Research Article

Extracellular vesicles concentration is a promising and important parameter for industrial bioprocess monitoring

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Extracellular vesicles (EVs) are membrane vesicles that are produced by cells to be released into their microenvironment. In this study, we present the EV concentration as a new factor for optimization of industrial bioprocess control. The release of EVs depends on many cell properties, including cell activation and stress status, and cell death. Therefore, the EV concentration might provide a readout for identification of the cell state and the conditions during a bioprocess. Our data show that the EV concentration increased during the bioprocess, which indicated deteriorating conditions in the bioreactor. This increase in EV concentration in the fermentation broth was the consequence of two different processes: cell activation, and cell death. However, the release of EVs from activated living cells had a much weaker impact on EV concentration in the bioreactor than those released during cell death. EVs and cells in the bioprocess environment were quantified by flow cytometry. The most accurate data were obtained directly from unprocessed samples, making the monitoring of the EV concentration a rapid, easy, and cheap method. These EV concentrations reflect the conditions in the bioreactor and provide new information regarding the state of the bioprocess. Therefore, we suggest EV concentration as a new and important parameter for the monitoring of industrial bioprocesses.

Supporting information available online

1 Introduction

Membrane vesicles are produced by cells for their release into the extracellular microenvironment. Upon their release, vesicles carry molecules, including proteins, lipids and nucleic acids. These vesicles can then fuse with target cells, or be taken up by them; in this way, vesicles are used to transfer biologically active molecules between cells. Through the transfer of their membranes and cargo molecules, which are enriched in specific proteins and mRNAs, vesicles are important for the metabolism of the target cells [1–3]. Such information exchange between cells is essential for cell differentiation and the coordination of different cell types in multicellular systems, and also for the control of population density in unicellular organisms. It is generally recognized that released vesicles act as multifunctional signaling complexes for the control of fundamental cellular and biological functions [4], as they are involved in the control of coordinated processes that are important for the general homeostasis of cells and cell populations [5]. These vesicles mostly have a role in communication with the neighboring cells, but some are released into biological fluids to enable communication between cells over long distances. Such vesicles are detectable in human biological fluids and cell culture supernatants [6].

Vesicles can be released into their microenvironment continuously, or they can be released according to the diverse physiological conditions of the system [5, 7]. Indeed, they are rapidly released by cells during cell

activation, such as during mechanical [8] and oxidative stress [9], carbonyl stress [10], and elevated intracellular Ca^{2+} levels [11, 12], during temperature elevation through physical exercise or exposure to external heat [13, 14], and during exposure to low temperatures [15]. Such conditions can result in a significant increase in vesicle numbers in blood samples or cell culture supernatants. On the other hand, many vesicles of various sizes are released from dead and dying cells, because apoptotic cells undergo intense morphological changes, with cell contraction and membrane blebbing.

On the basis of their size and the cell compartment from which they originate, these vesicles can be divided into exosomes (30 nm – 100 nm diameter; secreted from multi-vesicular bodies) and microvesicles (MVs; 100 nm – 1 μm diameter; through budding from the plasma membrane). During apoptosis, other vesicles known as apoptotic bodies (ABs; 500 nm – 5 μm diameter) arise from blebs in the cell membranes. Indeed, it can be difficult to distinguish between exosomes, MVs, and ABs; therefore, all of these classes of vesicles are collectively referred to as extracellular vesicles (EVs) [16, 17]. In this study, we focused on vesicles that can be detected by flow cytometry. Due to their small size, we were not able to detect exosomes using flow cytometry; however, the general term EVs is used.

Precise monitoring and control of bioprocesses is highly desirable; however, the most commonly measured variables during a bioprocess (e.g. pH, temperature, dissolved oxygen, cell density, cell viability) provide some information regarding the conditions within the bioreactor, but little information regarding the cell fitness. As the release of EVs depends on various properties of a cell, including cell activation, stress status, and cell death, we hypothesized that the amount of EVs released within a bioprocess system can be used for the determination of the cell state and the conditions during a bioprocess. Therefore, EV quantification might represent an ideal tool for bioprocess monitoring. Our data show that EV concentrations increase during a bioprocess, and that these reflect the conditions present in the bioreactor.

2 Materials and methods

2.1 Cell culture

A proprietary cell line expressing a recombinant monoclonal antibody was used. The cell line was derived from a Chinese hamster ovary (CHO) cell line, as one of the most commonly used cell lines in the pharmaceutical industry. Cells were grown in a stirred bioreactor tank with a working volume of 5 L. A proprietary chemically defined medium with D-glucose as the main carbon source was used. The CHO cell seeding density was $0.4 \pm 0.1 \times 10^6$ cells/mL. The bioreactor was run at 36.5°C, pH 7.0, and 50% dissolved oxygen. A feed that comprised glucose and amino acids was added to the culture on days 3, 5, 7, 9, and 11. As the cells were grown in serum-free medium, an ultra-centrifugation step at $100\,000 \times g$ [18] to separate out the vesicles from serum EVs was not necessary.

2.2 Sample collection and processing

Samples were obtained from the bioprocess on several cultivation days. The total cell numbers and cell viability were measured using a Vi-Cell device (Beckman Coulter). Additionally, the cell viability of some bioprocesses was determined by standard procedure of flow cytometry and propidium iodide (Sigma-Aldrich) staining.

The cells were removed from the cell culture supernatant by multi-step centrifugation (Eppendorf 5810R): $200 \times g$ for 10 min at 35°C, $500 \times g$ for 10 min at 35°C, and $3000 \times g$ for 15 min at 35°C. The cells were removed with care immediately after the collection of the samples. For the samples with concentrated EVs, 5 mL supernatant was further centrifuged at $26\,000 \times g$ for 30 min at 18°C. The pelleted EVs were washed in 4 mL phosphate-buffered saline (PBS), pelleted again by centrifugation, and resuspended in 500 μl PBS.

To identify EVs, PKH67 green fluorescent kits were used (Sigma-Aldrich). The intercalating dye PKH67 was experimentally determined as providing the brightest labeling of the vesicles [19]. The pelleted EVs were resuspended in 10 μL PBS and 40 μL diluent C was added. The solution with EVs was added to 50 μl diluent C with 0.75 μL PKH67. After a 3-min incubation, 400 μL Iscove's modified Dulbecco's medium with 10% filtered bovine serum albumin was added. Then, 4 mL PBS was added, and the samples were centrifuged at $26\,000 \times g$ for 30 min at 18°C. The labelled EV pellets were washed in 4 mL PBS, pelleted again by centrifugation, and resuspended in 500 μl PBS.

2.3 Flow cytometry analysis

For optimal monitoring of EVs, a special flow cytometer optimized for nano-sized particles is recommended, or at least a cytometer with strong lasers that enable increased intensity of signals. Most of the flow cytometric data acquisition and analysis were performed using an Altra flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA), with a high-power 488-nm laser (200 mW, water-cooled), a standard optical filter set-up, and the forward scatter (FS) threshold set at low. The FS and side scatter (SS) parameters were set at logarithmic gain. A minimum of 1×10^4 events was collected for each sample. The Beckman Coulter software EXPO32 was used for analysis of the data. These data are given as two different ratios: the ratio between the number of events

corresponding to the EVs and the number of events corresponding to the beads, and the ratio between the number of events corresponding to the EVs and the number of events corresponding to the cells.

The concentrations of EVs in the samples were measured according to MACS Quant (EVs/µl) or according to Altra (the concentration of EVs, relative to the beads). Twenty microliters of calibrating beads at a known concentration (1.05×10^6 beads/mL) and of a known size (10 µm; Flow Count, Beckmann Coulter) were added to samples of 380 µL.

3 Results

3.1 The most accurate determination of the concentration of extracellular vesicles (EVs) in the fermentation broth were obtained by unprocessed samples

Dying cells release significant numbers of vesicles of various sizes, and therefore, dead and dying cells strongly affect the quantity and quality of EVs present in the samples. As it is very difficult to distinguish these vesicles from live-cell-derived EVs by current protocols, we analyzed them together with live-cell-derived EVs, as a marker for bioprocess monitoring.

Most studies of vesicles in cell culture supernatant involve centrifugation of the samples to remove the cells and to concentrate the vesicles. However, high-speed centrifugation can affect cells, and large vesicles might eventually break up into smaller vesicles. Therefore, we measured EVs in three sample types:

(i)   direct samples from the fermentation broth without any processing;
(ii) cell culture supernatant after the cells had been removed by centrifugation;
(iii) samples of isolated and concentrated EVs.

All of these methods gave the same patterns in terms of the increased EV concentrations during the bioprocess. However, sample type (i) was the sampling of choice, as it was rapid and easy. Sample type (ii) included three centrifugation steps, and sample type (iii) included five centrifugation steps; each additional centrifugation step adds time onto the analysis, and leads to a reduction in the EV concentration in the samples. Our data show that after the two additional centrifugation steps in the concentrated samples (iii) there were about 80% less EVs/µL than in the supernatant samples (ii). Although the amounts of EVs in the samples were measured relative to the beads, it can be seen from Supporting information, Fig. S1 that greater difference of the amount of EVs was detected between bioprocess day 6 and bioprocess day 12 by sample type preparation (i) then by sample type preparation (ii) or (iii).

3.2 The concentration of extracellular vesicles (EVs) increases during the bioprocess

Samples were harvested from the bioreactor or a shaking flask on different days through the duration of the bioprocess. Our data show that the absolute number (and hence concentration) of EVs in the fermentation broth increased significantly during the bioprocess. Similar results were obtained in six independent experiments, and representative data are shown in Figs. 1 and 2. The profiles of the critical bioprocess parameters (total cell density, viable cell density, pharmaceutical product) in relation to the EV concentrations are shown in Fig. 1. In comparison to the beads, the EV concentration increased ~37-fold from cultivation day 6 to cultivation day 14. The cell concentration also increased significantly during the bioprocess; however, the EV concentration increased much more. The data for the simultaneous detection of EVs and cells during the bioprocess using flow cytometry are shown in Fig. 2. In comparison to the cells, the EV concentration increased seven to 10-fold from cultivation day 6 to cultivation day 14 (Fig. 2B). On bioprocess cultivation days 6, 7, and 8, the ratio of the EV concentrations to the cell concentrations increased only slightly; but the ratio of the EV concentration to the cell concentration increased significantly by the end of the bioprocess. However, the concentration of dead cells increased significantly by the end of the bioprocess; therefore, we compared the EV and dead-cell concentrations (Fig. 2C). The relationship between EVs and dead cells was low and constant (approximately 3.5 EVs/dead cell) by the end of the bioprocess (days 11, 12, 13, 14). In comparison to the concentration of dead cells, the concentration of EVs was high in the earlier days of the bioprocess (days 6, 7, 8; approximately 10 EVs/dead cell), which had further
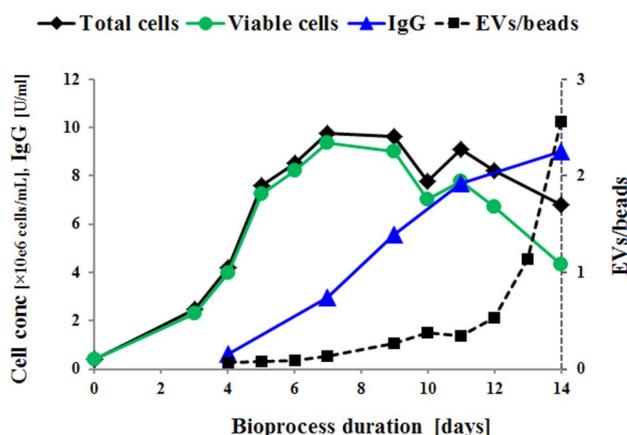

Figure 1. Growth profile (total cell density and viable cell density) and recombinant monoclonal antibody (mAb; IgG) production during batch cultivation in relation to the concentration of extracellular vesicles (EVs). The EV concentration is given by the ratio between the number of events corresponding to EVs and the number of events corresponding to beads (EV/beads). Representative data are shown.
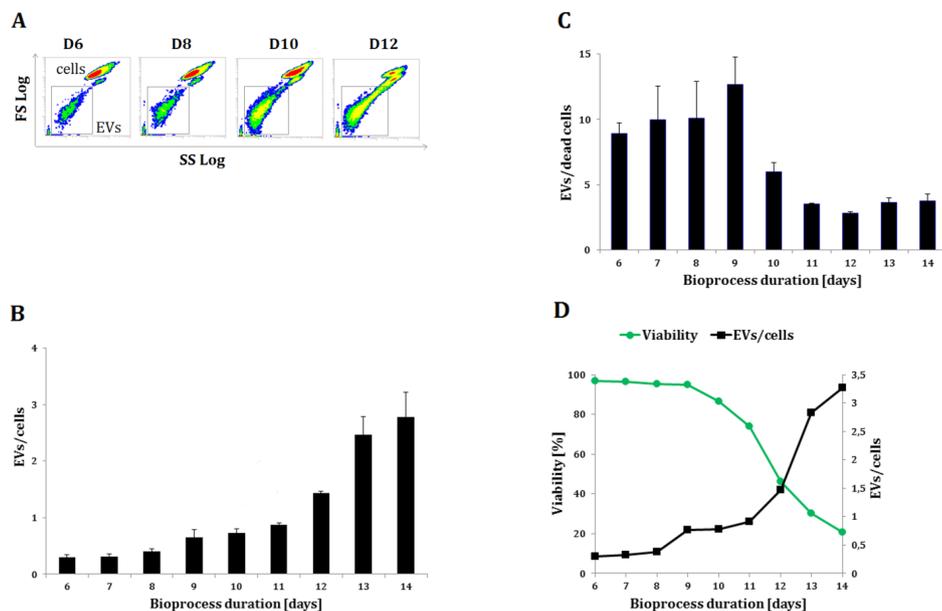
Figure 2. Flow cytometry analysis of samples from the bioprocess. The measurement of unprocessed samples from the fermentation broth is shown. (**A**) Representative forward scatter (FS) versus side scatter (SS) plots of the samples from the bioprocess are shown, as seen across day 6 (D6) to day 12 (D12). (**B**) Quantification of EV concentration relative to cell concentration (EVs/cells) through the duration of the bioprocess. Data are means ± standard deviation (SD) from three independent experiments. (**C**) Quantification of EV concentration relative to dead-cell concentration (EVs/dead cells) through the duration of the bioprocess. Data are means ± SD from three independent experiments. (**D**) Quantification of EV concentration relative to cell concentration (EVs/cells) in comparison to cell viability through the duration of the bioprocess. Representative data are shown.

increased by day 9 (~13 EVs/dead cell). This increase in the EV concentration was not the result of cell death, as the dead-cell concentration increased significantly only for bioprocess cultivation days 10, 11, and 12. Therefore, the formation of EVs appears to have been triggered by some other ongoing process in these cells. The graph presenting the decrease of cell viability in comparison to the increase of concentration of EVs relative to cell concentration through the duration of the bioprocess further confirmed that the increase of EV concentration is observed earlier than the drop in cell viability (Fig. 2D).

The process that led to this increase in EV concentration was the activation of the cells, as it has been known for a long time that activated cells release increased numbers of EVs. It would thus appear that the cells were activated due to lower cell fitness. However, this process that resulted in an increase in the EV concentration for the earlier bioprocess days had a much weaker impact on EV formation then the death of the cells, which was the cause of the large increase in EV concentration at the end of the bioprocess.

3.3 Confirmation of extracellular vesicles (EVs) by membrane labeling dye

FS/SS-based flow cytometry detection of EVs can be confounded by EV contaminants like protein aggregates or calcium phosphate microprecipitate [20]. To discrimi-

nate between EVs and other particles, identification of EVs using PKH67 was used. In all of the samples from the different time points through the bioprocess, and also for the samples taken on cultivation day 14, at least 95% of the events in the EV gate were fluorescent (Fig. 3). This confirmed that mostly EVs were measured by the monitoring system, and not some other EV-mimicking signals.
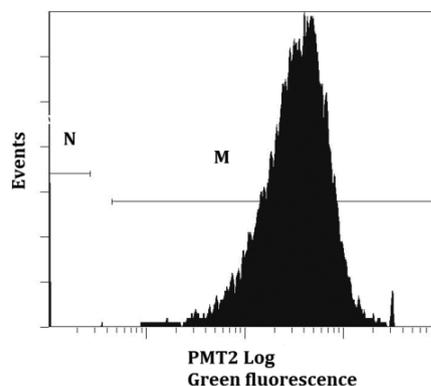

Figure 3. Flow cytometric analysis of extracellular vesicles (EVs) labeled with membrane labeling fluorescent dye (PKH67). Representative histogram of the sample from bioprocess cultivation day 12 (D12) is shown. The events in the EV gate were analyzed for fluorescence intensity of the green channel: 98% of the events in the EV gate were labeled with PHK64 (gate M), while only 2% of events in the EV gate were not fluorescent (gate N).

4 Discussion

In this study, we established the measurement of the EV concentrations as an innovative approach for bioprocess monitoring. Beside fundamental roles in the regulation of biological processes, EVs have important roles in disease pathogenesis, including many thrombotic and inflammatory conditions, and cancers, infectious diseases and neurodegenerative disorders. Recognition of their role in a variety of diseases has mostly stimulated investigations into their clinical purposes, at the diagnostic and therapeutic levels. However, here, we present EVs from a different point of view, as a tool for cell condition measurements during bioprocesses.

4.1 Improved control of bioprocesses is desirable

Precise control of bioprocesses is required to develop, optimize, and maintain biological reactors at maximum efficiency. Therefore, bioprocess monitoring represents a key aspect of the never-ending need for bioprocess optimization within industry. The conditions of the cells and the fluctuations in their growth are the results of continuous changes in the physicochemical conditions in the bioreactors. Due to the broad fluctuations in cell growth and product formation in bioprocesses, cell fitness monitoring can be highly complex. Process Analytical Technology (PAT) launched by the US Food and Drug Administration (FDA) in 2003 was introduced as a concept for improving the understanding of bioprocesses, with the tendency to develop different approaches to provide enhanced views of bioprocesses. Therefore, new parameters are highly desired for improved bioprocess control.

4.2 Concentration of extracellular vesicles as a new parameter for bioprocess monitoring

The release of EVs reflects cell activation and stress status, and death of the cells. Therefore, the EVs released can be used for the identification of the cell state and the conditions inside bioreactors. Our data showed that the EV concentration increased significantly through the duration of the bioprocess. The cell concentrations also increased during the bioprocess, although the EV concentrations increased much more. The most significant increase in the EV concentration relative to the cells occurred at the end of the bioprocess, when there were a lot of dead cells. At the end of the bioprocess, the EV concentration was proportional to the number of dead cells. Therefore, at the end of the bioprocess, when there were a lot of dead cells, the dead-cell concentration was the factor with the strongest impact on the EV concentration. In the earlier days of the bioprocess (i.e. days 6, 7, 8), the ratio between the EV and dead cell numbers was high (Fig. 2C). The EV concentration relative to the dead cells then increased further on bioprocess day 9,
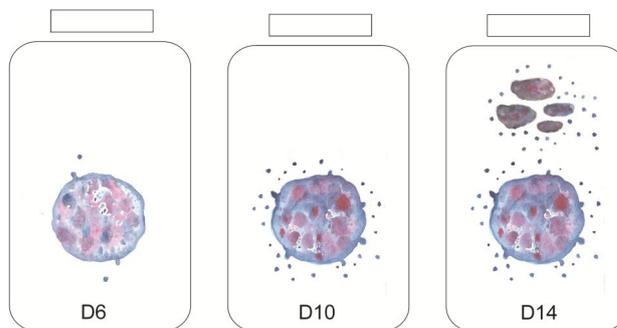

Figure 4. Schematic illustration of the increased concentrations of extracellular vesicles (EVs) during the bioprocess.

just before the increasing cell death (i.e. days 10, 11, 12). Therefore, this increase in EV concentration was not just the result of cells dying, but was the result of EV-budding processes that were triggered by the stress conditions. The increase of EV concentration is observed earlier than the significant increase of cell death (Fig. 2D) and this increase in EV concentration in the early phase of the bioprocess probably represents the prediction of the significant drop in cell viability. Throughout the duration of the bioprocess, the conditions in the bioreactor became worse because of the increased level of waste products and also because of fluctuations in nutrients. Therefore, some cells would have become stressed and they formed increasing numbers of EVs to remove misfolded proteins and metabolic waste from the cell. The stressed cells could exchange information with other cells in the bioreactor by EVs; as a consequence, the metabolism of target cells could be modified.

However, the formation of EVs from activated cells had a much weaker impact on the EV concentration in the bioreactor than when the cells were dying, which was the cause of the large increase in EV concentration at the end of the bioprocess. Our data thus show that increasing EV concentrations in the bioreactor are the consequence of the two processes, decline in cell fitness and enhanced cell death, which both indicate the deteriorating conditions inside the bioreactor. The method described here allows the measurement of a new biological parameter that provides new information regarding the cell and bioprocess conditions (Fig. 4). By combining this methodology with current monitoring methods, enhanced bioprocess monitoring can be achieved. As the release of EVs is a general characteristic of cells (e.g. for normal cells of multicellular organisms, for malignant cells, and for unicellular organisms), the measurement of EVs can be used under different cell culture conditions, and for all types of cell lines and microorganisms.

4.3 Flow cytometry as an approach for measuring the amount of extracellular vesicles (EVs)

Flow cytometry is an ideal technique for high-throughput quantification and multiparameter characterization of individual cells and particles [21], and it is the most widely used method to detect and quantify EVs. Although EVs smaller than 200 nm and exosomes cannot be detected by most conventional flow cytometers, as they cannot be distinguished from instrumental noise, it is possible to measure the larger EVs. The size, number, and content of these larger EVs can thus be analyzed by flow cytometry, defining this as a highly usable methodology. The complexity of bioprocesses requires frequent and rapid insight into their dynamic processes to allow efficient and effective control. Also, as for other flow systems, EV monitoring by flow cytometry can be adapted for continuous measurements, such that online monitoring can be achieved.

4.4 Monitoring of the concentration of extracellular vesicles (EVs) is a rapid, easy, and cheap method

For the analysis, two methods of sample preparation were also tested in comparison with measurements of the unprocessed samples: EVs from the supernatant following cell removal, and the isolated and concentrated EVs. Neither the cell removal nor the isolation of the EVs provided new information over the analysis of the unprocessed samples. Therefore the unprocessed samples can be measured directly by flow cytometer. This is a big advantage over the use of clinical blood samples, where isolation of the EVs is necessary because of the complexity of the samples. At the same time, a large number of EVs was lost during centrifugation necessary for EV isolation and concentration, which further supports the measurement of these samples without any further processing. This measurement of unprocessed samples directly from the fermentation broth makes the monitoring of EV concentration a very rapid, easy, and cheap method.

4.5 Concluding remarks

Thus, in this study we present a new method for optimized bioprocess control: bioprocess monitoring via EV quantification using flow cytometry. We have used the concentration of released EVs for identification of the cell conditions during the bioprocess. Our data show that the EV concentrations reflect the conditions in the bioreactor and provide new information on the state of the bioprocess. Therefore, we suggest that EV concentration can be used as a new and important parameter for ongoing assessment of such bioprocesses.

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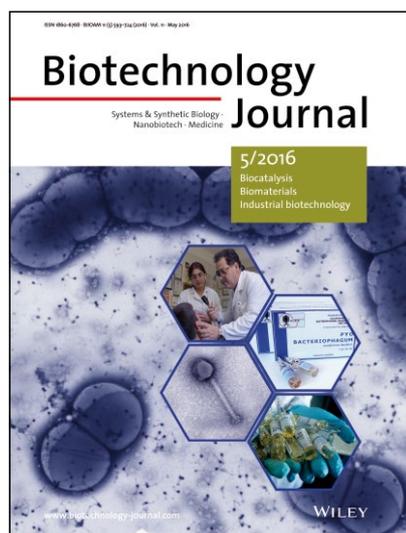
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