Recent capillary electrophoresis applications for upstream and downstream biopharmaceutical process monitoring

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1. Introduction

The biopharmaceutical market is one of the fastest growing biotechnology markets. In order to ensure affordable and reliable therapeutics, the biopharmaceutical process has to be closely monitored. Capillary electrophoresis (CE) has proven to be a valuable technique for the analysis of product concentration, critical quality attributes, product and process-related impurities, and nutrients and metabolites in the cell culture medium. Capillary zone electrophoresis, capillary gel electrophoresis, and capillary isoelectric focusing are extensively used for product concentration, and size or charge heterogeneity determination. CE has a number of benefits for the analysis of upstream and downstream process-intermediates, including the ability to handle highly complex matrices found in process-intermediates, high resolving power, little sample preparation requirements, rapid analysis, and low solvent and sample consumption. The small sample volumes (nL range) are especially beneficial for microbioreactor analysis or clone selection experiments. The simple setup and the possibility for miniaturisation and automation using microchip CE provides great opportunities for on-site, real-time monitoring of the process. This review discusses CE applications in upstream and downstream processing of the last decade.

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product safety, efficacy, and quality. Each of those properties is referred to as a critical quality attribute (CQA) for the PoI [2], and the CQAs of therapeutic proteins could for example include the glycosylation pattern, charge variants, or aggregation and fragmentation [3], and are often defined through in vitro testing of the PoI. To ensure affordable and reliable treatments, it is critical that the biopharmaceutical production is cost and time effective with minimal variation between batches. For effectivization, quality by design (QbD) can be implemented, which is “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management” (ICH Q8(R2), 2009) [2]. QbD increases the manufacturers as well as the regulatory agencies understanding of the impact of raw material and process parameters on the CQAs and helps with identifying product and process understanding and process control, which are parameters whose variability has a certain impact on CQAs. Critical process parameters should be monitored and controlled to maintain the quality of the PoI, and this can be achieved by incorporating process analytical technology (PAT) into the bioprocesses.

PAT includes online, in-line, at-line, and offline analysis performed on-site in (near) real-time, and can be incorporated into the whole bioprocess stream. Online analyses (online, in-line, at-line) include automated sampling and analysis, while for offline analysis (offline, at-line), samples are taken manually before analysis. Traditional process monitoring techniques include in-line analysis using probes for e.g. pH, pO2, pCO2, and cell density by impedance, and at-line analysis where samples drawn from the process stream are analysed directly on the process floor using spectroscopic or other dedicated sensors i.e. glucose, lactate, glutamine and ammonia [5]. Since the PAT initiative was launched by the FDA [6], the number of PAT platforms for the monitoring of the bioprocess using more complex analytical techniques like liquid chromatography (LC), capillary electrophoresis (CE), and mass spectrometry (MS) has increased [7].

CE is an excellent technique to implement in PAT since it uses small sample volumes (nl range), which makes it a great technique to use for microbioreactors with limited volumes or for process monitoring. CE has short analysis times with high resolutions, negligible waste, the possibility of automatization, and minimal sample preparation requirements compared to other liquid-based separation techniques. In CE, analytes are separated due to their charge-to-size ratio under the influence of an electric field within a narrow capillary. In the electric field, charged molecules have a mobility which is the sum of their electrophoretic mobility and the mobility of the electroosmotic flow (EOF). The EOF can be controlled by adjusting parameters of the background electrolyte (BGE), such as the pH, ionic strength, and viscosity, as well as by the temperature, magnitude of the electric field, and dynamic or permanent coatings of the capillary wall. Controlling the EOF is important to obtain robust CE separation methods. By adding certain additives to the BGE, different modes of separation can be achieved, which can be used for analysis of therapeutic protein CQAs. Capillary zone electrophoresis (CZE), the simplest CE mode, where a buffering BGE solution with or without additives is used, can be applied directly for the analysis of intact therapeutic proteins or with any top-down, middle-up or bottom-up approaches. CZE can provide information about glycosylation patterns and charge heterogeneity at the protein level. Capillary gel electrophoresis (CGE) is created by adding a gel or a polymeric network at low concentration to the BGE. For SDS-CGE, the sample can be both reduced and non-reduced providing different information. For apparent pl determination of a protein and levels of acidic, main,
and basic variants as well as impurities, capillary isoelectric focusing (cIEF) or imaging cIEF (icIEF) mode can be used. In (ic)IEF, ampholytes within a specific pH range are used to create a pH-gradient in the capillary to separate proteins based on pl differences [8]. Another advantage of CE is the possibility to transfer the separation methods to microchip devices (microchip capillary electrophoresis, MCE), which are miniaturised versions of CE where the capillary is replaced with a chip capillary device. The microchip device can be fabricated using various materials, e.g. glass, quartz, polymers, or even paper, which facilitates its broad area of applications [9,10]. MCE uses less volume than CE, often has shorter analysis times, is more portable, can be designed to have several channels for higher throughput, or multiple analytical separations occurring at the same time, and could have integrated sample preparation on the MCE device [11,12].

The application of CE in the biopharmaceutical industry with the focus on final product characterisation has been reviewed extensively (e.g. Refs. [13,14]). This review focuses on the application of CE and MCE for analysis of USP and DSP process-intermediates during the last decade.

2. Discussion

2.1. Determination of product concentration in upstream and downstream processing

The final product yield of a biopharmaceutical process is limited by the protein expression in the host cells. This expression is affected by a range of cell culture conditions. To optimise the production yield, and to understand the effect of cell culture conditions on protein expression, the protein expression should be monitored. After protein expression in USP, the Pol is purified in DSP. Loss of product during purification should be avoided and therefore, the product concentration should be analysed in every step of the USP and DSP processes.

For mAb titer determination in USP directly in cell culture medium (CCM) supernatant, Wang et al. [15] developed an MCE-MS method. The limited sample preparation consisted of only a desalting and a reduction step. A stable isoform-labelled mAb was added as internal calibrator prior to sample reduction. Heavy chain (HC) and light chain (LC) were separated with the CE method, and as no N-glycosylation variants were expected on the LC, the mAb concentration was determined using the relative signal intensity of the LC versus the isoform-labelled LC. The same method was further applied for glycosylation characterisation (section 2.2.3). Van der Burg et al. showed the monitoring of mAb concentration in USP in CCM supernatant without the need for any sample preparation using a simple CZE platform method [16]. By using UV detection rather than MS detection, desalting the sample was not needed. And by analysing intact mAb, using an external mAb calibration for quantification, no reduction was required either. Samples could be analysed directly in CCM supernatant without interference of matrix components. This shows the great capabilities of CE in handling complex sample matrices. A dynamic triethanolamine coating prevented adsorption from matrix components or mAb to the capillary wall, and the low pH BGE employed in this method allowed the analysis of a large variety of mAbs.

Another example of the capability of CE to handle various complex matrices is the adenovirus type 26 (Ad26) particle concentration determination with CE developed by Van Tricht et al. Applying analytical quality by design (AQbD), they developed and implemented this CZE method for routine intact Ad26 particle (150 MDa) quantification throughout USP and DSP [17,18]. Sample matrices varied and could contain DNA, proteins, surfactants, adjuvants, salts, and/or cell debris. No sample preparation was required, except for samples containing DNA, which required a simple sample pretreatment with benzonase. Adsorption of matrix components or viral particles was prevented by using a polyvinyl alcohol coated capillary, and by the addition of the neutral surfactant polysorbate-20 to the BGE. The Ad26 particles were detected without interference of matrix components in all process-intermediates with a total run time of 3.5 min (Fig. 1). This method was applied for a large variety of applications. It was applied in USP for determining seed Ad26 concentration, in DSP to study lysing agent effects on Ad26 yield and HCP and host cell (HC) DNA release, to study HC-DNA clearing agent effects on Ad26 particles, and to investigate the root-cause of high anion-exchange chromatography filtration pressure. It was also applied for chloride content determination, Ad26 content release testing, and for Ad26 stability testing [19].

Protein analysis in biopharmaceutical samples is often performed using traditional Western Blot. However, the electrophoresis, electrical blotting, antibody probing, and signal development are labour-intensive. In 2006, O'Neill et al. [20] used the principles of western blotting performed in a capillary; they developed an cIEF immunoassay (IA) method employing photochemical immobilisation and antibody probing of proteins in the capillary, followed by chemiluminescent detection. Now there are commercially available capillary western instruments and analysis kits for size-based (SDS-CGE-IA) or charge-based (cIEF-IA) separations. SDS-CGE-IA is used for protein concentration determination in USP and DSP. As an example, this technique has been employed for the monitoring of pertactin concentration in vaccine samples throughout DSP [21]. Pertactin was quantified in different USP and DSP process-intermediates from CCM to drug product, with good accuracy and no matrix interference. SDS-CGE-IA has also been applied for vaccine protein monitoring throughout DSP in Ebola vaccines [22]. Ebola viruses infect host cells through interactions mediated by viral glycoprotein. SDS-CGE-IA was used to monitor the types and quantities of glycoprotein variants generated throughout the process: after viral harvest, depth filtration, enzymatic digestion, ultra-filtration, addition of Tris and rHSA (drug substance), and final dilution in Tris and rHSA (drug product). Only the sample taken after viral harvest contained an unknown peak migrating between the glycoproteins, which was filtered out after depth filtration. The method resulted in similar glycoprotein profiles as obtained with manual Western blot, but with better reproducibility, more accurate quantitation, and improved ease of use.

Xu et al. used SDS-CGE-IA for biopharmaceutical protein concentration determination directly in cell culture harvest (CCH) and drug substance samples [23]. The therapeutic protein was analysed as three peptide peaks of 100, 130, and 210 kDa. The total peak area of the three peaks was used to determine the titer with good precision and accuracy. This method was also applied for high molecular weight (HMW) species determination (section 2.2.1). They also applied SDS-CGE-IA for the quantification of Fc-fusion protein in DSP intermediates [24]. The Fc-fusion protein consists of two polypeptides: fusion-Fc and single chain Fc. The peak from fusion-Fc polypeptide was used for quantification against a calibration curve from purified drug substance in the wide linearity range of 24 μg/mL to 6 mg/mL. This method was also used for quantification of product related impurities in DSP intermediates (section 2.4).

Conventional SDS-CGE is also a valuable technique for protein concentration determination. SDS-CGE was employed to investigate the effect of process parameters on production and quality of recombinant human β-glucuronidase [25]. CCH was concentrated and buffer exchanged, and bovine serum albumin (BSA) was added as internal calibration standard for molecular weight and concentration determination. The effect of different process parameters on
production was then determined by analysing the concentration upon harvest using SDS-CGE under reducing conditions. Commercially available SDS-CGE applications were generally developed for mAb analysis, but are also frequently used for non-mAb proteins; however, they cannot always be readily applied. Adjustment and optimisation of the SDS-CGE application is often required. Geurink et al. [26] described a general four-step approach for SDS-CGE method development for the quantification of viral proteins. First, factors most influencing method performance, critical method parameters (CMPS), were identified. Sample preparation could be affected by incubation time and temperature, pH, and reagent concentrations, and separation could be affected by the gel buffer and capillary effective length. For SDS-CGE, often a commercialised proprietary gel buffer is used. The composition of the gel buffer impacts the pore size, viscosity, and ionic strength, which in turn impact the amount of sample injected, sample stacking, separation, sensitivity, and analysis time. The magnitude of these effects depends on capillary temperature. The selected CMPS for method development/optimisation therefore included incubation time and temperature, pH, and reagent concentrations for sample preparation, and capillary effective length, gel buffer dilution, and capillary temperature for separation. A four-step approach was set up for quick method development. The approach included 1) assessment of feasibility of the default conditions and need for sample preparation and separation, 2) multivariate design of experiments (DoE) optimisation of sample preparation CMPS and 3) multivariate DoE optimisation of separation CMPS, and 4) method validation. This approach was applied for the development of SDS-CGE methods for the determination of influenza group 1 mini-haemagglutinin glycoprotein, and the determination of polio virus particle proteins from an inactivated polio vaccine. Using the same development approach, two different methods were developed that met the analytical target profile requirements within week(s), considerably reducing method development time.

In SDS-CGE, sample injection is commonly performed electrophoretically. The conductivity of the sample therefore affects the amount injected; at higher conductivity, less sample is injected. Desalting to concentrations <50 mM is recommended [27]. As process-intermediates often have high salt concentrations, desalting is often required. Sample preparation of process-intermediates include the removal of impurities such as host cell proteins and cell debris, and buffer exchange. Also, to accommodate for a wide range of sample concentrations from different in-process steps, concentration normalisation is required. This results in labour-intensive sample preparation. To overcome this, Le et al. developed an automated robotic platform for sample preparation for both the monitoring of LMW species by reduced SDS-CGE and the monitoring of partial reduction by non-reduced (NR-SDS-CGE) in DSP-intermediates [28]. The platform normalises sample concentrations, removes salts and other contaminants, and adds the required SDS-CGE reagents. The use of a protein A purification purifies the sample, but also allows for buffer exchange, and, as the Pro-A columns have a finite number of protein binding sites which will be saturated above a specific sample concentration, allows for normalisation of the protein concentration. After elution, sample buffer was added. Samples were then manually aliquoted and incubated before CE analysis. An alternative to Le’s approach is to use hydrodynamic injection, which is less prone to sample matrix effects so extensive sample preparation can be avoided [29].

The utility for monitoring LMW species by rCE-SDS in purification intermediates was demonstrated across a wide range of sample matrices. Normalisation of sample concentrations was achieved without biasing the distribution of species. Reproducibility studies show that the precision is similar to that with manual preparation. Application of this sample preparation technology to NR CE-SDS was demonstrated with samples from the harvest operation to monitor partial reduction.

2.2. Critical quality attribute monitoring in upstream and downstream processing

All product characteristics influencing the safety, efficacy, and quality of the product are considered CQAs. In order to control CQAs early on, and to optimise the biopharmaceutical process to produce well defined therapeutic proteins, characterisation of CQAs during production is essential. This chapter describes CE applications for the characterisation of the most common CQAs: size heterogeneity, charge heterogeneity, and glycosylation.

2.2.1. Size heterogeneity

Size heterogeneity comprises product related aggregates (HMW species), and fragments (low molecular weight (LMW) species). The
efficacy of therapeutic proteins is highly dependent on their structure. Aggregation of therapeutic proteins can cause loss in function and increase the risk of immunogenic responses, causing adverse effects ranging from protein neutralisation to anaphylaxis [30]. To minimise HMW and LMW size variants, the USP and DSP steps must be controlled, and size variants should be characterised throughout the process. The characterisation of size variants is frequently performed using SDS-CGE.

In one study, the effect of USP parameters on process-induced antibody disulfide-bond reduction was investigated using NR-SDS-CGE [31]. CCH samples were directly loaded on the robotic automated platform for SDS-CGE sample preparation developed by Le et al. [28] as described in section 2.1. After sample preparation, the main antibody was separated from LMW species and the relative area of the LMW species peaks and the main peak were used to monitor interchain disulfide reduction in various harvest experiments. The data showed that the type of mAb and the type of cell line have a large impact on reduction. This increased understanding allows for screening of cell lines and cell culture conditions to minimise process-induced reduction.

The SDS-CGE-IA method from Xu et al. [23] (section 2.1) was also employed for the determination of HMW species in CCH and drug substance samples. The largest of the three peptides detected, the 210 kDa peptide peak, is from the HMW species. The ratio of the HMW peak to the total peak area was used to determine the HMW percentage and could be determined with a precision of <5% RSD.

CE was employed for the optimisation of the DSP purification process. Due to the similarity in biochemical properties of HMW and LMW species to the target protein, their clearance in DSP can be challenging. It is therefore beneficial if these species could be removed early on. Depth filtration is commonly used during cell culture harvesting. Depth filtration purifies both by filtration and by adsorption of soluble impurities onto the filter surfaces. To minimise the burden on chromatographic purification steps further in DSP, the adsorption of HMW and LMW species on depth filters was studied [32]. Size exclusion chromatography was employed for HMW and LMW quantification, and NR-SDS-CGE for LMW quantification, which gave higher resolution and more accurate LMW determination. Employing these techniques increased the understanding of the depth filtration step; the results indicated formation of LMW species during filtration, which could be reduced by using lower temperatures. This allowed the design of a control strategy, which significantly reduced HMW and LMW levels.

2.2.2. Charge heterogeneity

Many PTMs influence the charge heterogeneity of therapeutic proteins. PTMs could result in more acidic species, for example by sialylation, deamidation, or glycation, or in more basic species, by for example succinimide formation or incomplete removal of C-terminal lysine. Depending on the position of the PTM, it could affect antigen binding affinity, potency, secondary structure, and aggregate formation [33]. Charge variant analysis is therefore important.

Charge heterogeneity is commonly determined using ion exchange chromatography (IEC), cIEF, icIEF, or CZE. A CZE method developed by He et al. [34], using a dynamic triethylenetetramine coating and high ε-amino-caproic acid concentration to prevent protein adsorption, is widely used for mAb charge variant analysis and has proven to be robust [35]. The robustness of cIEF [36] and icIEF [37] was also shown by intercompany studies. These studies, however, were employed on relatively clean samples of drug product.

For charge heterogeneity determination in USP and DSP process-intermediates, Michels et al. [38] developed a cIEF-IA method. They optimised the ampholyte solution to obtain a broad linear range of pH 5.5—9.8 in order to use the method as a platform method. Platform methods are desired for process development support, as they save a lot of time in reoptimizing method parameters for each product. In contrast to conventional cIEF, in cIEF-IA the ampholytes were removed from the capillary before detection, resulting in less background noise. The cIEF-IA required only 0.2 µg/mL protein, 1000-fold less than conventional cIEF. The method was evaluated for matrix interference of CCH. Three mAbs with considerably different pl s were spiked into CCH. No interference of matrix components was observed, and the relative distribution of charge variants determined was similar to those determined in samples in formulation buffer. The ability to characterize mAbs with diverse pl values without sample purification reduces analysis time and hands-on labour significantly, especially for clone screening where hundreds to thousands of samples are generated.

The method could be employed for determination of charge variants or titer in USP samples and for the determination of charge variants to support DSP formulation development.

2.2.3. Glycosylation

N-glycosylation and O-glycosylation are the most common forms of protein glycosylation. As the vast majority of therapeutic proteins contain N-glycosylation, that is the focus of this review. N-linked glycans consist of a biantennary structure containing a pentasaccharide core of two N-acetylgalactosamines (GlcNAc) and three mannoses, and a variable addition of monosaccharides. The latter results in large heterogeneity (Fig. 2). The glycosylation pattern considerably influences many factors such as biological activity, stability against proteolysis, pharmacokinetics, serum half-life, immunogenicity, and the antibody effector functions: complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated phagocytosis (ADCP) [39]. These effector functions are important for the efficacy of antibodies for cancer treatments. While most N-glycans are core fucosylated [40], afucosylation greatly enhances ADCC. The presence of bisecting GlcNAc also enhances ADCC to a lesser extent. CDC is affected by terminal galactose, GlcNAc, and mannos residues. Sialic acid or galactose at the terminus of N-glycans enhance anti-inflammatoryary functions, which is important for autoimmune disease treatment [39,40]. As the glycosylation pattern has such a large impact on the antibody function, it is a CQA that must be controlled and monitored. Glycosylation should be monitored early in the process for better process understanding and the possibility for glycosylation optimisation.

N-glycosylation analysis of therapeutic proteins can be performed at different levels; at the intact protein level, subunit protein level, as glycopeptides, or as released glycans. Each level provides different information, with the intact level providing information on the most abundant glycoform pairs, the subunit level on individual glycoforms, including low abundant glycoforms not detected on the intact level, glycopeptides provide information on the location of glycosylation, and released glycans on each individual glycan [41].

Released glycans are derivatised to enable detection, commonly with the fluorescent label 8-aminopyrene-1,3,6-trisulfonic acid (APTS). An example of this is the CGE-LIF method from Reusch et al. [42] for the monitoring of the N-glycosylation of mAbs in USP CCM samples for bioprocess development and characterization. A small-scale purification was performed by filtering the samples and applying them on a protein A column. The purified mAb was thereafter transferred automatically using a robot on the ultrafiltration plate for desalting and deglycosylation in ultrafiltration 96-well plates. CGE-LIF analysis was performed on a DNA analyser equipped with a 48-capillary array, allowing for high throughput analysis. All expected glycans could be detected; the high abundant
G0F, G1F, and G2F and the lower abundant G0, G2, G0F–N, and Man5. Separation of the isomeric structures of G1 and G1F was also achieved. The glycans Man5 and G0F–N co-migrated, which should be considered when, for example, afucosylation has to be determined, as this peak contains both the core-fucosylated G0F–N and the non-core-fucosylated Man5. During process development, often large datasets have to be analysed. An automated approach to data analysis could save a lot of time. Walsh et al. [43] developed a semi-automated approach for large data set analysis and applied this for the monitoring of the glycosylation of antibodies produced under 11 different USP culturing conditions with 3 replicates over 12 days. Sampling each day resulted in 391 samples (5 sampling errors). APTS-labelled released glycans were analysed by CGE-LIF. A triple internal standard was used instead of an external maltoligosaccharide ladder to calculate the glucose unit (GU). Glycans were identified through GU glycan databases. GU glycan databases for APTS-labelled glycans include GUcal [44] and GlycoStore [45]. For quantifying the glycan peaks, HappyTools [46] was employed. However, fluctuating migration times due to day-to-day variability of the method and the different culture conditions and non-Gaussian peaks limited automated quantitation. By grouping electropherograms with similar migration times using a clustering algorithm before applying automated non-Gaussian area calculation in HappyTools, manual data analysis time was reduced from 2–3 days to 1–2 h for first-time analysis, and down to minutes for repeated analysis.

Both released-glycan methods described above allow high-throughput analysis, which is beneficial for large amounts of samples in, for example, USP bioreactor optimisation or clone selection experiments. The sample preparation protocols for released-glycan analysis are, however, labour- and time-consuming. For real-time monitoring, short analysis times through limited sample preparation are required. Sample preparation is reduced when analysing glycans at the subunit or intact protein level where deglycosylation and derivatisation steps are avoided. The MCE-MS method from Wang et al. [15] (section 2.1) was also applied for monitoring glycosylation directly in CCM supernatant at the subunit level. Sample preparation was limited to a desalting and a reduction step to reduce mAb to HC and LC. After a charge-based separation of HC and LC, the deconvoluted MS spectra of the HC were used to determine glycosylation patterns. The MCE method was capable of separating minor charge variants of the HC and LC, such as aglycosylated HC and glycated LC components, potentially allowing for improved detection of low abundant species [15]. The method was applied to follow changes in glycosylation over the time course of the cell culture process by quantifying the glycoforms Man5, G0F–N, G0F, G1F, and G2F, and the level of aglycosylation in CCM supernatant samples after various days of cultivation. For afucosylation determination, a deglycosylation step was added using GlycINATOR enzyme. This enzyme removes N-linked carbohydrates after the first GlcNAc, leaving only core-fucosylated or afucosylated GlcNAc intact. Using one CE-MS method and two sample preparation strategies, mAb glycoforms, the level of afucosylation, and mAb concentration could be monitored in USP. Sample preparation was completely avoided by analysing glycosylation on the intact mAb level [47]. CE-MS analysis was performed directly on CCM supernatant samples. Two MS systems were compared in this study. Although the sensitivity was better using an Orbitrap, the five most abundant glycoforms pairs (G0/G0F, G0F/G0F, G1F/G0F, G0F/G2F or G1F/G1F, and G1F/G2F) could be detected with both a QTOF and the Orbitrap with high sensitivities of 50 μg/mL and 25 μg/mL, respectively. The better sensitivity of the Orbitrap is partly attributed to the in-house two-capillary CE-MS interface used, which functioned as a valve and prevented separated matrix components and salt from entering the ESI source. The possibility to inject sample to 26% of the capillary volume adds to the high sensitivity. The reason for maintaining efficient separation with such large injection volumes is probably transient isotachophoretic stacking because of the high salt concentration in CCM supernatant, which illustrates the
possibilities for on-capillary concentration with CE. The method was compared to an HPLC-MS method, showing sharper and narrower peaks using CE. The desalting by CE led to better spectra quality and the glycoform G1F/G2F was better resolved using CE-MS.

2.3. Metabolites and nutrients monitoring in upstream processing

Cell cultivation processes for the expression of therapeutic proteins require CCMs that support growth and production. The composition of the CCM is important as it affects cell growth, viability, productivity, and CQAs. All CCMs require similar basic nutrients, such as sources of carbon, nitrogen, phosphate, amino acids, fatty acids, vitamins, trace elements, and salts. The concentrations of these nutrients in the CCM provide important information on cell metabolism and thus the state of the cell culture. To optimise cell culture conditions to form well-defined therapeutic proteins, information on titer, CQAs, and nutrients and metabolites concentrations should be combined. The concentration of nutrients and metabolites should be closely monitored.

2.3.1. Amino acids

Amino acids (AAs) are essential nutrients for the cultivation of cells. They are the building blocks of proteins, and are therefore the primary constituents of all proteinaceous material of the cells, including the cytoskeleton, enzymes, receptors, and signalling molecules. Furthermore, AAs are required for cell growth and maintenance. Both a lack and an excess of AAs could lead to undesired effects [48]. Monitoring of AAs provides information on the state of the culture, and is important to identify optimal AA concentrations and to be able to adjust their concentrations to these optima.

As most AAs lack UV absorbance or fluorescent groups, other detection strategies should be employed, such as LIF detection after derivatisation, contactless conductivity detection (C²D), or MS. Mikkonen et al. [49] developed a CE-C²D method for the monitoring of free AAs directly in CCM supernatant samples. The advantage of C²D detection is that no derivatisation step is required, simplifying sample preparation. The EOF was suppressed, and adsorption from matrix components was prevented by adding 0.1% -CD and b-CD, and were investigated. These CD are bucket-shaped molecules with a hydrophilic outer surface and a hydrophobic cavity. Based on the hydrophobicity of the amino acids, they can undergo different degrees of complexation by the CD cavities. As the neutral CDs have no electrophoteric mobility, CD-complexed AAs migrate slower. This additional separation mechanism enhanced the selectivity. As baseline resolution of 20 AAs in one single method was not achieved due to the difference in physical chemical properties, two methods using different CDs at different concentrations were combined to separate all 20 AAs. The two methods were applied for the analysis of AAs in CCM supernatant samples after different days of cultivation. No interference of matrix components was observed. By combining the results of the two CE-C²D methods, 17 AAs could be detected in the CCM supernatant samples, with no other sample preparation than dilution. Arginine and histidine overlapped due to the high concentration of arginine, and cysteine was not detected in CCM samples, likely due to the low concentration and its conversion to oxidized dimer.

For reduced analysis time and minute sample volumes, and for the possibility of automation, MCE becomes increasingly popular. An MCE-LIF method for the monitoring of AAs in USP was developed employing fluorosence derivatisation with NDA [50]. NDA derivatises primary amines, present in all AAs except proline, which could therefore not be detected using this method. Due to the differences in physical chemical properties of the AAs, two methods were used for the separation of all 19 AAs; one micellar electrokinetic chromatography (MEKC) method for the more polar AAs, and one solvent modified MEKC method containing 4% 1-butanol for the less polar AAs, both using SDS as micellar surfactant. MCE separation showed to be similar to capillary separation, although resolution between a few AAs was lost. The methods were applied to spent CCM supernatant after different days of cultivation. Limited matrix component peaks were observed, and by combining the results from the two methods, 17 AAs could be quantified. In another study, derivatisation was avoided by employing MCE-MS [51]. Using isotope-labelled AA standards for internal calibration, 16 AAs could be quantified in less than 2 min. Due to the mass-specific detection of CE-MS, AAs that comigrated in CE (methionine and threonine, and phenylalanine and proline), could be resolved with MS. The method was applied on spent CCM samples after various days of a CHO cultivation process, without AAs supplementation, to determine the AAs consumption rate. The correlation of the reduction level of certain AAs with reduced cell viability was demonstrated. This information could be used for the optimisation of feed strategies. Although MCE-MS is rapid, MS requires heavy vacuum systems using turbomolecular pumps, making MS instrumentation large, and thus more difficult to implement in rapid, on-site analysis. To overcome this, the group of Ramsey developed a miniature cylindrical ion trap-based mass spectrometer operating at high pressure (≥1 Torr), eliminating the need for turbo pumps, and coupled this high-pressure MS (HPMS) to MCE [52]. The MCE-HPMS system was employed for the monitoring of AAs consumption rate in an Escherichia coli cell cultivation process [53]. 18 of the 20 AAs were detected with limited sample preparation in less than 3 min. The smaller AAs glycine and alanine were not detected, likely because these AAs are scattered more easily by collisions with the buffer gas due to their small size. The comigrating AAs methionine and threonine, asparagine and proline, tryptophan and glutamine, and glutamic acid and cysteine were easily resolved with MS. Relative consumption rates were determined based on the peak areas normalised to the internal standard of isotope-labelled valine. Elliott et al. [54] employed this MCE-HPMS for the monitoring of AAs, vitamins, and dipeptides in microbioreactors stressed with varying levels of ammonia (unstressed, 10, or 30 mM NH3). No sample preparation other than diluting the CCM supernatant samples was required. The data were correlated with cell viability data from the viability analyser and glucose, lactate, IgG, and NH3 concentrations measured using a Cedex Bioanalyzer. A large difference in mAb production for the different NH3 stresses was found. A difference in cell viability, lactate, and glucose levels was observed for the 30 mM NH3-stressed bioreactor after approximately 132 h into the process, while AA changes could be observed even earlier in the process. To fully understand the mechanism of AA changes and therapeutic protein production, more frequent sampling is required. These rapid MCE methods allow for at-line, real-time monitoring of AAs in cell cultivation processes.

2.3.2. Saccharides

Commonly, glucose is the primary energy and carbon source in CCMs. Glucose consumption usually leads to pyruvate accumulation and increased lactate concentration. The latter can inhibit cell growth in mammalian cell cultures. Strategies to prevent this involve using low glucose concentrations or employing alternative energy sources, such as galactose, mannose, and fructose. The replacement of glucose, or the addition of saccharides to glucose-containing CCMs, influences lactate production and consumption, and influences glycosylation of the protein [55]. To fully understand...
the effect of different saccharides and their concentrations on cell viability and glycosylation, a CE and MCE-LIF method was developed for the monitoring of glucose, mannose, galactose, fructose, and lactose directly in CCM supernatant [56]. As these saccharides do not possess chromophores or fluorophores, their detection was ensured by fluorescent derivatisation with APTS. Sample preparation was developed to aid automation; no sample clean-up was required and common sample drying steps were avoided. The derivatisation reaction and BGE composition were optimised using AQbD to result in a robust method. The CE method could readily be transferred to MCE. CCM supernatant after different days of cultivation could be analysed directly without any interference of matrix components in less than 6 min.

2.3.3. Nucleotides

Another factor in affecting glycosylation is the intracellular level of nucleotide and sugar nucleotides. Sugar nucleotides are monosaccharides bound to a nucleotide, and are precursors for glycosylation. Nucleotides play important roles in nucleic acid synthesis, cellular growth, and energy metabolism. Bucsella et al. [57] developed a CE method for the quantification of nucleotides and sugar nucleotides in CHO cell extracts to optimise the CCM condition needed for robust routine testing. The EOF should be controlled and adsorption avoided. This was achieved by employing a dynamic successive multiple ionic layers using the ready-to-use polycation and polyanion buffers from Czefix. When applying the coating, the migration time repeatability improved from 6% RSD to 0.3% RSD compared to no capillary coating. The separation was further improved by optimizing the pH, BGE concentration, capillary temperature, and applied voltage. Nucleotides and sugar nucleotides were extracted from CHO cells using cold acetonitrile, no concentration or derivatisation steps were required. Eleven nucleotides and sugar nucleotides were separated and quantified.

2.3.4. Vitamins

Vitamins are essential nutrients and play important roles in cell growth, cell viability, and productivity [58,59]. Vitamins are instable molecules, sensitive to a range of external factors, such as light, oxygen, low or high pH, and temperature, as well as to interactions with other cell culture medium components [60], challenging accurate analysis.

Although vitamins are essential nutrients which should be monitored, there is a lack of methods for in-process vitamin analysis. One CZE method was developed for the analysis of five B-vitamins (thiamine, nicotinamide, pyridoxine, pyridoxal, and pyridoxamine) directly in CCM samples [61]. To prevent adsorption of matrix components to the capillary wall, a dynamic triethanolamine coating was employed. The vitamins could be analysed in CCM with good precision and accuracy. Later, van der Burg et al. developed an MEKC method employing SDS for the analysis of ten B-vitamins and vitamin C in CCM [62]. There is a need for more methods analysing vitamins in process–intermediates.

2.4. Impurities analysis in downstream processing

After expression of biopharmaceuticals in host cells, the Pol must be purified from components such as cell debris, HCPs, HDNA, residual proteins, etc. During the purification in DSP, other components can be added, such as lysing agents, HC-DNA clearing agents, and eluents for chromatographic purification steps, which also have to be removed. The removal of all these impurities must be followed throughout DSP, both to improve the process and to prove clearance.

The SDS-CGE-IA method for Fc-fusion protein concentration determination developed by Xu et al. (section 2.1) was also employed for the evaluation of the DSP process [24]. The Fc-fusion protein consists of two polypeptides: fusion-Fc and single chain Fc. The CCH contained an excess of single chain Fc polypeptide, which had to be removed by the purification process. Also, HCP and co-expressed enzyme had to be removed. The clearance of single chain Fc polypeptide, co-expressed enzyme, and a specific HCP, heat shock protein 70 (HSP70), was quantified throughout the DSP process in CCH, eluate of first column process, and drug substance samples (Fig. 3). Different primary antibodies were used for Fc polypeptide, HSP70, and enzyme antibody probing.

For vaccine production, fetal bovine serum could be used as CCM. BSA is a major component of fetal bovine serum, and could cause allergic reactions in humans. Therefore, the WHO requires a limit of ≤50 ng per vaccine dose. To monitor these low levels in vaccine samples, sensitive analytical tools are required. For this purpose, a SDS-CGE-IA method was developed [63]. Quantification was performed using an external BSA calibration curve. BSA protein loss due to adsorption was avoided by dilution in sample buffers containing SDS. Analysing BSA resulted in monomer, dimer, and trimer peaks. A significantly higher amount of dimer was detected with SDS-CGE-IA compared to SDS-PAGE or size exclusion chromatography. It is suggested that this is because the primary antibody used preferentially recognises dimer BSA over monomer BSA.

![Fig. 3](image-url) (A) Removal of excess single chain Fc. Left: gel-like image and right: electropherograms of fusion-Fc and Fc polypeptides detection in (1, green) CCH, (2, silver) first column eluate, and (3, pink) drug substance using HRP-conjugated anti-Fc antibody. (B) Removal of co-expressed enzyme. Left: gel-like image and right: electropherograms for the first column eluate (2, blue) and drug substance (3, red) probed by anti-enzyme antibody, and drug substance probing with HRP-conjugated anti-Fc antibody as control (1, green). The three polypeptides single chain Fc, fusion-Fc and enzyme are assigned with #, *, and v, respectively. Reprinted from Ref. [24], with permission from Wiley.
Since BSA aggregates were typically not observed in the tested vaccine samples, monomeric BSA was used for quantitating residual BSA. The method was applied to various drug product samples as well as to different DSP-intermediates to monitor the clearance of BSA with a lower limit of quantification of 5.2 ng/mL.

HCPs present a safety risk because they could cause immunogenicity, adjuvant activity, or proteolytic activity. Therefore, it is essential to confirm HCP clearance during purification in DSP. Challenges in HCP quantification include the low concentration, the wide dynamic range, and their detection in the presence of large excess of therapeutic protein. Removing the therapeutic protein before HCP analysis reduces the background signal from the therapeutic protein itself, however, it increases the number of sample preparation steps and time, and it induces the risk to lose HCP during sample preparation. Analysing HCP without therapeutic protein removal simplifies the workflow and eliminates loss of HCP during purification. Zhang et al. [64] evaluated the effect of mAb depletion before HCP analysis and showed that less proteins and peptides were found in a nondepleted sample, due to suppressed identification of low abundance HCP by high abundance mAb peptides. However, the non-depleted sample included 24 proteins that were not present in the depleted sample. Most of these proteins were expected as “hitchhiker” proteins that are removed along with the mAb by purification. The peptides showed a broad pl distribution, therefore, an online pH gradient fractionation using a strong cation exchange SPE monolith prepared at the tip of the capillary was introduced. Five successive elutions using buffers at various pHs were injected into the capillary. The complexity of the injected sample was decreased which improved the detection of low abundance peptides, and increased the number of proteins and peptides identified.

The removal of HC-DNA could be performed using dimophen bromide. As bromide is an anticonvulsant and sedative, it has a set low abundance peptides, and increased the number of proteins and peptides in the injected sample was decreased which improved the detection of bromide in DSP process-intermediates [19]. The high concentration of chloride present in some samples caused electromigration dispersion of the bromide peak. However, a simple water dilution of the sample was sufficient to improve peak efficiency and reduce electromigration dispersion, and bromide could be quantified accurately with an LOQ of 0.2 µg/mL.

2.5. Downstream process support

An MCE application for the determination of the physicochemical properties required for chromatographic purification is used to aid DSP process development. MAbS can be purified using affinity interactions with protein A, but for other therapeutic proteins, such an affinity purification step is not at hand. For these therapeutics, often chromatography is used for purification. To aid faster chromatographic process development, mechanistic models for process simulation can be used. This allows for large numbers of experiments to be carried out in silico, having the advantage of low developmental time and costs, and increased process understanding. In order to apply these mechanistic models, the physicochemical properties of the therapeutic proteins have to be known. For IEC purification, the steric–mass action (SMA) parameters such as equilibrium constant, protein characteristic charge, and steric factor are important. These factors can be determined by the retention volumes of single proteins in linear salt gradient IEC of different gradient lengths. Due to the high complexity of DSP process-intermediates, retention volumes for single proteins cannot be readily determined from IEC alone. To determine the SMA parameters of a single protein in a cell lysate, Kröner et al. analysed IEC fractions with microchip CGE. Per IEC run, 83 fractions were collected, desalted, and concentrated [65]. Similar to SDS-CGE, proteins were given a uniform charge-to-size ratio using the surfactant lithium dodecyl sulfate (LDS). Microchip LDS-CGE analyses were performed under reducing conditions on a LabChip GX-II device using the HT Protein Express 200 assay (~45 s/sample), allowing high throughput analysis. Quantification of single proteins in each fraction was performed using an internal calibration standard. The single proteins were assigned to the retention volume belonging to the fraction number, and the exact retention volume of the protein was determined by Gaussian peak fitting of the concentrations and the retention volumes. The SMA parameters could be determined using the retention volumes of single proteins for all gradient lengths. By using IEC and microchip LDS-CGE, relevant factors for in silico prediction of chromatographic behaviour for DSP purification of proteins in complex cell lysate were characterised.

2.6. Towards complete upstream process monitoring

The rapid expansion of the use of biopharmaceuticals requires efficient bioprocessing. In-dept understanding of the UPS and DSP process is essential to optimise the process in terms of production yield, CQAs, and production robustness. To understand how various process variables affect the CQAs or yield of therapeutic proteins, these different variables (e.g., metabolites, nutrients, pH), together with cell viability, CQAs, and titer have to be monitored continuously during the UPS process. On-line sensors for pH, pO2, pCO2, or cell density monitoring are frequently used. However, monitoring of important nutrients and metabolites is limited to only a few, including glucose, lactate, glutamine, and ammonia. The monitoring should be expanded to a broader range of nutrients and metabolites. Real-time, continuous monitoring requires on-line sampling, analysis, and data processing. CE has proven to be a valuable tool for the monitoring of titer, CQAs, and nutrients and metabolites in process-intermediates, and the possibility for miniaturisation makes it suitable for PAT.

Alhusban et al. [66] developed an on-line, near-real time analysis setup for the monitoring of UPS. In this setup, employing sequential injection-CE, samples were automatically taken from a bioreactor, led through a microscope for cell counting, and directed to an injection valve through an H-filter to prevent clogging. Valves were used to direct sample and BGE solutions to the capillary or the waste and allowed for hydrodynamic sample injection and flushing of the capillary. The setup is depicted in Fig. 4. The CE method was developed for the analysis of glucose, glutamine, leucine/isoleucine and lactate. For electrophoretic separation of glucose, a strongly alkaline BGE (pH > 12) was required. To allow for the use BGEs with such high pHs, polymethylmethacrylate (PMMA) capillaries were used. The capillary was coated with a polyelectrolyte coating. All target analytes could be separated without interference of matrix components. Over the course of 4 days, measurements were performed every 30 min. Only 8.1 mL sample was used for the total of 200 measurements, which could potentially be further reduced by optimizing dead volume and recycling of the medium solution back into the bioreactor after the H-filter.

The MCE-HPMS developed by Ramsey’s group [52–54] was commercialised as CCM analyser “REBEL” by 908Devices. The “REBEL” can analyse over 30 nutrients and metabolites in near-real-time. Although lacking the possibility for automated sampling or sample preparation, the analysis of this large number of nutrients in only minutes is a great step towards complete process monitoring. The European Union financed Horizon2020 IMI2 project iConsensus works to take it a step further, by developing a completely automated analytical control and sensing platform for continuous monitoring of the upstream process, including automated sampling, sample preparation, analysis, data processing, and
data transfer to a monitoring information database and models for automatic feedback control (https://www.iconsensus.eu/). This platform aims to correlate data from on-line sensors and at-line miniaturised quantification techniques, including MCE applications [50,56] (Fig. 5).

3. Concluding remarks

In the past decade, CE has been extensively used in biopharmaceutical upstream and downstream processing. In particular SDS-CGE and CZE showed powerful for size and concentration determination in process-intermediates. Especially CZE has proven to be very tolerable against samples with complex matrices such as CCM samples. Although in particular cIEF, icIEF, and CZE, are valuable tools for protein charge determination, only a few applications are published for charge determination in process-intermediates. For increased process understanding, analysing charge variants in process-intermediates is essential. Considering the success of CE for process-intermediate analysis, the area of CE for charge variant analysis in process-intermediates should be further explored.

The capability to handle various complex matrices, limited sample clean-up requirements, low sample volumes, rapid analysis, and possibilities for miniaturisation and automation makes CE suitable for offline process monitoring as well as for PAT on-site, real-time monitoring of the process. The small sample volumes required is especially advantage for analysis of micro-bioreactors or during clone selection. CE applications, using MCE in particular, will be further explored and developed for the continuous monitoring of USP.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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