effects due to the addition of glucose feed-solution.

2.4 | Specific rates of amino acids and darbepoetin alfa

The concentration of nine amino acids (ALA, LEU, SER, ASN, MET, PHE, GLN, HIS, TRP) was measured by gas chromatography (GC). We used the EZ:faastTM-Kit (Phenomenex, Aschaffenburg, Germany) and a ZB-AAA ZebronTM Amino Acid GC-column 10 m x 0.25 mm (Phenomenex, Aschaffenburg, Germany). The measuring range for each amino acid was determined, the samples adequately diluted, and three technical replicates were measured each. Specific rates were calculated according to the following equation;

$$q = \frac{\frac{[\Delta c]}{[\Delta t]}}{[(N_6 - N_3) / (lnN_6 - lnN_3)]} \quad (1)$$

Where Δc is the change in product / amino acid concentration during exponential growth (concentration between day 3 and 6; in (nmol mL^{-1}), Δt the time period in days, N_6 and N_3 the VCD on day 6 and day 3 in cells per mL, and ln the natural logarithm.

For the quantification of DPO production, a commercial ELISA (Quantikine IVD ELISA, Biotechne, Wiesbaden, Germany) was used. The ELISA was developed for the quantification of human erythropoetin (EPO) but could readily be used for the quantification of DPO and was performed according to the manual instructions. Specific DPO productivity (q_{DPO}) was determined according to the equation above.

2.5 | Isoform and N-glycan analysis

For the analysis of isoforms, DPO from the cell culture broth was purified in a single affinity purification step (Toyopearl AF-Blue HC-650 M, Tosoh Bioscience, Griesheim, Germany). The column was equilibrated with 20 mM Tris/HCl pH 6,9, 100 mM NaCl, 5 mM $CaCl_2$. DPO was bound and subsequently eluted with 20 mM Tris/HCl pH 6,9, 2 M NaCl, 5 mM $CaCl_2$, 10% (v/v) isopropanol. After that, the protein was concentrated and desalted with double distilled water using Vivaspin 20 concentrators (Sartorius, Göttingen, Germany) to a concentration of 5 mg mL^{-1}. Samples were microfiltrated (0.2 µm) and analyzed at pH 5.5 with capillary zone electrophoresis (CZE) using a 104 cm fused silica capillary (diameter: 50 µm; Agilent, Waldbronn, Germany) and a 7100 Capillary Electrophoresis System (Agilent, Waldbronn, Germany). The analysis was performed according to previously published methods.[29,30]

For the analysis of N-glycans using HPLC with high performance liquid chromatography (HPLC), purified DPO was concentrated to 5 mg mL^{-1} using centrifugal filters. N-glycans were released from DPO enzymatically using PNGase F (NEB, Frankfurt, Germany). 200 µg of DPO was denatured with 0.5% SDS (sodium dodecyl sulfate) and 40 mM DTT (dithiothreitol) for 10 min at 95°C, subsequently 3 µL of PNGase F were added to a final reaction volume of 80 µL, 1% NP-40 and a 50 mM sodium phosphate buffer at pH 6. In the case of a simultaneous digest with Sialidase S (Prozyme, Ballerup, Denmark), the digest was prepared as for the PNGase F digest, with 3 µL of Sialidase S in addition. The released glycans were separated from the protein using cold ethanol (3-fold volume) precipitation and labeled (Signal 2-AB plus Labelling Kit, Prozyme) with 2-aminobenzamide (AB). Excessive 2-AB label was removed via solid phase extraction (Glyko S Cartridges, Prozyme). For the analysis with hydrophilic interaction liquid chromatography (HILIC)-HPLC, the 2-AB labelled glycans were analyzed with an amide column, XBridge Glycan BEH Amide 130Å, 2.5 µm, 2.1 × 150 mm (Waters, Eschborn, Germany) using gradient elution, 20% buffer (80% acetonitrile) to 66% buffer in 56.62 min at a flow rate of 0.34 mL min^{-1}. Buffer (10 mM ammonium formate,

pH 4.45) and acetonitrile were premixed, keeping the ammonium formate concentration at constantly 10 mM. The column temperature was at 60°C, excitation was set to 330 nm and emission to 420 nm. For peak identification, glycan standards and the GU method (glycan units method) were used. Retention times were expressed relative to a 2-AB glucose homopolymer standard in glucose units.[31]

In the case of the analysis with strong anion exchange chromatography (SAX-HPLC), a simultaneous digest with Sialidase S was not performed during the preparation. The N-glycans were reconstituted in distilled de-ionized (DDI) water and analyzed with a SAX HPLC column (ProPac SAX-10 LC Column, 2 × 250 mm, Thermo Scientific). The 2-AB labeled N-glycans enabled fluorescence detection (FLD) at an excitation wave length of 260 nm and an emission wave length set to 430 nm. Chromatograms were processed by the Galaxie (Varian) software. Sample preparation, glycan analysis and quantification of the relative amounts of mono-, di-, tri-, and tetrasialylated glycans were performed by Biofidus Analytical Services (Bielefeld, Germany).

2.6 | Advanced glycation end products (AGEs)

For the analysis of advanced glycation end products (AGEs), a commercial competitive ELISA kit (OxiSelect AGE Competitive ELISA Kit, Cell Biolabs Inc. San Diego, USA) was used. The ELISA was performed according to the instruction manual. DPO samples were adjusted to a concentration of 2 mg mL^{-1} and compared to an AGE-BSA standard with concentrations ranging from 0.39–100 µg mL^{-1}. The absorbance of the AGE-BSA standard was plotted, fitted to a logarithmic function and the AGE level of the sample calculated accordingly.

3 | RESULTS

3.1 | Effects of different glucose feed-back control on cell growth and metabolism

An optical glucose biosensor was used to establish a custom-made automated glucose feeding system, depicted in Figure 1A. This system was tested for its ability to continuously monitor and maintain glucose levels at different concentrations (Figures 1C–E.) These figures show the comparison of the setup with a commercial at-line monitoring system (CedexBio) and with a manual hexokinase assay in maintaining glucose concentrations at specified levels. Figures 1 B to E show four typical biosensor profiles, with different glucose feeding strategies and offline reference measurements. (Figure 1C–E). On average, mean sensor deviations from offline signals (hexokinase assay) were between 5–15% (Figure 1C-E).

Having established a platform for continuous glucose monitoring and control, this system was employed to produce the protein DPO in CHO cells to assess different cultivation conditions without compromising product quality.

For this purpose, three different glucose feeding modalities were used and compared. A batch experiment with 40 mM glucose starting concentration, and two different continuous fed-batch modalities: one at 0.8 mM low glucose (LG) concentration, and the other at 40 mM high glucose (HG) concentration (Figure 2A). Cell growth and viability were determined in all three modalities by periodical offline measurements. Cell growth in the LG fed-batch experiment was lower than the batch and high glucose modalities (Figure 2B), which was also noticeable in a second set of experiments (Figure S1). Specific growth rates μ between day 3 and day 6 were 0.48 ± 0.035 day^{-1} and 0.46 ± 0.04 day^{-1} in the first and second set of the fed-batch LG experiments, respectively. In the first set of experiments, the growth rates in the batch and fed-batch HG cultures (μ = 0.51–0.53 day^{-1}) were not significantly higher, however, in the second set of experiments growth rates were significantly increased (μ = 0.59–0.64 day^{-1}) at higher glucose concentrations. The viability was comparable for all three glucose feeding conditions (Figure S1B). In all three experimental conditions, the pH value was manually controlled by the addition of base and was maintained close to pH 7.

The two sets of experiments from different inoculum cultures showed some differences in cell growth: In the second set, cell growth was generally lower but the duration of the experiment longer, i.e. cell viabilities stayed longer on a higher level (compare Figure 2 and Figure S1). Experiments were terminated when the viability dropped below 80% to avoid a potential negative impact of e.g. sialidases in the supernatant on product quality at lower viability.[12] In batch experiments, the process was terminated after 8 days, in the fed-batch LG experiments after 8 or 9 days, and in the fed-batch HG flasks after 7 or 9 days (Figure 2C and Figure S1B). Hence, cell culture duration varied but most likely not due to differences in the glucose feeding strategy.

Next, several metabolites and amino acids were measured in the three modalities by gas chromatography. At low glucose feed-back control, an increase in the consumption rate of seven amino acids was observed (Figure 2D), including glutamine. This increased consumption of amino acids in the low glucose branch correlated with an increased rate of ammonia production (Figure 2E) indicating a metabolic shift and utilization of amino acids in energy metabolism. Alanine was produced at similar levels in all three different glucose feeding experiments. Lactate was produced at lower rates in the fed-batch LG experiment compared to the batch and fed-batch HG flasks. The reduced level of lactate under low glucose feeding was significant ($p < 0.05$) in the second set of experiments (Figure S1D). Absolute lactate concentrations did not exceed 20 mM (data not shown).

Interestingly, specific productivity and absolute darbepoetin alfa concentration were higher in the second set of experiments (Figure 2F), where cell growth was generally lower. Consistent with the specific protein productivity, no significant or reproducible changes in absolute protein concentrations were observed in any of the three modalities. (Figure S3B).

3.2 | Investigating the glycosylation and glycation of darbepoetin alfa

The glycosylation pattern of DPO was analyzed using two orthogonal methods, capillary zone electrophoresis (CZE) and high-performance

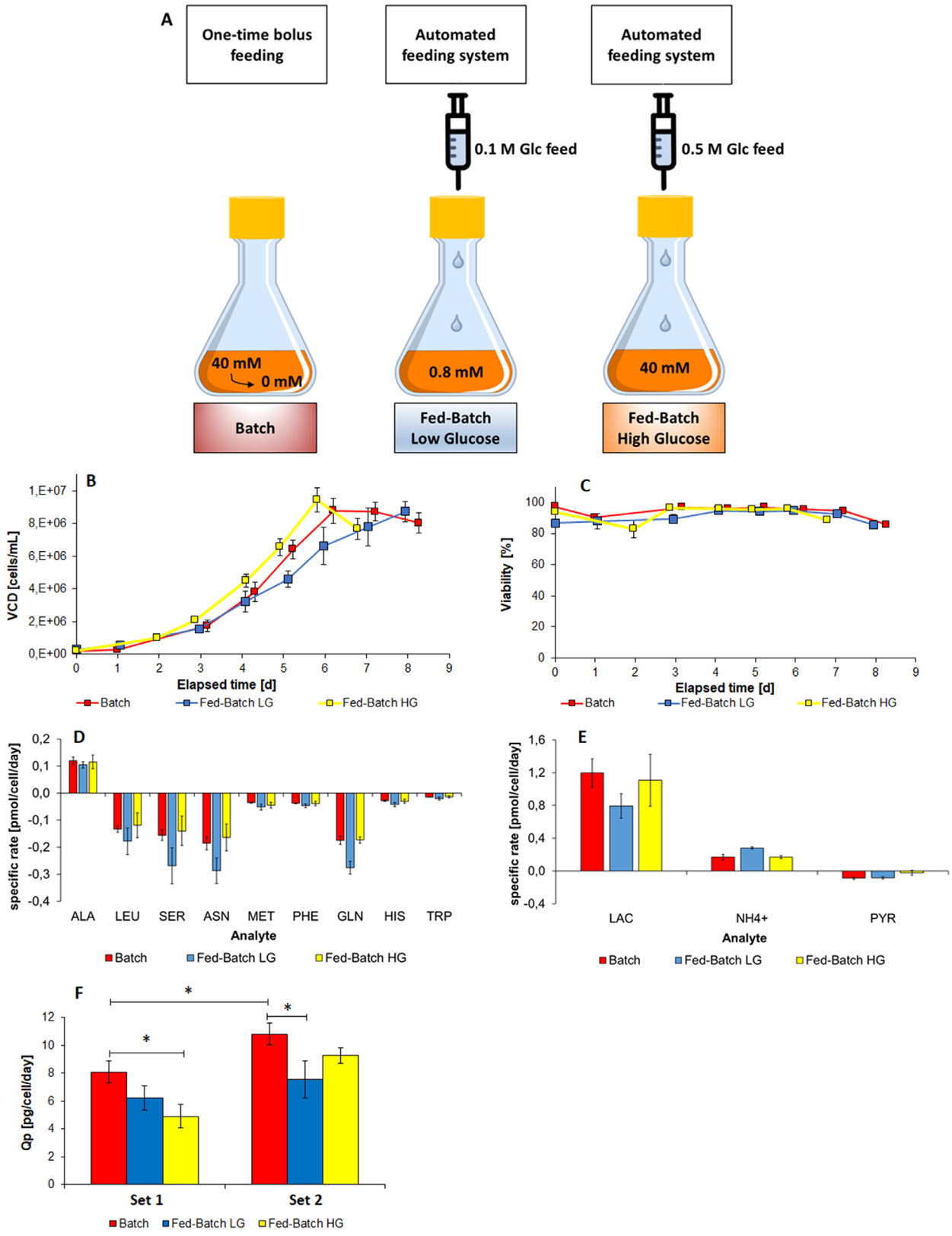

FIGURE 2 Continuous glucose feeding during first set of experiments. (A) Experimental set-up of the shake flask experiments with different glucose feeding strategies. Batch cultivations were compared to fed-batch experiments at low or high glucose control (LG, HG). For the viable cell density (VCD) (B) and viability (C), mean values of $N = 3$ or $N = 4$ biological replicates were plotted with the error bar corresponding to the standard deviation. Cell counts (VCD) in the fed-batch experiments were corrected for dilution effects. For the specific rates (D and E) error bars correspond to the variation of $N = 3$ technical replicates. In (B–E), data from the first set of experiments is shown. In (F) specific DPO production of both sets of experiments are displayed as bars that correspond to means of three biological replicates (flasks). The error bars correspond to the standard deviation. $^*p$-value < 0.05; two-sided t-Test, unpaired

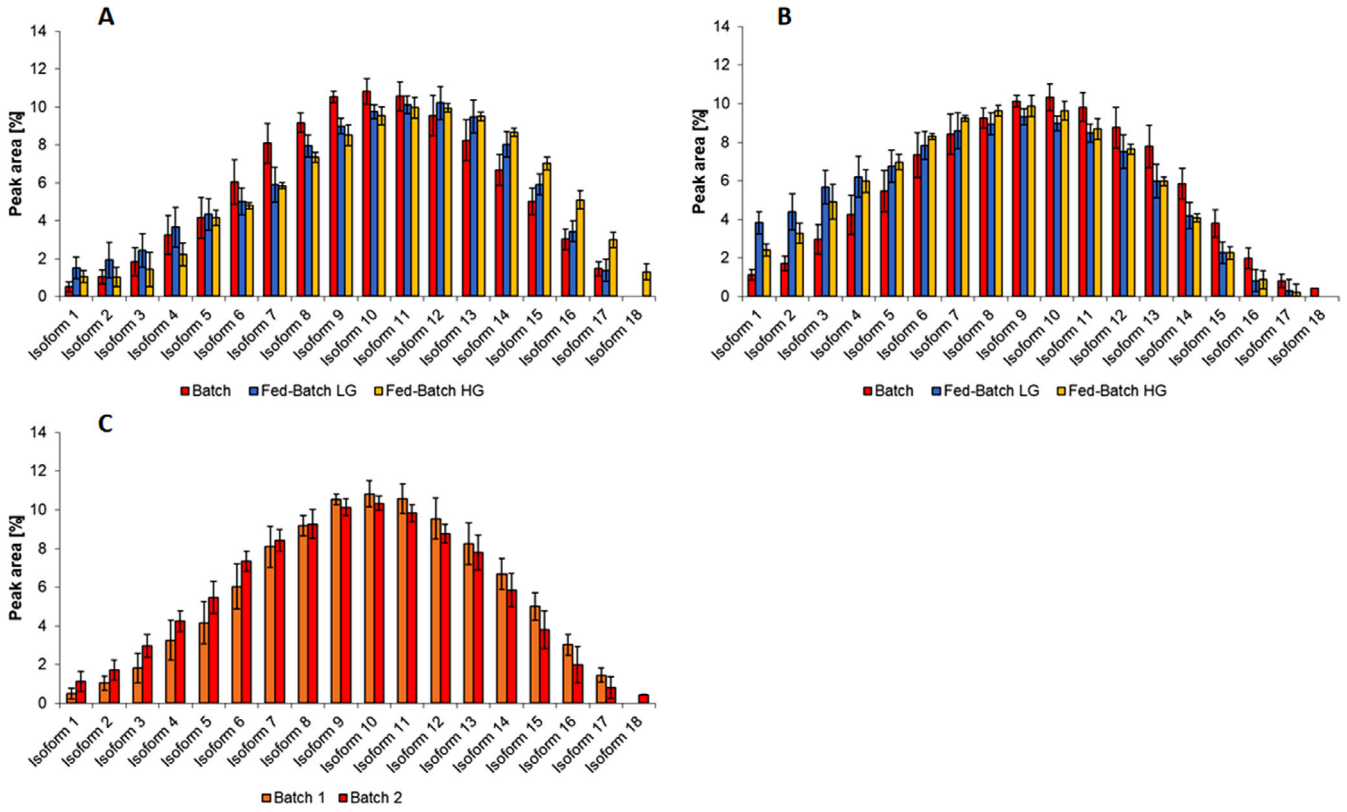

FIGURE 3 Isoform distribution of acidic DPO. DPO isoforms measured with CZE were plotted using relative peak areas of the electropherograms. DPO isolated from the different glucose feeding experiments (batch, fed-batch low and high glucose control) were compared. In (A) samples from the first set of experiments is shown, in (B) samples from the second set of experiments. In (C), the DPO isoforms from batch flasks of both experimental sets were plotted in one graph for direct comparison. Means of $N = 4$ biological replicates, for fed-batch HG flask $N = 3$, were plotted with the error bar corresponding to the standard deviation

liquid chromatography (HPLC). CZE analyzed the distribution of the intact glycoprotein isoforms, which differ in the degree of sialylation and therefore can be separated due to their varying charge. In the first set of experiments using different glucose feeding modalities, a slight shift in the DPO isoform distribution was observed (Figure 3A). DPO isoform 10 was the predominant type purified from batch flasks, whereas isoforms 11 and 12 were most abundant in both LG and HG fed-batch experiments. Under HG conditions, the relative abundance of isoforms 15–17 was significantly higher ($p < 0.05$) than in LG flasks and batch flasks (Figure 3A). However, in the second set of experiments, this effect could not be reproduced (Figure 3B); instead, isoform distribution appeared uniform. With LG feed-back control, the abundance of less acidic DPO isoforms (1–3) was slightly elevated in contrast to batch flasks and fed-batch HG flasks. When comparing batch flasks of both sets of experiments, it became apparent that the variance in DPO isoform distribution was rather small even though cell growth showed differences between the two sets of experiments (Figure 2, Figure S1).

In an additional experiment, DPO isoforms produced under hypothermic conditions were investigated. The reduction of the cultivation temperature to 31°C at constant glucose levels (1, 5, and 8 mM) led to elevated appearance of lower, less acidic DPO isoforms (Figure S2).

To validate the findings from CZE, SAX-HPLC was performed for glycan analysis. For the SAX-HPLC analysis, N-glycans were enzymatically cleaved from DPO using PNGase F. In the first set of experiments, the relative amount of mono- and disialo-N-glycans was slightly higher in the batch culture as compared to both fed-batch cultures (Figure 4A). Accordingly, tri- and tetrasialo glycans were less abundant in batch cultures than in both fed-batch cultures (high and low glucose control). However, DPO N-glycans from batch cultures of the second set of flask experiments showed the opposite trend (Figure 4B). At hypothermic conditions, the level of monosialylated N-glycans increased by 15–20 %, whereas the level of tri- and tetrasialylated N-glycans decreased by 15–20% (Figure 4C).

In addition to SAX-HPLC, HILIC-HPLC was performed. Here, the terminal sialic acids were removed from the cleaved N-glycans by incubation with sialidase S. Different glucose feeding regimes and hypothermic conditions revealed only marginal differences comparing the N-glycan profile (Figure 4D).

The glycation of isolated DPO was measured with a commercial competitive ELISA kit. The glycation was < 0.2 % and did not differ markedly between the samples (Figure S3A).

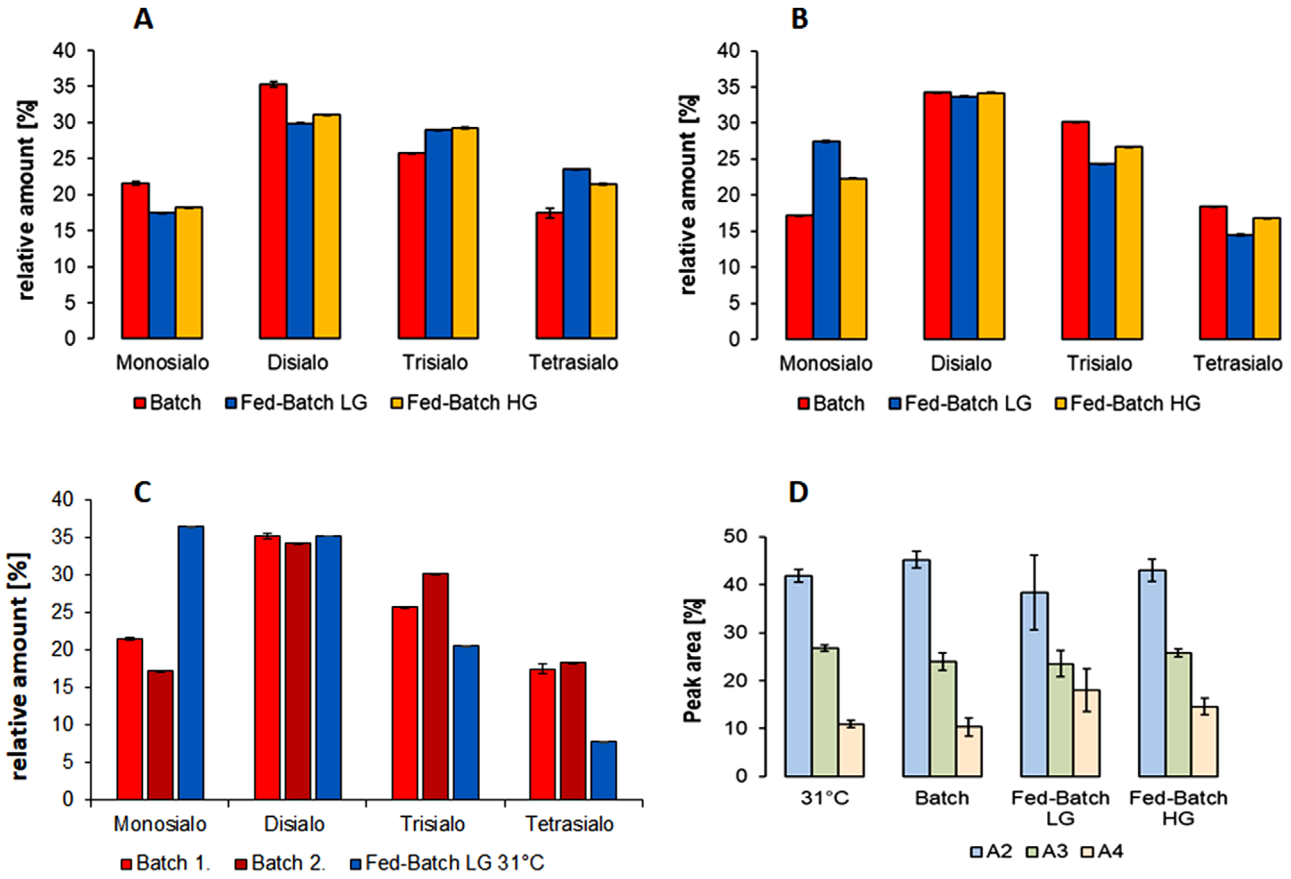

FIGURE 4 Comparison of DPO N-glycan profiles using SAX and HILIC-HPLC. The relative amount of DPO N-glycans from the first set (A) and second set (B) of flask experiment is shown. N-glycans were grouped into mono- di-, tri-, and tetrasialylated N-glycans. In (C) relative amounts of N-glycans from the two sets of batch experiments are compared to DPO N-glycans from a culture at low glucose feeding (1 mM, Fed-Batch LG) and hypothermic conditions (31°C). Each bar corresponds to the mean of two technical replicates from one biological sample, and the error bar corresponds to the standard deviation. Biofidus AG Analytical Services performed the SAX-HPLC measurements in (A–C). In (D), the terminal sialic acid from the first set of experiments was cleaved from the N-glycans and analyzed using HILIC-HPLC. DPO N-glycans obtained from different processes were quantified using relative peak areas. Glycans were categorized into diantennary (A2), triantennary (A3), and tetraantennary (A4) structures. The means of $N = 3$ biological replicates ($N = 2$ for 31°C) were plotted with the error bar representing the standard deviation

4 | DISCUSSION

4.1 | Continuous glucose feeding in cell culture bioreactors

A major drawback in the case of glucose monitoring is a lack of robust and reliable in-line probes, therefore standard practice is sampling from the cultivation vessel and subsequent at-line or off-line analysis. In this study, we demonstrated the applicability of an in-line glucose biosensor, which was recently developed for mammalian cell culture bioprocesses.[9] Using the biosensor in combination with an automated feeding system enabled us to control glucose at very low levels (< 1 mM), which would not have been possible with discontinuous manual bolus feeding. The main advantage of the optical glucose biosensor used here is the easy-to-use plug-and-play principle, which does not necessitate complex chemometric data evaluation as in the case of spectroscopic methods.

Several systems for on- and in-line glucose measurements have been presented in the past. Nevertheless, with respect to chemometry-free, enzymatic/amperometric measurements, very few alternatives to offline measurement exist: Trace Analytics (Braunschweig, Germany) offers glucose monitoring using immobilized enzyme technology connected to the bioreactor by sets of tubing/pumps and dialysis or filtration probes in an online configuration whereas C-CIT Sensors AG develops in situ enzymatic glucose sensors.

Optical systems pose the advantage of being non-invasive, and therefore Raman spectroscopy-based probes are promising for the in-process control of nutrients and metabolites.[17] Berry and colleagues reported an average percent signal error of 13.3% at a set point of 12.5 mM glucose when comparing predicted values to actual offline measurements,[17] which is comparable to the results obtained with the glucose biosensor used in this study. However, the group discovered several challenges with their probe. Ambient room light leaking into the reactor caused spectral interference, and the signal errors typically increased at a later stage during culturing. An increasing level of

cell debris and the accumulation of waste products were found to be the predominant reasons for the growing sensor deviation over time [17)]. The observed sensor deviations during this study were due to a slow enzyme activity loss.[9] This error can be easily compensated by a one-point-recalibration, which is usually only required after more than a week of continuous measurement and was therefore not necessary during this study.

4.2 | The effect of continuous glucose feeding on cell growth, metabolism and protein production

In this work, we employed the sensor in a case study with DPO producing CHO cells. In these cells, the accumulation of lactate can be reduced significantly when using a low glucose feed-back control at 0.8 mM compared to batch and fed-batch feeding at high glucose levels. This finding is in accordance with other reports in the literature and of particular interest given that excessive accumulation of lactate is known to hamper bioprocess performance, causing inhibition of cell growth and protein production at high concentrations.[32,33] At low glucose feeding levels, significantly more glutamine was consumed and more ammonia produced compared to the batch and fed-batch HG experiments. The increased uptake of glutamine and other amino acids under glucose limitation has been attributed previously to the increasing demand of the cell to replenish TCA cycle intermediates.[34] With respect to this effect, particularly the consumption rate of amino acids like glutamine, serine, histidine, methionine, phenylalanine and leucine has been described to increase in CHO cells at glucose concentrations below 0.54 mmol L^{-1},[28] which is in accordance with our findings. The differences between the two sets of experiments were most likely due to the fact that different cell bank vials and medium lots were used. Such "batch effects" are unfortunately not unusual in cell culture and are one of the reasons for independent experiments.

4.3 | The effect of continuous glucose feeding and temperature on protein quality

The complete analysis of DPO and DPO-released N-glycans revealed no substantial differences in our three glucose-modalities, while a reduced cultivation temperature markedly changed the N-glycan pattern. The latter is in accordance with other reports in the literature.[26,35]

In most studies where glucose shortage was responsible for incomplete or aberrant protein glycosylation, discontinuous feeding strategies were employed.[18,36] In contrast to such previous studies, our glucose biosensor enabled the maintenance of glucose levels at constantly low levels, without intermittent periods of starvation. Surprisingly and contrary to reports for MABs,[18,36] we found that in our case, the DPO isoforms produced with continuously low glucose (< 1 mM) control were not substantially different compared to those produced at either single feed (batch) or at continuously high glucose concentrations (~40 mM), suggesting that either the cell used here is non responsive to different glucose levels with respect to product quality or that the discontinuous nature of glucose feeding in previous studies contributed to alterations in product quality.

In our first set of experiments, an increased level of higher sialylated N-glycans in (particularly high glucose) fed-batch cultures was measured by SAX-HPLC and CZE. Conversely, the second set of experiments showed a trend towards higher isoform distribution for batch cultures. Because trends observed with CZE analysis were consistent with SAX-HPLC analysis, one can conclude that the variation of DPO glycosylation comparing both sets of flask experiments is largely due to variability in the cell culture processes rather than analytical inconsistencies. In this case, a different batch of cryopreserved cells might be the cause of the observed differences in sialylation, since other parameters like cell growth, pH and the levels of the metabolic waste products lactate and ammonia did not show significant correlations with the degree of DPO glycosylation.

Contrary to SAX-HPLC, the analysis of asialo-N-glycans by HILIC-HPLC revealed uniform patterns, even under hypothermic conditions. This indicated that the observed shifts of DPO CZE isoform patterns in hypothermic experiments were due to differences in sialylation rather than due to changes in glycan antennary.

Combining both analysis methods, HILIC and SAX-HPLC, one can confirm whether glycan antennarity or sialylation is the limiting factor for the final protein sialylation. In our study and in contrast to,[28] the overall amount of N-glycan antenna seemed more limiting for DPO sialylation than sialylation per se on the level of microheterogeneity.

It has been shown that feeding at low glucose concentrations with a glucose feedback control system can reduce protein glycation.[16,17] In our case, the proportion of DPO glycation relative to the amount of total DPO was determined by an ELISA, developed to quantify advanced glycation end products (AGEs). The level of glycation was very low (< 0,2%) in all processes irrespective of the glucose feeding strategy, thus no significant improvement could be achieved using continuously low glucose levels.

Taken together, no effects on the product quality with respect to glycation, glycosylation or sialylation patterns were observed using low glucose feedback control, arguably because glucose starvation was prevented. The product quality under hypothermic condition, however, was decreased. The effect of mild hypothermia on product quality is cell line, protein and temperature dependent see e.g.[37] Often positive but also negative effects were reported. Sou et al. found a decreased proportion of more processed glycan structures in a monoclonal antibody at mild hypothermia;[38] Trummer et al.[35] describe reduced sialylation levels of an EPO-FC fusion protein at lower temperatures.

5 | CONCLUSION

In summary, our novel in-line glucose biosensor proved to be easily applicable in standard cell cultivation vessels. It does not need any chemometric data evaluation. Deviations to offline measurements were small (5–15%) when sensors were operated at glucose levels

0–40 mM. The optical glucose biosensor is a single-use device and can be disposed of after use, making it attractive for the growing market of single-use bioreactors.

In the present study, lactate production was reduced when applying a low-level feed-back control of glucose. However, the DPO-producing CHO cell line used in this study was not sensitive to lactate levels (20 mM) that accumulated at higher glucose feeding rates, and thus no influence on specific productivity was observed. Nevertheless, the set up would be highly advantageous for systems that are negatively affected by lactate accumulation. Glycation levels were below 0.2% under all conditions, not reaching critical numbers even at high glucose feeding rates. Effects on the sialylation of darbepoetin alfa was observed under hypothermic conditions (31°C), but not under continuously controlled glucose concentration at a low or a high glucose level. To conclude, the glucose control system presented here can open up possibilities of feed regime and process optimization without hampering product quality.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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