Application of the Bradford Assay for Cell Lysis Quantification: Residual Protein Content in Cell Culture Supernatants

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Frequently measured mammalian cell culture process indicators include viability and total cell concentration (TCC). Cell lysis, an additional important process characteristic that substantially contributes to the overall product purity profiles, is often not addressed in detail. In the present study, an inexpensive and simple application of the Bradford assay is developed to determine the residual protein content (RPC) in cell culture supernatants. The reliability and reproducibility of the method are tested in a long-term study and compared with lysis quantification via the DNA measurement. The results show that its performance is more robust and accurate over time and the respective concentration range. Additionally, both methods are used for cell lysis process monitoring in a recombinant Chinese hamster ovary fed-batch process. In the presented process, by applying the established assay, the lysis rate \( k_{\text{CL}} \) is determined to be constant over time at \( 4.6 \times 10^{-4} \) lysed cell concentration (LCC) per TCC and time (LCC/TCC/h). In contrast, DNA data did not confirm the constant lysis rate due to variations of the content per cell during cultivation. Thus, information on the RPC can facilitate the determination of the optimal harvest time point with respect to purity and in improving process characterization.

1. Introduction

Total cell concentrations (TCCs) and viable cell concentrations (VCCs) are typical process performance indicators for mammalian bioprocesses. These, their time derivatives, and their respective rates are commonly used to describe the state of bioprocesses. Viability is a commonly derived key process indicator used to illustrate the vitality status of cell culture processes, but it is dependent on the magnitude of the lysis rate.\(^{[13]}\) However, cell lysis and the amount of host cell proteins (HCPs) in the culture broth are often not determined during cultivation although these variables significantly affect further process steps.\(^{[2]}\) Consequently, the amount of living, dead, and lysed cells must be considered to achieve a comprehensive picture of the process.

The lysis of a mammalian cell is defined as the loss of an intact membrane and the release of the intracellular content. Therefore, lysed cells cannot be directly detected via cell counting methods. Thus, they are usually indirectly measured through the detection of the released internal constituents, such as DNA,\(^{[3,4]}\) lactate dehydrogenase,\(^{[3]}\) or via the detection of cell debris.\(^{[10]}\) The change in rheological characteristics represents an additional opportunity to account for cell lysis;\(^{[11,12]}\) however, this is only applicable for microbial systems or high cell density cell culture systems.

An additional lysis research opportunity involves the detection of HCPs in the supernatant.\(^{[13]}\) Although the HCP content is regarded as a critical quality attribute of the final product,\(^{[14]}\) the implementation of HCP as a routine process variable in mammalian cell culture has not been reported to date. Nevertheless, for integrated or continuous processes, monitoring and control of HCP content could be of particular importance,\(^{[15]}\) such as in avoidance of fouling or blocking in chromatography resins\(^{[16,17]}\) during downstream operations.

Enzyme-linked immunosorbent assay (ELISA) is a common method used to quantitatively determine the HCP content of a given sample though it lacks coverage of the complete spectrum of HCPs and is likely to miss weak or nonimmunogenic species.\(^{[18,19]}\) Moreover, this procedure is costly and can result
in a huge work, particularly when many samples need to be analyzed. The application of ELISA for the routine tracking of HCP generation during a bioprocess to uncover cell lysis can be regarded as not economically feasible. This is also true for 1D- or 2D-polyacrylamide gel electrophoresis or any type of mass spectrometry-based methods.[14]

In the present work, we describe a simple and inexpensive Bradford assay method to determine the HCP content in culture supernatants, which is referred to as residual protein content (RPC). The present study provides critical considerations regarding the reliability and precision of the developed RPC method in comparison to the standard DNA technique to estimate cell lysis. Furthermore, we demonstrate that RPC data allows the identification of the process state and provides additional information for the understanding and modeling of the process.

2. Experimental Section

2.1. Fed-Batch Process

For the fed-batch model process, a recombinant monoclonal Chinese hamster ovary (CHO) cell line (The Antibody Lab GmbH, Austria) producing an anti-tumor necrosis factor-α (TNF-α) immunoglobulin G1 (IgG1) was used. The cell line was generated by applying the Rosa26 bacterial artiﬁcial chromosome expression strategy[20] to a serum−/−free adapted host cell line derived from CHO-K1 (American Type Culture Collection [ATCC] CCL-61). A working cell bank, where each vial contained 5 × 10^6 cells, served as the starting material for all experiments. Cells were thawed in chemically deﬁned culture medium (Dynamis AGT, A26175; Thermo Fisher Scientiﬁc, USA) supplemented with 8 mM 1-glutamine (25030081; Sigma-Aldrich, Germany), 3 mM L-1 phenol red solution (RNBD642; Sigma-Aldrich, Germany), 1:1000 anti-clumping agent (0010057DG; Thermo Fisher Scientiﬁc), and 1 mg mL⁻¹ G418 (G8168; Sigma-Aldrich, Germany).

Subculturing was performed three times (every third or fourth day) using the aforementioned media without any anti-clumping agent and G418. Fed-batch experiments were performed in shake flasks (#431147; Corning, USA) with a starting volume of 300 mL and an initial cell density of 2.5 × 10^6 cells per mL. The medium for the batch was additionally supplemented with 0.1% v/v antifoam C (A8011; Sigma-Aldrich, Germany) to mimic the typical large-scale cultivation conditions. A 2D (glucose and process temperature), three-stage, factorial design of experiments was used to capture the design space. To the feed medium, 0.1% antifoam (CHO CD EfficientFeed A AGT Kit, A1442002; Thermo Fisher Scientiﬁc) was added along with 10 g L⁻¹, 20 g L⁻¹, or 30 g L⁻¹ glucose (G7021; Sigma-Aldrich, Germany). Pulse feeding started at day 3 and lasted until day 13, which involved a linear pulsed feed rate with a total added volume of 33% v/v with respect to the end volume. The process starting temperature of 37 °C was changed at day 4 to 31 °C or 34 °C, or kept constant at 37 °C. Sampling was performed once per day, and the experiments were terminated after cell viability dropped below 70%. All cultivations were conducted in a humidified CO₂ incubator (Heracell VIos 160i; Thermo Fisher Scientiﬁc) with 5% v/v CO₂ in ambient air and at 200 rpm on an orbital shaker (MaxQ 2000 CO2 Plus; Thermo Fisher Scientiﬁc). For the mock control experiment, the serum-free host cell line was cultivated at a constant temperature of 37 °C using the feed containing an additional 30 g L⁻¹ glucose. Further fed-batch conditions were as previously described.

All cell culture experiments were performed in duplicate.

2.2. Cell Cycle Staining

Two samples of 1.5 × 10⁶ cells from the cell broth were taken and centrifuged (10 min, 180 × g); and the pellets were washed two times with 1.0 mL of phosphate-buffered saline (PBS) (91501; Roth, Germany). The remaining cell pellet was dissolved in 1.0 mL of ice-cold 70% high-purity ethanol, added dropwise. The fixed cells were stored at 4 °C.

Prior to analysis, the samples were centrifuged and washed twice. Then 1.0 mL of Tris buffer (T3253; Sigma-Aldrich, Germany) was added dropwise. In the last step, cells were resuspended in 500 μL of Tris, including 1 μg mL⁻¹ 4,6-diamidino-2-phenylindole (DAPI) (10236276001; Sigma-Aldrich, Germany), incubated for 30 min, and subsequently measured using the Gallios Flow Cytometer (B43620; Beckman Coulter, USA) (FL9, number of events is equal to 10000). The percentages of cells in the G1/G0 and G2/M phases were determined by calculating the respective peak areas.

2.3. Cell Lysate Generation

For the cell lysate generation, a fed-batch process with the producing cell line was carried out as described in the previous section. The process temperature was set to 34 °C, and the feed with an additional 20 g L⁻¹ glucose was used. In the stationary phase (day 12), a sample was drawn for lysate generation. A total of 1.5 × 10⁶ cells were washed with PBS and resuspended in 0.7 mL radioimmunoprecipitation assay (RIPA) lysis buffer (20-188; Merck, Germany) supplemented with an ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor (COED-TAF-RO Roche; Sigma-Aldrich, Germany). The solution was then incubated for 1 h at 4 °C, centrifuged at 8000 × g for 10 min; the supernatant was stored overnight at 4 °C.

2.4. Antibody Titer Quantification

The antibody titer was determined via bio-layer interferometry (BLI) using the Octet system (Octet QK; Pall ForteBio, USA) with protein A tips (18-5010; Pall ForteBio). For sample preparation, the culture broth was centrifuged at 180 × g for 10 min at room temperature. The supernatant was stored at −20 °C until measurement. All dilutions were performed in PBS with 1% Tween 20 (P2287; Sigma-Aldrich, Germany). The binding to protein A was measured at 30 °C, and the resulting binding rate was calculated using Octet data analysis software 6.4 (Octet QK; Pall ForteBio). Sample readings were quantified using an IgG calibration curve in concentrations ranging from 10.0 µg mL⁻¹ to 50.0 µg mL⁻¹.

2.5. Residual Protein Content

The RPC in the bioprocess supernatants of CHO cells was determined using the Bradford assay (B6916; Bio-Rad Laboratories, USA) and the absorbance of Coomassie brilliant blue G
(CBB) was measured at 595 nm. Sample preparation was performed as previously described for the titer quantification. Measurement was performed using a plate reader (Infinite M1000; Tecan, Switzerland) in 96-well plates (P7366; Nunc, USA). Bovine serum albumin (BSA) (5000002; Bio-Rad Laboratories) and IgG (Lot-No. 046M4855V, 12511; Sigma-Aldrich, USA) were measured in a concentration range of 81.0–520.0 µg mL\(^{-1}\) and 40.6–325.0 µg mL\(^{-1}\), respectively, along with the analysis of each unknown samples.

2.6. DNA Assay

Double-stranded DNA (dsDNA) concentration in the fermentation supernatants of CHO cells was determined by fluorescence measurements using Picogreen dsDNA reagent (P7581; Thermo Fisher Scientific). The dye was excited at 480 nm, and emissions were measured at 520 nm. All dilutions were performed in Tris-EDTA buffer (10 mm Tris-HCl, 1 mm EDTA, pH 7.5). Two different DNA standards were used: Lambda DNA standard (P7589; Thermo Fisher Scientific) and Calf Thymus DNA standard (1702482; Bio-Rad Laboratories). A calibration curve was recorded for concentrations ranging from 13.13 ng mL\(^{-1}\) to 1680 ng mL\(^{-1}\). Measurements were performed using a plate reader (Infinite M1000; Tecan) in a 96-well black bottom plate (NNC#237108; Thermo Fisher Scientific).

2.7. Statistics

Statistical analyses were performed using SigmaPlot13.0 software (Systat Software Inc., USA). Residual distributions were tested on normality (Shapiro–Wilk test) and on constant variance (Levene’s median test). The confidence bands, prediction bands, and variances were calculated using the delta method.'

The mean deviation of the parameters \(k\) and \(d\) was calculated according to Equation (1):

\[
\text{mean deviation(\%)} = \frac{x_{\text{target}} - x_{\text{actual}}}{x_{\text{target}}} \times 100
\]  

3. Results and Discussion

3.1. RPC: Method Development and Evaluation

A recombinant CHO-K1 cell line consists of roughly 70 wt% protein,\(^{[22]}\) which is released when a cell undergoes lysis. For the measurement of the RPC in cell culture supernatants of recombinant cell lines cultivated in serum-free media, the secreted recombinant product must be considered. It is well known that the CBB dye interferes differently depending on the protein composition.\(^{[24]}\) In the present study, we used a set of experiments to elucidate this interference.

The cell lysate from the producer cell line was obtained by incubating in the RIPA lysis buffer; the RPC was determined using the Bradford assay. The cell lysate contained 676 µg mL\(^{-1}\) protein on average. This corresponds to 315 \(\pm\) 16 pg protein per cell, which is fairly a reasonable value, in the view of the reported values for mammalian cells such as 180 pg per cell for HEK293T,\(^{[24]}\) 246 pg per cell for a producing CHO-K1 cell line,\(^{[22]}\) and 410 pg per cell for Madin–Darby canine kidney cells.\(^{[25]}\) A recent study even >500 pg of protein per cell was reported for a CHO cell line, which was cell size-dependent.\(^{[25]}\)

Figure 1. A) Absorbance of the BSA standard (•) and polyclonal IgG standard (▼) as a function of the respective concentration (n = 10). Measured triplicates of the total protein content of a cell lysate (○) and the anti-TNF-α reference standard (△). Linear function is depicted. B) Linear regression of the calculated BSA value versus the theoretical values of different spike-in IgG/BSA mixtures (n = 7).
The binding of CBB to proteins obeys the Lambert–Beer law.[26] Accordingly, the absorbance $A$ of a sample is related to its concentration and the path length. As more species are present, the total absorbance $A$ can be described as a linear function of each individual absorbance $A_i$ (Equation (2)):

$$A_{\text{total}} = A_{\text{RPC}} + A_{\text{IgG}} + A_{\text{Blank}}$$  (2)

This implies that the absorbance of the residual protein ($A_{\text{RPC}}$) content should be quantifiable by subtracting the medium ($A_{\text{Blank}}$) and the IgG blank ($A_{\text{IgG}}$), derived from the BLI measurements and recalculated using the extinction factor, from the total absorbance value. The residual protein concentration is then subsequently calculated via the BSA calibration.

To support this relationship, different sets of BSA/IgG mixtures were measured (Figure 1B). The experiment contained 45 measurements in total. The IgG concentration was set from 40 µg mL$^{-1}$ to 218 µg mL$^{-1}$, and 81 µg mL$^{-1}$ to 280 µg mL$^{-1}$ for BSA. The absorbance values ranged from 0.15 AU to 0.62 AU, respectively. The BSA concentration was recalculated after subtraction of the resulting absorbance value of a given IgG concentration from the total absorbance (calibrations used are depicted in Figure 2A). The slope of calculated and added concentrations of BSA was 1.04, representing a good recovery of the added BSA. The coefficient of variation (CV) had an average of 8%, an initial indicator for the error of this analysis.

Matrix effects were not observed. Different concentrations of BSA, from 65 µg mL$^{-1}$ to 130 µg mL$^{-1}$, were spiked into two host cell culture supernatants, from the beginning of fed-batch and from the harvest time point, respectively. The recovery was 94.6 ± 9.2% ($n = 18$).

Furthermore, the media and feed used exhibited the same absorbance value as the PBS buffer. As expected, smaller peptide molecules and single amino acids did not have any impact on the CBB absorbance.[23]

The experiments conducted demonstrated that the total absorbance can be separated into two absorbance attributes, which are linearly related (Equation (2)). As a result, the absorbance of a known substance, calculated via its extinction coefficient, can be subtracted from the total absorbance to obtain the concentration of remaining constituents.

### 3.2. RPC: Long-Term Assessment

The consistency of this method was tested in a long-term study of over two years by three different operators. For each assay, a set of standards was measured and a linear regression was performed. The limit of quantitation (LOQ) and limit of detection (LOD) were calculated using the standard deviations of the blank responses and were 70.9 µg mL$^{-1}$ and 23.4 µg mL$^{-1}$, respectively.

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**Figure 2.** A) Obtained mean deviation from the slope $k$, B) the intercept $d$ derived from the BSA standard measurements, and C) the mean deviation to the target concentration at 0.45 AU, calculated according to Equation (3) during a time course of 690 days. The solid line represents the average value. The gray dashed line and the dashed dot line represent 95.4% and 99.7% of the sample population ($n = 10$), respectively. The mark “a” indicates where the operator had changed. D) Maximal error as a function of the measured absorbance, which could be obtained through the deviations of $k$ and $d$. 

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The mean deviations (see Equation (1)) of the resulting values of the slope \( k \) and intercept \( d \) were monitored throughout the study (Figure 2A,B). The first ten values were taken to calculate the average and standard deviation of those parameters. If \( k \) or \( d \) was outside of the value \( \bar{y} \pm 3\sigma \), 99.7% of the sample population was within, the assay was discarded. Consequently, the method was stable and robust over the time course. No obvious trends occurred that could indicate any altering effects of the standard or the CBB dye. Also, the change in the operator had no effect on the analysis performance.

While \( k \) resulted in low variation, \( d \) exhibited larger deviations; however, considering the absolute values it did not affect the measurements to a great extent. Therefore, the possible impact on the determination of the variances of \( k \) and \( d \) was evaluated. This was done by the addition of the absolute deviation of \( k \) and \( d \) to the linear function and then calculating the concentration for different absorbance values along the calibration range (Equation (3)). The resulting value was then substituted into Equation (1) to assess the mean deviation compared to the target concentration \( x_{\text{target}} \):

\[
(x_{\text{target}} + \Delta x) = \frac{y_{\text{target}} - (d + \Delta d)}{k + \Delta k}
\] (3)

For the limits of the chart plots, the first ten assays were again taken to gather a statistically meaningful population for determining the average and standard deviation. The resulting values can be considered as having maximal possible error that could occur if the stated calibration was invalid (Figure 2C,D).

For most of the calibration range, the error was below 20% (Figure 2D). The intercept \( d \) affects the determination in the low-concentration region though the maximal possible error at higher absorbance values is dependent on the slope \( k \). At low concentrations, higher variation had to be accepted. Therefore, when a larger quantity of protein is in the supernatant, particularly at a later stage of a process, the method is more accurate. At the beginning of a bioprocess or during the batch phase, RPC results with higher variances could be estimated.

Error propagation within this methodology can be obviated, since the antibody titer quantification via the BLI is a robust and reliable procedure. The standard prediction error of this measurement was in the range of \( \pm 9.096 \mu g \) (equivalent to \( \pm 0.003 \text{ AU in the RPC method} \). It was apparent that the impact of the variance of antibody determination was very small and thus did not have a significant influence on RPC estimation.

### 3.3. Picogreen Method Evaluation and Long-Term Assessment

As a comparative method for cell lysis determination, DNA was quantified using the Picogreen assay. The calibration range was established to be 52.5 ng mL\(^{-1}\) to 840 ng mL\(^{-1}\) (Figure 3A). Both the ordinate and the x-axis were logarithmized to pass the constant variance and normal distribution test and, evidently, to establish a linear regression \( (n = 10) \). The LOD for this method was 9.4 ng mL\(^{-1}\) and LOQ was 28.4 ng mL\(^{-1}\).

Similar to the RPC method, mean deviations (see Equation (1)) from the slope \( k \) and intercept \( d \) were monitored over a time course of over 690 days (Figure 3B,C); both exhibited variation under 20%. The change in the operator had a greater impact on the parameter distribution, indicated by “a”; as such, a new calibration was necessary.

The impact on variations of \( k \) and \( d \) on the evaluation of the DNA concentration was recalculated (Equations (1) and (3)). Observation six, indicated by an arrow, was regarded as an outlier (Figure 3D).

With increasing fluorescence, maximal error decreases and will be less than 30%. DNA quantification using the Picogreen assay is highly sensitive and thus susceptible to errors. Consequently, it is unremarkable that such deviations occurred during this long-term assessment. Data obtained from blank measurements substantiated these observations due to the fact that the CV was approximately 32%, which is a reasonable level of background variation.

Matrix and dilution effects were also occasionally apparent. DNA spiked into a mock supernatant demonstrated that the spiked DNA amount was more accurately estimated at a higher dilution factor. For the last five dilution steps, a recovery rate of 93.4 ± 13.4% was achieved. In contrast, DNA spiked into fresh media was not heavily influenced by the dilution. The achieved recovery rate was 110.1 ± 13.9%.

### 3.4. RPC and DNA as a Cell Lysis Marker

To compare both methods, the concentration range was transferred into the lysed cells per mL by assuming that a cell contained 315 pg of protein and 5.60 pg of DNA. The working ranges for both methods were different. The RPC method covered a range from 2.6 × 10\(^5\) to 1.7 × 10\(^6\) lysed cells per mL, whereas the DNA method spanned a broader range from 9.4 × 10\(^3\) to 1.5 × 10\(^5\) lysed cells per mL. While DNA is more sensitive, RPC offers the advantage of being more accurate. The RPC showed a reasonable error level of below 20% over nearly the entire working range. Furthermore, the RPC longtime robustness, without repeated calibration effort, makes this tool useful for both research and industrial applications, as no additional calibration was required.

Furthermore, the characteristics of both methods could be easily monitored. The mean deviations of slope \( k \) and intercept \( d \) are practical and reasonable indicators.

### 3.5. Improved Process Characterization Based on Cell Lysis Monitoring

The developed RPC and DNA method was applied during a CHO shake flask study, where a 3\(^2\) full factorial experimental design was conducted. It was assumed that variations in the cell size throughout the course of the fed-batch cultivation had no impact on the protein content per cell. The two independent parameters included the glucose concentration in the feed and the process temperature. In total, 18 experiments were conducted and 180 samples measured. Increasing the glucose content in the feed had no influence on the VCC, HCPs, or the product titer. Therefore, the complete data set for
each particular temperature profile was summed up and averaged (Figure 4). The duration of the process was controlled via the viability of the cell culture (stopped at <70%).

Depending on the temperature, the fed-batch processes reached TCCs of between $1.2 \times 10^7$ cells per mL and $2.5 \times 10^7$ cells per mL, with product titers ranging from $400 \mu g \, mL^{-1}$ to $600 \mu g \, mL^{-1}$. Specific productivity $q_p$ decreased over time and achieved maximum values of approximately 12 pg cell$^{-1}$ d$^{-1}$. The processes at 31 °C lasted 1.6- and 1.3-fold longer compared to the experiments at 37 °C and 34 °C, respectively, until the stop criterion (viability <70%) was reached (Figure 4A,B). At lower temperatures, more cells were in the G1/G0 phase and the growth slowed or even stopped (Figure 4C,D), which is why temperature shifts are widely used for proliferation control.[31]

Figure 3. A) DNA concentration as a function of RFU. The first calibration set is depicted ($n = 10$). B) The mean deviation of the slope $k$, C) intercept $d$, and D) the mean deviation to the target concentration at $3.8 \log$ RFU, calculated according to Equation (3), over a time course of 690 days. The mark “a” indicates when the operator changed and when a new calibration was performed. The solid line represents the average value. The gray dashed line and the dashed dot line represent 95.4% and 99.7% of the sample population ($n = 10$), respectively. E) The maximal error for the first calibration as a function of the measured fluorescence, which could be obtained through the deviations of $k$ and $d$, RFU, relative fluorescence units.

Apparantly, cell lysis accounted for a maximum of 8.8% of the total produced biomass in the system (Figure 5A), whereas the HCP content reached values up to $500 \mu g \, mL^{-1}$, corresponding to 50% of total protein in the supernatant for this model process. Despite this small fraction of lysed biomass, the resulting impurities were rather substantial.

Since dead and living cells can undergo cell lysis, the ITCD was calculated (Equation (4)), which we defined as

$$\text{ITCD} = \int_{t_0}^{t_{\text{end}}} \text{TCC} \, dt$$

This was plotted against the RPC (Figure 5B). The fed-batch process exhibited a constant specific protein release rate of $1.5 \times 10^{-7} \mu g/X_{\text{total}}/h$, which corresponds to a constant lysis rate.
$k_{DL}$ of $4.6 \times 10^{-4}$ lysed cell concentration (LCC)/TCC/h, and is subsequently temperature-independent. It is proposed that the majority of lysis in this process is derived from dead cells since cell damage in bioprocesses is largely bubble-associated.\[^{32}\]

The amount of DNA in cultivations at $31^\circ C$ was lower than those in processes at $34^\circ C$ and $37^\circ C$ cultivation temperature (Figure 5C). The two linear regressions exhibited different slopes, $k_{34+37^\circ C}$ of 4.8 pg per cell and $k_{31^\circ C}$ of 2.0 pg per cell, for the combined data set of $37^\circ C$ and $34^\circ C$ and for the process at $31^\circ C$, respectively. In the process at $31^\circ C$, substantially more cells were in the G1/G0 phase\[^{31}\] (Figure 3C,D). Since the cells in the G1/G0 phase (N) have a different amount of DNA compared to the cells in the S G2/M phase (N to 2 N), the lysis of a G1/G0 cell releases a lower amount of DNA into the supernatant than an S G2/M cell. In this respect, it was remarkable that $k_2$ was approximately one half of $k_1$. However, the derived amount of DNA per cell was lower than expected.\[^{10}\]

Therefore, DNA would be inappropriate for cell lysis estimation in the type of experimental setup used in the present study.

### 3.6. RPC as a Key Process Indicator

In the best case, the cell culture supernatant should exclusively contain the target protein. This would provide the best starting conditions for ensuring high purification performance. Conventionally, viability or a death marker protein is used as a performance indicator and harvest criterion.\[^{13}\] However, from the downstream perspective, cell viability is only relevant when the amount of RPC and DNA increases, which only occurs during cell lysis. This could also be of significant relevance for the yield, longevity, and overall performance of the subsequent protein purification strategies.\[^{2,16}\] In general, reducing the soluble impurity level can simplify early-stage downstream processing.\[^{34}\] It is known that certain HCP species affect the downstream process more than others. HCP constituents can associate with the monoclonal antibody (mAb) product or they might even co-elute due to noncharacteristic binding to the chromatography resin. Among others, clusterin, actin, or nidogen-1, are some of the most prominent proteins interacting with mAbs. Most of these sticky proteins are intracellular proteins.\[^{17}\] Hence, cell lysis leads to an increased level of these proteins in the supernatant, which increases the probability of their association to the mAb and their sticking to the chromatography resins. Owing to the wash and cleaning in place (CIP) procedures of the protein A columns, HCP precipitates and deposits on the resin, which contributes to fouling. Moreover the diversity of HCP species also changes over the lifetime of the protein A resin, which can lead to clearance problems in the subsequent purification steps.\[^{35}\]

In terms of purity, the ratio of RPC to mAb, and also other overexpressed active pharmaceutical ingredients, could be used to identify an optimal and a consistent stop criterion for a cell culture
process. Although the fed-batches were all harvested with similar viability, the supernatant quality differed (Figure 5D). The process using the lowest temperature exhibited the worst ratio of impurities to the target protein, and could not accomplish similar ratios to the other two processes. The results clearly demonstrate that the RPC/mAb ratio could be an alternative process indicator for CHO cell cultures. It can give an appropriate impression of the impurity level in the supernatant. However, to understand the dynamics and the complexity of HCPs, a qualitative analysis must be further applied.\[15,19,36\]

4. Conclusions

Cell lysis is an important parameter to correctly characterize bioprocesses. Lysed cells have a substantial impact on the upstream and downstream performance. Information regarding the RPC is beneficial for establishing and maintaining a consistent process performance. Notably, RPC determination adds great value for further improvement to integrated bioprocesses as well as to the development of process models for process control purposes.

One particularly important aspect is that a producing CHO cell consists largely of proteins (>70%), whereas DNA remains a minority (1.4%). Therefore, changes to the protein spectrum will not have a great impact on the overall protein content, since the majority of the proteins are necessary for cell maintenance and protein production.\[37\] Indeed, HCP production can be very similar throughout the different growth phases.\[1\] We showed that the DNA content subsequently depends on the phase of the cell cycle. A cell in the S or G2/M phase contains more DNA than a cell in the G1 or G0 phase. Notably, the amount of cells at a certain cell stage will differ depending on the process conditions.

With this established methodology, the user has a very precise, fairly accurate, and robust tool for depicting the state of a cell culture bioprocess, apart from relying only on the viability of a cell population. The presented assay could be implemented as a high-throughput technique, as it can be performed in multiwell plate format and thus the use of pipetting robots may also be feasible. Furthermore, the method described in this study does not include light-sensitive or time-dependent steps; it is easy to implement and cost effective, and thus it could be applied to arbitrarily large experimental settings. The generated data are most suitable for establishing soft sensors and process models. Moreover, RPC\[14\] can now be monitored inexpensively and easily throughout the entire process, which makes it an interesting approach also for continuous processing.

Acknowledgements

The authors would like to acknowledge the entire PAT-Plant project team, but especially Benjamin Bayer and Florian Bacher for their efforts in conducting the fed-batch experiments. They also thank the Austrian...