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RESEARCH ARTICLE

Scale-down of CHO cell cultivation from shake flasks based on oxygen mass transfer allows application of parallelized, non-invasive, and time-resolved monitoring of the oxygen transfer rate in 48-well microtiter plates

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Abstract

Cultivating Chinese hamster ovary (CHO) cells in microtiter plates (MTPs) with time-resolved monitoring of the oxygen transfer rate (OTR) is highly desirable to provide process insights at increased throughput. However, monitoring of the OTR in MTPs has not been demonstrated for CHO cells, yet. Hence, a CHO cultivation process was transferred from shake flasks to MTPs to enable monitoring of the OTR in each individual well of a 48-well MTP. For this, the cultivation of an industrially relevant, antibody-producing cell line was transferred from shake flask to MTP based on the volumetric oxygen mass transfer coefficient (k_La). Culture behavior was well comparable (deviation of the final IgG titer less than 10%). Monitoring of the OTR in 48-well MTPs was then used to derive the cytotoxicity of dimethyl sulfoxide (DMSO) based on a dose–response curve in a single experiment using a second CHO cell line. Logistic fitting of the dose–response curve determined after 100 h was used to determine the DMSO concentration that resulted in a cytotoxicity of 50% (IC50). A DMSO concentration of 2.70% ± 0.25% was determined, which agrees with the IC50 previously determined in shake flasks (2.39% ± 0.1%). Non-invasive, parallelized, and time-resolved monitoring of the OTR of CHO cells in MTPs was demonstrated and offers excellent potential to speed up process development and assess cytotoxicity.

KEYWORDS

CHO cells, oxygen transfer rate, μRAMOS, real-time cytotoxicity, scale-down

1 | INTRODUCTION

The general application potential for small-scale bioreactors to culture mammalian cells has been reviewed by Bareither and Pollard, 2011.[1] For scale-down of suspension cultures, including Sf-9 insect cells, so-called TubeSpin reactors, the use of which was first published in a scientific journal in 2004, are frequently used.[2,3] For TubeSpin reactors, successful scale-down from stirred tank reactor cultivations has already been demonstrated for mammalian cell cultures.[4] For these orbitally shaken cylindrical bioreactors, which are typically not baffled,

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exceptionally high gas transfer rates were determined.[5,6] Beside TubeSpin reactors, microtiter plates (MTPs) are among the cultivation systems used for mammalian cell cultivation. Compared to shake flask cultures, cultivation in MTPs enhances throughput and enables the parallel variation of several different parameters (e.g., media ingredients) at the same time, ultimately increasing process efficiency.[7,8] Additionally, costs for media are reduced, as filling volumes in MTPs tend to be several factors lower than in shake flasks. Moreover, by utilizing robotic platforms, seeding, feeding, or sample withdrawal from MTPs can be automated.[9,10] Consequently, the cultivation of Chinese hamster ovary (CHO) cells has been carried out in different MTPs to enhance process development and characterize cell lines. Mora et al., 2018 utilized 24-deep well plates to enhance cell line development of different monoclonal antibody (mAb)-producing CHO cell lines.[11] Deshpande and Heinzle et al., 2004 used 96-well plates to determine the cell-specific oxygen consumption rate and establish a viability assay for CHO cells.[12] Markert and Joeris et al., 2019 utilized 24-well MTPs in fed-batch experiments for clone selection using CHO-K1 cells.[9

For prolonged cultivation in MTPs, evaporation needs to be considered. Differences regarding temperature distribution and evaporation are found between the center wells of the MTP and the outer wells. These are called edge effects.[14] Evaporation in general and differences in evaporation caused by edge effects have been cited as issues during the cultivation of CHO cells in MTPs.[15] Evaporation can be reduced by choosing an appropriate cover or sealing foil.[16] In addition, for 96-round well plates and 96-square deep well plates, problems with the oxygen supply of CHO cultures have been reported.[17,18] Moreover, a comparably high critical shaking frequency is required in 96-well plates to overcome the surface tension.[7] However, high shaking frequencies result in high hydromechanical stress.[19] Consequently, a trade-off between throughput, hydromechanical stress, and evaporation usually needs to be found. As a result, 48-well MTPs might represent a suitable choice for CHO cells as filling volumes are larger than in conventional 96-well plates, but throughput is increased compared to 24- and 6-well plates. In addition, 48-well plates have been suggested as a compromise between 96-well plates and shake flasks for microbial cultivation.[20

1.1 | Oxygen transfer is a critical scaling parameter

Results obtained from cultivation in 48-well MTPs need to be comparable to later process development stages. However, it is typically impossible to keep all engineering parameters constant during scale-up.[21] Consequently, the critical parameter that will influence, for example, the product yield most, needs to be identified and kept as constant as possible.[16] Oxygen transfer has been recognized as one of the critical scale-up parameters for mammalian cells.[22–24] Oxygen transfer is described by the maximum oxygen transfer capacity (OTR_{max}) [mol L^{-1} h^{-1}] and the volumetric oxygen mass transfer coefficient (k_La) [h^{-1}]. At the OTR_{max}, the dissolved oxygen tension (DOT) is close to 0%. Consequently, the measured OTR becomes a horizontal line when a culture has reached the OTR_{max}. To avoid an oxygen limitation, which often induces undesirable metabolic effects, such as byproduct formation, or, in more severe cases, cell death, the DOT is typically controlled (e.g., kept at 30% or above) in stirred tank reactors. However, the DOT is typically not controlled in shaken small-scale cultivations. Accordingly, knowledge about the OTR reached by the culture and the reachable OTR_{max} in small-scale cultivations is necessary. With knowledge of the OTR_{max} [mol L^{-1} h^{-1}], it is possible to calculate the k_La [h^{-1}], if the oxygen solubility (L_{O2}) [mol L^{-1} bar^{-1}], the oxygen concentration in the gas phase (y_{O2}) [–] and the absolute pressure (p_R) [bar] are known (Equation 1).

$$k_La = \frac{OTR_{max}}{L_{O2} \cdot p_R \cdot y_{O2}{}^*} \tag{1}$$

Scale-up based on a constant k_La is a key scaling criterion for mammalian cell cultures[22] and using OTR_{max} is a well-known critical scale-up parameter from shake flasks to stirred tank.[25] Besides, model-based workflows have been established to scale CHO cell cultivation from 24-deep well plates to shake flasks.[26

1.2 | Determination of OTR_{max} and k_La in shake flasks and microtiter plates

An empirical correlation to calculate the OTR_{max} in shake flasks for a wide range of operating conditions was presented for microbial cultivations.[27] The respective formula is given below (Equation 2) with comprising osmolality (Osmol) [Osmol kg^{-1}], shaking frequency (n) [rpm], filling volume (V_L) [mL], shaking diameter (d_0) [cm], maximum flask diameter (d) [mm], reactor pressure (p_R) [bar], and oxygen mole fraction in the gas phase (y^*_{O2}) [mol mol^{-1}].

$$OTR_{max} = 3.72 \cdot 10^{-7} \cdot Osmol^{0.05} \cdot n^{\left(1.18-\frac{Osmol}{10.1}\right)} \cdot V_L^{-0.74} \cdot d_0^{0.33} \cdot d^{1.88} \cdot p_R \cdot y^*_{O2} \tag{2}$$

Characterization of the gas–liquid mass transfer in 48-well MTPs with a round geometry was performed for a wide range of operating parameters.[20] From these data, an empirical equation for calculation of the k_La [h^{-1}] depending on the filling volume (V_L) [μL], the shaking frequency (n) [rpm], and the shaking diameter (d_0) [mm] was later derived (Equation 3).[28

$$k_La = 4.39 \cdot 10^{-4} \cdot V_L^{-0.68} \cdot n^{2.35} \cdot d_0^{0.95} \tag{3}$$

The equation is valid for filling volumes of 300–600 μL well^{-1}, shaking frequencies of 100–1000 rpm, and shaking diameters of 2–50 mm. Further, an equation to determine the critical shaking frequency required to achieve an increase of the OTR with increasing shaking frequencies, was determined for 96-well plates.[7] This equation was demonstrated to be also applicable for 48-well MTPs.[20] Later, an equation for calculating the OTR_{max} and the k_La in 48-well MTPs with different geometries was determined.[29] For calculation of the OTR_{max} [mol L^{-1} h^{-1}], the relative diameter of the well (Peri = 1

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for 48-well MTPs with round geometry), the shaking frequency (n) [s^{-1}] and the filling volume (V_L) [L] were considered at a fixed shaking diameter (d_0) of 3 mm (Equation 4).[29] From the OTR_{max}, the k_La can again be calculated using Equation (1).

$$OTR_{max} = 2.5 \cdot 10^{-7} \cdot Peri^{6.0} \cdot V_L^{-0.64} \cdot n^{2.37} + 3.3 \cdot 10^{-4} \quad (4)$$

In addition, a CFD-based k_La model that agreed well with experimental data was established to estimate k_La values and describe shear stress in 96- and 6-well MTPs.[18]

The increased throughput of MTP cultivations compared to shake flasks will only prevail if the wells do not have to be sacrificed for manual sampling. In addition, either the sterile barrier needs to be breached, or the whole plate must be removed from the shaker for sampling under aseptic conditions. Thus, combining cultivation in MTPs and non-invasive monitoring techniques is especially desirable.

1.3 | Time-resolved monitoring of the OTR in microtiter plates

Non-invasive monitoring leaves the culture undisturbed while ideally providing time-resolved and quantitative information on culture behavior. For monitoring mammalian cells, the oxygen uptake rate (OUR) is an essential parameter to provide insights into culture behavior.[30] If the oxygen concentration in the liquid does not significantly change during measurement, the OUR equals the oxygen transfer rate (OTR). The validity of this assumption for CHO cell culture cultivations in shake flasks has been discussed previously.[31] Monitoring of the OTR has been established for different CHO suspension cultures in shake flasks using the Respiration Activity Monitoring System (RAMOS) and the Transfer-Rate Monitoring (TOM) system.[31–33] In both devices, electrochemical sensors are used.[34,35]

Monitoring of the OTR was introduced for microbial cultivation in 48-well MTPs with a round geometry and termed "μRAMOS."[36] Here, an oxygen-sensitive fluorescent dye is embedded in a matrix.[36] This "sensor spot" measures the oxygen partial pressure in the gas phase. The luminescence of the fluorescent dye is reduced in the presence of oxygen depending on the partial pressure.[37] The Stern–Volmer equation links the luminescence quenching and the oxygen partial pressure.[38] Each well of the MTP is separately gassed. A measurement and a gas flow phase are alternated to determine the OTR. During the gas flow phase, each of the wells is gassed with a specific aeration rate. An aeration rate of 0.125 vvm was previously found to result in similar CHO cell behavior compared to shake flasks passively ventilated by a cotton plug.[31] During the measurement phase, each well's inlet and outlet valve is closed.[36] The respiration activity of the cells results in a linear decrease of the oxygen partial pressure in the headspace of the well or shake flask.[34–36] This linear decrease is then used to calculate the OTR.[34,35]

Monitoring of the OTR in MTPs for mammalian cells, more particularly CHO suspension cells, is of interest for a wide variety of applications, including media development, cytotoxicity tests, and clone screening. Taking cytotoxicity tests as example, several replicates and concentrations are usually investigated, to accurately evaluate a test substance's cytotoxicity. Calculating the cytotoxicity of different test substances from time-resolved and non-invasive OTR data was previously demonstrated in shake flasks.[33] However, throughput in shake flasks is limited, as culture volumes are comparably high and the capacity of the incubator limits parallelization. Consequently, time-resolved determination of cytotoxicity in MTPs is highly desirable to enhance throughput and reduce costs for media. In order to apply OTR measurement in MTPs to CHO suspension cells, the culture conditions need to be scaled down to MTPs with culture behavior maintained to previously used shake flasks. Accordingly, this study aims to (i) perform a scale-down for two different CHO suspension cell lines from shake flasks to 48-well MTPs and (ii) determine the OTR time-resolved in 48-well MTPs. Finally, the cytotoxicity of dimethyl sulfoxide (DMSO) as a test substance is determined to give an application example. The overall motivation for this study is to enable time-resolved monitoring of the OTR of CHO cells in 48-well MTPs and to demonstrate its application potential with an example.

2 | MATERIAL AND METHODS

2.1 | Cell lines and culture media

Two different CHO suspension cell lines originating from different sources were used. The first cell line is an industrial cell line developed by Rentschler Biopharma SE.[39] The second cell line is a CHO suspension cell line obtained from Cell Lines Service (CLS) GmbH, Germany. CHO cell line one produces an mAb. Differences in mAb producing and non-producing CHO cell lines regarding growth and substrate consumption have been reported.[40] In previous experiments, both cell lines also showed a different susceptibility toward $CoCl_2$.[33]

Cultivation was carried out in serum-free, chemically defined liquid culture media for both cell lines. Cell line one was cultured in PowerCHO 2 serum-free, chemically defined medium (Lonza AG, Switzerland). Cell line two was cultured in sciNX serum-free medium (CLS GmbH, Germany). Both media were supplemented with 1% v/v PenStrep and 6 mM L-glutamine (cell line one) or 5 mM L-glutamine (cell line two) were added. Before use, media and supplements were preheated to the cultivation temperature for about 30 min in a water bath (VWB2 12, VWR, USA). The L-glutamine stock solution (Gibco Life Sciences, Thermo Fisher Scientific, USA) had a concentration of 200 mM, the PenStrep stock solution (Sigma–Aldrich, USA) contained 10,000 Units mL^{-1} penicillin and 10 g L^{-1} streptomycin.

2.2 | Cryopreservation, thawing, and cell passaging

Detailed information for cryopreservation, thawing, and cell passaging is given in Ihling et al., 2021 (cell line one) and Ihling et al., 2022 (cell line two). For cryopreservation, cells were mixed with the spent medium from cultivation (45% v/v), fresh culture medium (supplemented with

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TABLE 1 Overview of the experiments presented in this study.

Parameter	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5–8
Cultivation system	250 mL SF	250 mL SF and 48-round well MTP	250 mL SF and 48-round well MTP	48-round well MTP	250 mL SF
Medium	PowerCHO 2 with 6 mM L-glutamine and 1% PenStrep	PowerCHO 2 with 6 mM L-glutamine and 1% PenStrep	sciNX with 5 mM L-glutamine and 1% PenStrep	sciNX with 5 mM L-glutamine and 1% PenStrep	sciNX with 5 mM L-glutamine and 1% PenStrep
Figures	1	2, Figure S2	3, 4, 5, Figure S3	3, 4	Figure S4
CHO cell line #	1	1	2	2	2
Filling volume (V_L) [mL]	90	50 (SF)/1 (MTP)	50 (SF)/2 (MTP)	2	50
Shaking frequency (n) [rpm]	100	140 (SF)/650 (MTP)	140 (SF)/650 (MTP)	650	140
Shaking diameter (d_0) [mm]	50	50 (SF)/3 (MTP)	50 (SF)/3 (MTP)	3	50
Device for OTR acquisition	RAMOS (SF)	No OTR acquisition	TOM (SF) μRAMOS (MTP)	μRAMOS (MTP)	TOM (SF) RAMOS (SF)
Duration of measurement phase [min]	20	No OTR acquisition	18 (SF) 40 (MTP)	20	18 (Exp. 6–8) 36 (Exp. 5)
Duration of gas flow phase [min]	40	No OTR acquisition	42 (SF) 20 (MTP)	40	42 (Exp. 6–8) 84 (Exp. 5)
# of passages	2	4	2	7	Variable (4–21)

CHO, Chinese hamster ovary; MTP, microtiter plate; OTR, oxygen transfer rate; RAMOS, Respiration Activity Monitoring System; SF, shake flask; TOM, Transfer-Rate Monitoring.

L-glutamine and PenStrep) (45% v/v), and DMSO (10% v/v). Afterwards, cells were frozen under defined conditions using a Nalgene Mr. Frosty freezing container (Thermo Fisher Scientific, USA). Cells were stored in the vapor phase of liquid nitrogen until thawing. Cells from one cryovial were rapidly thawed and resuspended in the culture medium. One washing step was included to remove DMSO.

All passages were carried out in unbaffled 250 mL Corning polycarbonate flasks (Sigma–Aldrich, USA) with a filling volume of 50 mL (cell line one) or 20 mL (cell line two). The flasks were closed with a vent-cap and cultured at a temperature of 36.5°C. A shaking frequency of 140 rpm and a shaking diameter of 50 mm was used. Cultures were placed in an incubator with CO_2 and humidity control (ISF1-X, Kuhner AG, Switzerland). Set-points for CO_2 and humidity were 5% and 70%, respectively. The seed viable cell concentration (VCC) for subsequent passages was set to 0.2×10^6 mL^{-1} (cell line one) or 0.25×10^6 mL^{-1} (cell line two). Cells were passaged every 3–4 days.

2.3 | Main culture cultivation conditions

An overview of the experiments presented in this study is given in Table 1. The initial VCC was set to 0.2×10^6 mL^{-1} (experiment 1) and 0.5×10^6 mL^{-1} (experiments 2–8), respectively, and cultivation was carried out at 37°C in all cases.

For cultivation in shake flasks, if not stated otherwise, a shaking diameter of 50 mm, a filling volume of 50 mL, and a shaking frequency of 140 rpm were used. Cultivation was carried out in the RAMOS or TOM device described below to determine the OTR. Manual sampling and the determination of offline parameters were carried out from a flask cultured in parallel under the same conditions as the RAMOS flasks.

CHO cell line one was cultivated in 48-well MTPs with a round geometry (MTP-R48-B, m2p-labs GmbH, Germany). The plate was incubated in an incubator (ISFX-1, Kuhner AG, Switzerland) with the CO_2 concentration set to 5% and the relative humidity set to 70%. Each well of the MTP was filled with 1 mL of inoculated medium. The MTP was sealed with a sterile cover (F-GPR48-10, m2p-labs GmbH, Germany). A shaking diameter of 3 mm and a shaking speed of 650 rpm were applied.

Cultivating CHO cell line two in MTPs with monitoring of the OTR was carried out in 48-well MTPs with a total volume of 3.4 mL per well (MTP-R48-B, m2p-labs GmbH, Germany). The plate was sealed with a sealing foil (HJ 900371-T) (HJ-Bioanalytik, Germany) to ensure sterility. A filling volume of 2 mL per well was used for cultivation. Cultivation was performed at a shaking speed of 650 rpm and a shaking diameter of 3 mm.

2.4 | Simultaneous measurement of OTR and DOT in shake flasks

For a parallel measurement of the OTR and the DOT, the cultivation was carried out at 100 rpm and 90 mL filling volume to make sure that the oxygen sensor spots used for DOT measurement were covered with culture medium at all times. One oxygen sensor spot ("optode") (OXSP5) (PyroScience, Germany) was glued into each flask and the glass flasks were afterwards autoclaved for 20 min at 121°C. The sensor spots were then calibrated according to the manufacturer's

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guidelines using ambient air. As an oxygen meter, a FireSting-Pro device with a fiber optic was used for signal detection (PyroScience, Germany).

2.5 | Monitoring of the oxygen transfer rate

Cultivations in shake flasks were carried out with non-invasive monitoring of the OTR. Two different devices, an in-house built RAMOS and a commercial Transfer-Rate Online Monitoring (TOM) system (Kuhner AG, Birsfelden, Switzerland), were used. The working principle of both devices is similar. Cells in the RAMOS device were incubated in a temperature-controlled incubator (ISF1-X, Kuhner AG, Switzerland) and aerated from a gas bottle (5% CO_2 in synthetic air). Cultivation in the TOM device was carried out using the air from the incubator (70% relative humidity [r.H.] and 5% CO_2) (ISF1-X, Kuhner AG, Switzerland). Details for the duration of the measurement and gas flow phases are given in Table 1. OTR data from both devices were previously demonstrated to be comparable for the cell line used in this study.[32]

Monitoring the OTR in MTPs was carried out in a 48-well μRAMOS prototype device described previously.[36] Each well was gassed individually with 0.125 vvm of 5% CO_2 in synthetic air from a gas bottle using a gas-tight cover. The gas flow and measurement phase duration are given in Table 1. Even though gassing was slightly different between flasks and MTPs (monitored flasks and MTP in μRAMOS device: 5%CO_2, 19.5% O_2, 0% r.H., offline MTP in the incubator: 5% CO_2, 19.1% O_2, 70% r.H.), culture behavior in shake flasks at both conditions was previously demonstrated to be comparable.[32] All measurement values that deviated more than 30% from the previous measurement value were considered outliers and excluded from evaluation.

2.6 | Addition of DMSO for cytotoxicity evaluation

DMSO (≥99.7%, Merck KgA, Germany) was added from a sterile solution to the medium to reach the desired concentration. All concentrations are given as v/v. Afterwards, cell culture from a passage flask was added, and the inoculated medium was distributed into the different wells of the MTP.

2.7 | Determination of cytotoxicity and IC50 value

For comparison with previously published data from shake flasks,[33] the cytotoxicity was determined by dividing the OTR measured after 100 h of cultivation for the treated culture by the OTR measured after 100 h for the control culture (Equation 5).

$$\text{Cytotoxicity}_{t=100\,\text{h}}\,[\%] = \left(1 - \frac{\text{OTR}_{\text{treated},t=100\,\text{h}}}{\text{OTR}_{\text{control},t=100\,\text{h}}}\right) \cdot 100\% \quad (5)$$

A dose–response curve was obtained by plotting the DMSO concentration in logarithmic form against the calculated cytotoxicity. Afterwards, a 4-parameter logistic fitting was carried out (Equation 6) in OriginPro 2020 (OriginLab Corporation, USA) to obtain the concentration of DMSO at which a half-maximum response of the cells was observed (IC50).

$$\text{Cytotoxicity}\,[\%] = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2 \quad (6)$$

In Equation (6), A_1 is the minimum cytotoxicity in the absence of the test substance, A_2 is the maximum cytotoxicity reached, x_0 represents the test substance concentration that results in a cytotoxicity of 50% (IC50), and p represents the slope at the steepest part of the dose–response curve.

2.8 | Analysis of offline parameters

For analysis of offline parameters, either 1.5 mL of culture broth was removed under sterile conditions from the same flask (cultivation in shake flasks) or one complete well was sampled at each time point (cultivation in MTP). Analysis of VCC and viability was performed using culture broth. The culture broth was centrifuged (5 min, room temperature, 6000 rpm, mini centrifuge Rotilabo, Carl Roth, Germany). The cell-free supernatant was used for the determination of the pH value. The remaining cell-free supernatant was stored at −20°C until further analysis. For glucose, lactate, IgG, and glutamine analysis, the supernatant was thawed, sterile-filtered (0.2 μm cut-off) and analyzed.

2.8.1 | Determination of VCC and viability

The VCC was determined by the trypan blue exclusion method using manual counting in a Neubauer counting chamber (Counting chamber C-Chip Neubauer improved, Carl Roth, Germany). Live and dead cells were manually counted, and the values from four quadrants were averaged to determine the VCC and the viability.

2.8.2 | Value

The pH value was determined at room temperature with an InLab Easy pH electrode (Mettler Toledo, Germany). A CyberScan pH 510 meter (Eutech Instruments, Thermo Scientific, Germany) was used. The pH was determined after the sample was removed from the flask. As CO_2 quickly gasses out from the sample, the determined pH will be higher than experienced by the cells under cultivation conditions. However, the trend in pH over time is expected to be comparable, as the offset caused by gassing with CO_2 is assumed to be constant.

2.8.3 | Glucose and lactate

Glucose and lactate concentrations were determined by high-pressure liquid chromatography (HPLC). The filtered supernatant was trans-

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ferred into a vial. An organic acid resin column (Rezex ROA-Organic Acid H^+ [8%], 300 × 7.8 mm, Phenomenex Inc, USA) was used for separation. Analysis was carried out with a Thermo Scientific Ultimate 3000 RS system (Thermo Scientific, USA). The system was equipped with a refractive index detector (RefractoMax 520, Shodex, Germany). As the mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.8 mL min^{-1}. Separation was carried out at a temperature of 60°C.

2.8.4 | IgG and glutamine analysis

The supernatant for IgG and glutamine analysis was prepared as described in Section 2.8. IgG and glutamine concentrations were determined with the Cedex Bio metabolite analyzer (Roche, Switzerland).

Due to technical reasons and the low available sampling volume, the oxygen and carbon dioxide concentrations were not measured in manually taken samples.

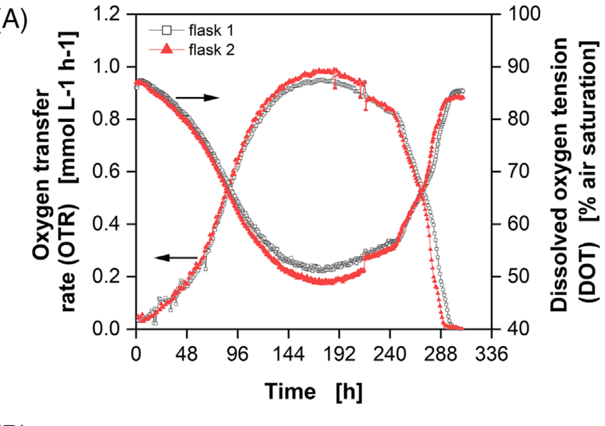
3 | RESULTS AND DISCUSSION

3.1 | Comparison of OTR$_{max}$ and k_La values in shake flasks and MTPs for selecting cultivation conditions in MTPs

To compare the OTR$_{max}$ at different cultivation conditions, the sulfite oxidation method has been extensively used and characterized.[41,42] For the cultivation conditions used (n = 140 rpm, V_L = 50 mL), the OTR$_{max}$ is calculated to 6.18 mmol L^{-1} h^{-1} (Equation 2, Table S1). This corresponds well to the OTR$_{max}$ determined experimentally (6.78 ± 0.29 mmol L^{-1} h^{-1}) (Figure S1). However, the OTR$_{max}$ reachable in cell culture medium may differ from the OTR$_{max}$ determined using the sulfite system. Possible reasons for this include different medium osmolalities, a reduced oxygen partial pressure in the cell culture cultivation, but also media components that affect oxygen transfer being present in culture medium.[43] Therefore, it was determined, how the OTR$_{max}$ calculated for the sulfite system differs from the OTR$_{max}$ in cell culture medium. By determining this "cell culture medium factor", OTR$_{max}$ values can easily be calculated or experimentally determined using the sulfite oxidation method and then corrected to culture medium using the cell culture medium factor.

To determine the OTR$_{max}$ for cell culture medium, the OTR and DOT were measured in parallel during cultivation of CHO cells in a serum-free culture medium (Figure 1). As the sensor spots used for DOT measurement had to be submersed in the culture medium the whole time to avoid erroneous measurement values, the shaking frequency was decreased to 100 rpm and the filling volume was increased to 90 mL in this experiment. Under these conditions, the OTR$_{max}$ of the sulfite system is calculated to about 2.78 mmol L^{-1} h^{-1} (Equation 2, Table S1).

Figure 1A shows the data for two shake flasks (open and closed symbols, respectively). Both cultures behave almost identical and the course of the DOT is the expected mirror image of the OTR. Under the



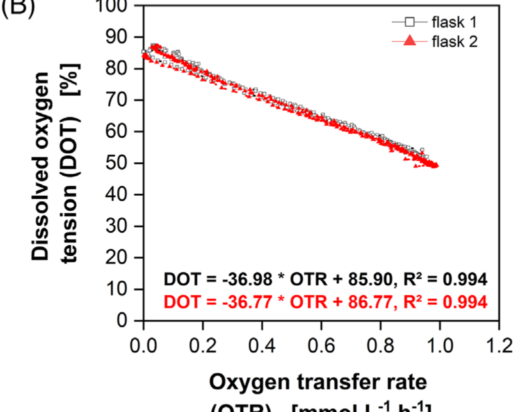


FIGURE 1 Simultaneous measurement of the oxygen transfer rate (OTR) and the dissolved oxygen tension (DOT) for Chinese hamster ovary (CHO) cell line one in shake flasks. (A) The OTR and the DOT were measured over time in two 250 mL glass shake flasks (shake flask 1: open black squares, shake flask 2: closed red triangles) with a filling volume of 90 mL and a shaking frequency of 100 rpm and a shaking diameter of 50 mm. (B) Correlation between OTR and DOT to calculate the OTR$_{max}$ (DOT = 0%). The OTR$_{max}$ is calculated to about 2.35 mmol L^{-1} h^{-1}. CHO cells with an initial viable cell concentration of 0.2 × 10^6 mL^{-1} were cultured at 37°C in PowerCHO 2 serum-free, chemically defined medium supplemented with 6 mM L-glutamine and 1% PenStrep. Data from experiment 1 (Table 1).

chosen cultivation conditions, the cultures were clearly not oxygen-limited as indicated by the DOT values, which stayed well above 40% during the whole course of the cultivation. As the sensor spots were calibrated at ambient air, but cultivation was performed in presence of 5% CO_2 and about 19.5% O_2, it is reasonable that the DOT at the beginning was at about 90%.

To estimate the OTR at which an oxygen limitation would be reached, the DOT was plotted against the OTR for both flasks (Figure 1B). A linear dependency is expected, if all other parameters are considered to remain constant (Supplementary Section "Relationship between OTR and DOT"). It should be noted that >300 data points were used for the correlation. The relationship between OTR and DOT is almost perfectly linear for both cultures. From the linear equation, the OTR$_{max}$ was calculated (DOT = 0%). The OTR$_{max}$ in culture medium under the given cultivation conditions (n = 100 rpm, V_L = 90 mL)

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was calculated to 2.35 mmol L^{-1} h^{-1}. This is about 15% lower than the OTR_{max} calculated for the sulfite system (2.78 mmol L^{-1} h^{-1}). Thus, for the original cultivation conditions (n = 140 rpm, V_L = 50 mL, $OTR_{max,\, sulfite\ system}$ = 6.18 mmol L^{-1} h^{-1}), the OTR_{max} is estimated to be about 5.25 mmol L^{-1} h^{-1} in culture medium.

Calculation of the k_La from the determined OTR_{max} values required the oxygen solubility (L_{O2}), the absolute pressure (p_R), and the oxygen fraction in the inlet gas (y_{O2}) (Equation 1). L_{O2} of the 0.5 M sulfite solution was 0.72 mmol L^{-1} bar^{-1} at 37°C,[44] for culture medium 0.91 mmol L^{-1} bar^{-1} were used, p_R was assumed to be the same as ambient air pressure (1.013 bar), and y_{O2} was 0.2095 (sulfite system) and 0.195 (culture medium), respectively. With the experimentally determined OTR_{max}, a k_La of ~42 h^{-1} was calculated for the 0.5 M sulfite system at 140 rpm and 50 mL in the shake flask (Table S1).

The k_La in the MTP is influenced by the shape of the well, the shaking diameter (d_0), the filling volume (V_L), and the shaking frequency (n).[29] For compatibility with the µRAMOS prototype for monitoring the OTR, 48-well MTPs with a round well geometry had to be used. As d_0 of the prototype device was also fixed to 3 mm, V_L and n could be varied to match the OTR_{max} reached in shake flasks. For V_L, a minimum of 1 mL was set to keep the relative evaporation in an acceptable range. For calculating the k_La of the sulfite system in MTPs, Equation (3) (model Sieben et al., 2016[28]) and Equation (4) (model Lattermann et al., 2014[29]) were used. As both equations were only validated for V_L up to 600 µL, data are extrapolated for a V_L of 1 mL in our study. Finally, the shaking frequency (n) was determined to 650 rpm to achieve k_La values of ~46 h^{-1} (model Sieben et al. 2016[28]) or ~40 h^{-1} (model Lattermann et al., 2014[29]), respectively. This determined shaking frequency is well above the critical shaking frequency. The critical shaking frequency, introduced by Hermann et al., 2003, was calculated to 288 rpm.[7] At shaking frequencies above the critical shaking frequency, the centrifugal forces are larger than the surface tension. With the determined parameters, the k_La in shake flasks and MTPs should be comparable and is expected to be around 40 h^{-1}, when calculation is based on the 0.5 M sulfite system. In addition, k_La values between 30 and 40 h^{-1} are in the same order of magnitude and at the upper end of the range of k_La values reported for TubeSpin reactors.[45]

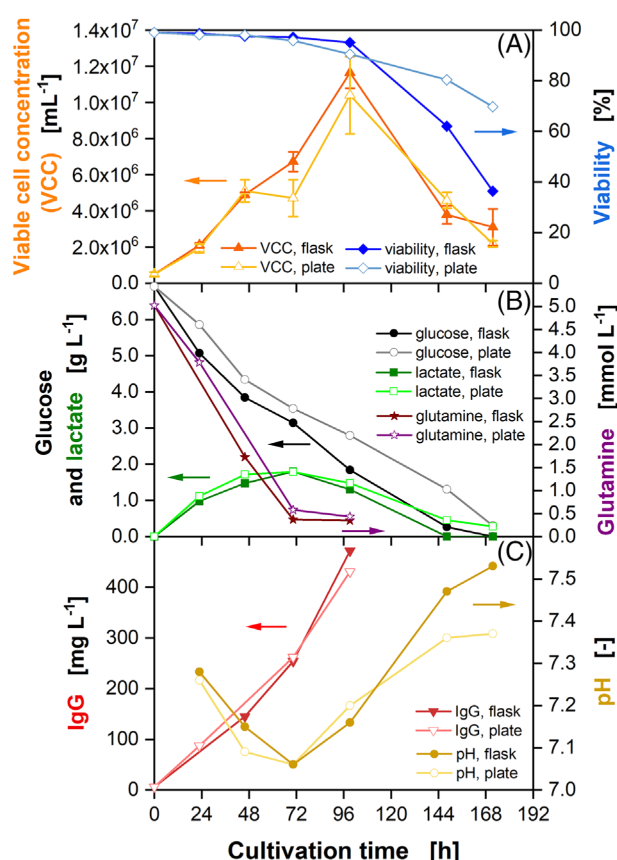

FIGURE 2 Comparison of growth behavior of Chinese hamster ovary (CHO) cell line one in a 250 mL glass shake flask (SF) and in a 48-round well microtiter plate (MTP). (A) Viable cell concentration (VCC) (SF: closed orange triangles, MTP: open orange triangles) and viability (SF: closed blue diamonds, MTP: open blue diamonds) are depicted over the cultivation time. (B) The glucose concentration (SF: closed black circles, MTP: open black circles), the lactate concentration (SF: closed green squares, MTP: open green squares) and the L-glutamine concentration (SF: closed purple stars, MTP: open purple stars) are depicted over the cultivation time. (C) The IgG concentration (SF: closed red squares, MTP: open red squares) and the pH value (SF: closed yellow circles, MTP: open yellow circles) are depicted over the cultivation time. CHO cells with an initial viable cell concentration of 0.5×10^6 mL^{-1} were cultured at 37°C in PowerCHO 2 serum-free, chemically defined medium supplemented with 6 mM L-glutamine and 1% PenStrep. Data from experiment 2 (Table 1).

3.2 | Cultivation of antibody-producing CHO cell line one in 48-well MTPs

After suitable cultivation conditions for 48-well MTPs were defined, cultivation of CHO suspension cell line one was carried out in glass shake flasks and 48-well MTPs in parallel to verify the comparability of the culture behavior. It was previously demonstrated that the material (glass or polycarbonate) did not affect culture behavior in shake flasks for this cell line in the same medium.[31] Figure 2 depicts the comparison of the offline measured parameters between the shake flask (closed symbols in Figure 2) and the 48-well MTP (open symbols in Figure 2).

As can be seen, the course of the VCC (Figure 2A) was well comparable in shake flasks (Figure 2A, closed orange triangles) and MTPs (Figure 2A, open orange triangles). The viability (Figure 2A, open and closed blue diamonds, respectively) was almost identical until 100 h. Afterwards, the viability in the shake flask (Figure 2A, closed blue diamonds) decreased faster than the viability in the MTP (Figure 2A, open blue diamonds).

In previous experiments, run under the same cultivation conditions and in the same culture medium as in this study,[31] the maximum VCC was reached after about 120 h, which is about 24 h later than in this study. The different times until the maximum VCC was reached may be attributed to different initial VCCs (VCC_0) used. While the VCC_0 was 0.2×10^6 mL^{-1} in the previous study, it was set to 0.5×10^6 mL^{-1} in this study. Given that the doubling time of CHO cells in serum free culture

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media can be in the range of 18–24 h,[46] it appears reasonable that the culture viability started to decrease earlier compared to the previous study. The glucose consumption was slightly faster in the shake flask (Figure 2B, closed black circles) than in the MTP (Figure 2B, open black circles). This slight difference in glucose consumption was likely the reason for the earlier decrease of the viability in the shake flask (Figure 2A, closed blue diamonds). Lactate production was comparable between the shake flask (Figure 2B, closed green squares) and the MTP (Figure 2B, open green squares). However, lactate consumption appeared to be slightly slower in the MTP. Consequently, the lactate concentration was higher in the MTP between 96 and 168 h. Glutamine consumption (Figure 2B, stars) was very comparable in both cultivation systems. For glutamine, the detection limit was reached for the values determined at 100 h. This indicated that glutamine was already depleted at this point. The slope of the glutamine concentration over the cultivation time also supports the assumption that glutamine was depleted after less than 100 h, most likely between 75 and 80 h.

The course of the pH value was almost identical during the whole cultivation (Figure 2C, circles). Toward the end of the cultivation, the pH in the shake flask (Figure 2C, closed yellow circles) increased a little higher than the pH in the MTP (Figure 2C, open yellow circles). The slower consumption of lactate in the MTP (Figure 2B, open green squares) explains the slightly lower pH value in the MTP toward the end of the cultivation. IgG formation between shake flask and MTP (Figure 2C, red triangles) was compared until 100 h, as the maximum VCC was reached around this time (Figure 2A, triangles). The amount of IgG formed was very similar (less than 10% maximum deviation), even despite different wells of the MTP being used for sampling at each time-point.

However, as the sampling intervals were quite irregular in the experiment, the OTR from two shake flasks monitored in parallel to the sampled flask was also evaluated (Figure S2A). The OTR was almost identical between both cultures. This strongly supports the assumption that the shake flask cultures were behaving reproducibly within the experiment.

As the monitored RAMOS flasks could not be sampled, previously established correlations to determine the VCC and the glucose concentration from OTR measurements were used (Figure S2B).[32] This way, in addition to the data from the manually sampled flask, VCC (orange squares and stars, respectively) and glucose concentration (black squares and stars, respectively) were obtained for the two additional flasks and plotted together with the data from manual sampling (Figure S2B). The calculated VCC showed the expected exponential increase that was also determined previously.[31] Given that the correlation for the VCC determined from OTR has a certain error and as manual cell counts can have errors in the double-digit range, the manually determined cell counts are in the same order of magnitude as those determined from OTR measurement.

Furthermore, the comparison of the glucose concentration calculated from the OTR in shake flasks and determined from manual sampling in shake flask and MTP (Figure S2B, black and gray curves) fits very well and the overall shape is well reflected. Even though the VCC measurements showed a large deviation in this experiment, the RAMOS data suggest that culture behavior was reproducible and that cell growth was as expected.

When comparing the OTR to the data from manual sampling, the increase of the OTR appears to flatten when glutamine is depleted. This observation needs to be investigated in further experiments to determine, if glutamine depletion may be determined from the OTR in a similar manner as demonstrated for VCC and glucose.

In summary, in the first 100 h of the cultivation, the cultivations in shake flasks and MTPs were very well comparable, when scale-down was performed based on k_La and the k_La was in the range of about 40 h^{-1}. This k_La is well comparable to that achieved in TubeSpin reactors, which are also known to provide good scalability to large scale reactors and achieving cell numbers of at least 1×10^7 mL^{-1}.[4,45] After 100 h, a slight deviation in glucose and lactate consumption occurred. The reason for this deviation is currently investigated.

With an expected OTR_{max} of 5.25 mmol L^{-1} h^{-1} reachable (Table S1) and a maximum OTR of 1.45 mmol L^{-1} h^{-1} reached (Figure S2), the DOT is expected to have been well above 50% during the entire cultivation. Additionally, a k_La value of about 8 h^{-1} was previously considered sufficient for culture densities in highly optimized stirred tanks.[45] Consequently, no oxygen limitation of the cultures was expected. Finally, culture behavior was found to be well reproducible within the experiment.

3.3 | Monitoring of the OTR of CHO cell line two in 48-well MTPs

For CHO cell line two, a thorough characterization of the OTR along with offline measured parameters has been presented previously for cultivations carried out in shake flasks.[32] Here, cultivation was transferred to MTPs.

A μRAMOS prototype device for monitoring the OTR in each well of a 48-round well MTP was used.[36] This prototype was adapted to cultivate and monitor CHO suspension cells for the first time. As the signal-to-noise ratio of the OTR depends on the ratio between filling volume and gas volume, the filling volume was increased to 2 mL in the MTP. First, the MTP was closed with a sealing foil to keep the culture sterile. Then, the sensor plate with the oxygen sensor spots was mounted on top of the MTP. During cultivation, each well was gassed with a specific aeration rate of 0.125 vvm (synthetic air with 5% CO_2).

Figure 3 depicts the OTR of CHO suspension cell line two in sciNX medium in two consecutive experiments in MTPs with the time-resolved acquisition of the OTR (Table 1). In experiment 3, three wells (Figure 3, closed cyan squares, open cyan triangles, closed cyan stars) were monitored. In experiment 4, five wells (Figure 3, closed black triangles, closed black circles, open black squares, open black stars, open black diamonds) were monitored. The monitored wells were randomly distributed on the 48-well plate to account for potential temperature differences between the wells. The duration of the measurement phase was set to 40 min (experiment 3) and 20 min (experiment 4), respectively (Table 1). As a high-flow phase followed each measurement phase to bring the oxygen partial pressure inside the well back to its level

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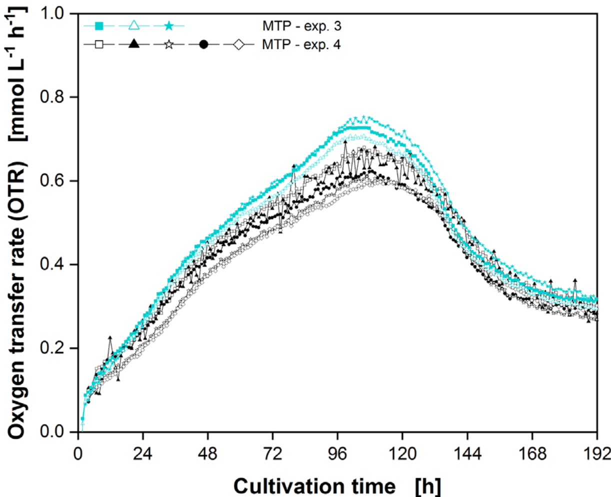

FIGURE 3 Cultivation of Chinese hamster ovary (CHO) suspension cell line two in a 48-well microtiter plate (MTP) with online monitoring of the oxygen transfer rate (OTR). The OTR was determined in triplicate (closed cyan squares, open cyan triangles, closed cyan stars) in experiment 3 (Table 1) and in quintuplicate (open black squares, closed black triangles, open black stars, closed black circles, open black diamonds) in experiment 4 (Table 1). Only every 2nd data point is shown. CHO cells with an initial viable cell concentration of 0.5×10^6 mL^{-1} were cultured at 37°C in sciNX medium supplemented with 5 mM L-glutamine and 1% PenStrep. A filling volume of 2 mL per well was used at a shaking frequency of 650 rpm and a shaking diameter of 3 mm. The plate was sealed with a sealing foil (F-GPR48-10 and gassed with an aeration rate of 0.125 vvm from a gas bottle (5% CO_2 in synthetic air).

before the measurement phase, the longer measurement phase in experiment 3 is not expected to have affected the culture behavior. However, the longer measurement phase led to a better signal-to-noise ratio of the OTR compared to experiment 4. Consequently, the average standard deviation between the replicates within one experiment over the whole cultivation time differed between 3.0% (experiment 3) and 9.1% (experiment 4). From the results, culture behavior in the MTP was regarded as quite reproducible within one experiment. In addition, culture behavior between subsequent experiments was also comparable, especially concerning the overall course of the OTR. Slight differences in the maximum OTR reached between the consecutive experiments (Figure 3) might be attributed to differences in the amount of passaged culture used to inoculate the main culture. Different amounts of passaged culture resulted in a slightly different dilution of the medium and different carryover of spent medium from the last passage. In addition, the media composition might vary slightly between different media lots. Moreover, different numbers of passages (Table 1) were carried out before inoculating the main culture. The different numbers of passages might also have affected culture behavior as was demonstrated for an antibody-producing cell line previously.[47]

The results from OTR measurement in MTPs (Figure S3, closed blue squares) were compared to a shake flask culture (Figure S3, closed blue triangles) to validate the transferability from shake flasks to MTPs. The average OTR from the two independent MTP experiments aligns well with the cultivation conducted in shake flasks. As the ratio of filling to gas volume was higher in MTPs (0.58) than in shake flasks (0.2), a better signal-to-noise ratio was obtained at the same duration of the measurement phase (Table 1).

As cultivation was only carried out in one flask and the OTR signal was rather noisy, the OTR was compared to previous and consecutive shake flask experiments (Figure S4). From five shake flasks cultivations conducted consecutively (three of them in biological duplicates) using TOM or RAMOS, it becomes apparent that differences between consecutive experiments in shake flasks were more prominent than within one experiment in shake flasks and MTPs (compare Figures S3 and S4). Differences in the value and time of the maximum OTR reached in consecutive shake flask experiments might be attributed to slight differences in the medium composition, differences in the lag phase as well as a different number of passages carried out prior to inoculation of the main culture. Differences in medium composition are likely, as experiments with a similar OTR progression (Figure S4, closed blue triangles and orange squares) were conducted in close temporal proximity.

3.4 | Monitoring of the OTR of CHO cell line two in 48-well MTPs at different DMSO concentrations for determination of the cytotoxicity

It was demonstrated previously that monitoring the OTR in shake flasks is suitable to determine the cytotoxicity of test substances.[33] The IC50, which represents the concentration of a test substance at which a half-maximum response of the cells is observed, was previously determined for different cell lines and test substances using monitoring of the OTR in shake flasks.[33] As pointed out previously, statistical analysis and a wide range of analyzed concentrations are desirable in cytotoxicity experiments to obtain reliable results. Hence, monitoring the OTR parallelized in MTPs represents an ideal opportunity for cytotoxicity testing. Consequently, the cytotoxicity of DMSO, which was previously investigated in shake flask scale,[33] was investigated using MTPs. For a more detailed discussion about the concentrations chosen, the reader is directed to ref. [33] The course of the OTR for different DMSO concentrations ($n = 3$–6) determined in MTPs is depicted in Figure 4. In the experiment, the OTR was very reproducible, as indicated by the error bars.

The culture with 1% DMSO (Figure 4, open blue diamonds) showed a very similar OTR progression as the control culture (Figure 4, closed cyan squares). However, for DMSO concentrations above 1%, the course of the OTR differed from the control culture already from the beginning. With increasing DMSO concentrations, the increase in the OTR was slower, and the maximum OTR reached was lower than the control culture. For 5% DMSO (Figure 4, open purple circles), the OTR decreased, indicating that the cells were not growing.

It was demonstrated previously that the cytotoxicity could be calculated by dividing the OTR of the treated culture by the OTR of the control culture.[33] In the previous study conducted in shake flasks, the ratio of the OTR values was calculated when the OTR of the control had

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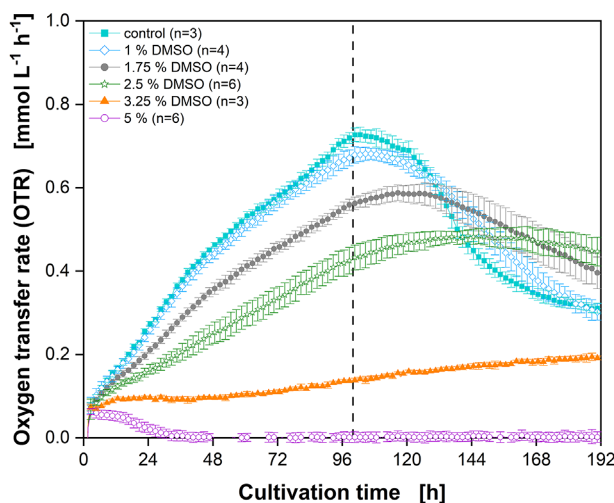

FIGURE 4 Influence of dimethyl sulfoxide (DMSO) on the oxygen transfer rate (OTR) of Chinese hamster ovary (CHO) cell line two in 48-well microtiter plates (MTPs). The OTR is depicted in presence of 0% DMSO (=control) (closed cyan squares), 1% DMSO (open blue diamonds), 1.75% DMSO (closed gray circles), 2.5% DMSO (open green stars), 3.25% DMSO (closed orange triangles), or 5% DMSO (open purple circles). The number of replicates (*n*) is given in brackets. For clarity, only every 3rd data point is shown. Data from experiment 3 (Table 1). Dashed vertical line marks a cultivation time of 100 h. CHO cells with an initial viable cell concentration of $0.5 \cdot 10^6$ mL^{-1} were cultured at 37°C in sciNX medium supplemented with 5 mM L-glutamine and 1% PenStrep. In the MTP, a filling volume of 2 mL per well was used at a shaking frequency of 650 rpm and a shaking diameter of 3 mm. The plate was sealed with a sealing foil (F-GPR-10). The MTP was gassed with an aeration rate of 0.125 vvm from a gas bottle (5% CO_2, 19.5% O_2).

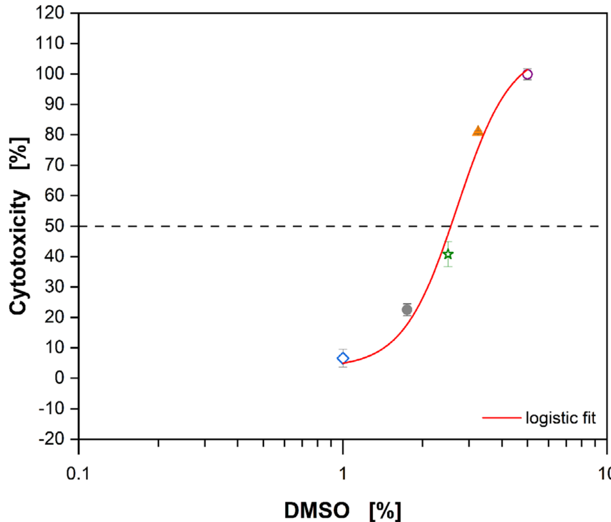

FIGURE 5 Dose–response curve for dimethyl sulfoxide (DMSO) for Chinese hamster ovary (CHO) cell line two determined from oxygen transfer rate (OTR) measurement in a 48-well microtiter plate (MTP). Cytotoxicity was calculated by dividing the OTR of the treated culture and the OTR of the control culture after 100 h (Equation 5). The course of the OTR over time is depicted in Figure 5. Data from experiment 3 (Table 1). Logistic fitting of the dose–response curve obtained from MTP data was carried out using Equation (6). Dashed line marks a cytotoxicity of 50%. An cytotoxicity of 50% (IC50) of 2.7% ± 0.25% was determined. The axes were scaled as in Ihling et al., 2022 to simplify comparison.[33]

reached its maximum.[33] In the MTP experiment, the control reached the maximum OTR after ~100 h (Figure 4, dashed line). Consequently, the cytotoxicity was calculated for each DMSO concentration at this time-point (Equation 5). From these data, a dose–response curve was determined using Equation (6) (Figure 5).

The obtained dose–response curve after 100 h showed the expected sigmoidal shape (Figure 5, open and closed symbols). In the next step, determination of the IC50 was performed by 4-parameter logistic fitting (Equation 6) (Figure 5, red line). The IC50 for DMSO was determined to 2.70% ± 0.25% from the experiment in the MTP. This IC50 is similar to the IC50 determined from the shake flask experiment (2.39% ± 0.1%).[33] Consequently, IC50 values determined in real-time based on the OTR are comparable in shake flasks and MTPs.

4 | CONCLUSIONS

This study demonstrates the successful scale-down of two CHO suspension cell cultures from shake flasks to 48-well MTPs. For an industrially relevant antibody-producing CHO suspension cell line, the cultivation from shake flask experiments was scaled-down to MTPs using the k_La as a scale-down criterion. The k_La in shake flasks was determined to ~42 h^{-1} using the 0.5 M sulfite oxidation method. Two different models previously established for calculating the k_La in MTPs were used to determine matching cultivation conditions in the MTP. Parallel cultivation was carried out in a shake flask and an MTP. The course of all offline measured parameters was comparable, but glucose consumption was slightly slower in the MTP. Nevertheless, final IgG titers were comparable (8.6% deviation). The media consumption for cultivation in MTPs was reduced 50-fold compared to shake flask experiments.

Cultivation in 48-well MTPs enabled monitoring of the OTR to follow culture progression. For a second CHO suspension cell line, the OTR was determined non-invasively and parallelized in MTPs. The course of the OTR was found to be well reproducible (average standard deviation below 10%) within one experiment. In addition, the course of the OTR was similar in consecutive experiments. Monitoring of the OTR in MTPs was used to determine the cytotoxicity of DMSO without sampling and in real-time. The DMSO concentration to reach a IC50 was determined to 2.70% ± 0.25%. This value compares very well with the IC50 previously determined for the same cell line in the same medium from the measurement of the OTR in shake flasks (2.39% ± 0.1%).[29] This way, the application potential for monitoring the OTR of CHO cells in MTPs was successfully demonstrated. Compared to the shake flask scale, the media required could be reduced at least 10-fold while keeping the temporal resolution the same. In the future, monitoring the OTR of CHO suspension cells in MTPs might also be used to rapidly optimize and screen different culture media.

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Further, the suitability of other online-monitoring devices established in this scale (e.g., the BioLector system) might be investigated for online monitoring of culture progression.

AUTHOR CONTRIBUTIONS

Nina Ihling: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing – original draft, writing – review & editing. Christoph Berg: Conceptualization, project administration, supervision, writing – review & editing. Richard Paul: Data curation, investigation, visualization, writing – review & editing. Lara Pauline Munkler: Data curation, formal analysis, investigation, visualization, writing – review & editing. Veronique Chotteau: Funding acquisition, project administration, resources, writing – review & editing. Jochen Büchs: Conceptualization, funding acquisition, project administration, supervision, writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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