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Experimental determination of maximum shear stress in Mobius® Breez perfusion microbioreactors and comparative analysis with stirred tank bioreactors

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ABSTRACT

Perfusion processes have experienced increased popularity in recent years due to their ability to sustain high cell densities and productivities in biopharmaceutical production, offering advantages over traditional batch and fed-batch cultivation methods. The Mobius® Breez microbioreactor significantly reduces experimental effort by downsizing the classical volume of perfusion bioreactors to the mL range and thus represents a valuable tool for process development. However, miniaturization has raised questions regarding comparability with traditional bioreactors in terms of the physical environment, such as hydrodynamic shear stress. Therefore, the maximum hydrodynamic shear stress, cultivation performance, and membrane-wall contact were evaluated to elucidate the system's behavior. Findings reveal two distinct operational conditions, distinguished by the presence or absence of membrane-wall contact, resulting in varying levels of hydrodynamic stress. Conditions lacking membrane contact demonstrate stress levels within safe operating thresholds for CHO cells, while those involving membrane contact exceed these thresholds, potentially leading to cell damage. Through the identification of critical frequencies of membrane motion, this study offers insights for optimizing microbioreactor operation and enhancing overall bioprocess efficiency.

1. Introduction

The ever-increasing demand for biopharmaceutical products has prompted the biotechnology industry to explore innovative approaches to improve the efficiency, productivity, and sustainability of biopharmaceutical manufacturing processes [1–3]. Process intensification, a multidisciplinary concept aimed at enhancing process performance while reducing the overall footprint, has emerged as a promising paradigm to address these challenges. In particular, perfusion for mammalian cell culture processes has become increasingly popular due to its ability to provide continuous and steady-state operation, enabling higher cell densities, extended culture durations, and enhanced yields. Additionally, this intensified approach offers greater process control, reduced operational costs, and increased flexibility in biopharmaceutical production, making it an attractive alternative to traditional batch and fed-batch processes [4,5].

However, the development of perfusion processes is time-consuming and the miniaturization of cell retention systems challenging [6,7]. The extensive use of single-use systems, despite their undeniable advantages, introduces often additional discrepancies in vessel geometry during scale-up or when transitioning from one supplier to another. As a result of difference in vessel geometries and mechanical forces introduced by the pumping device of the retention system during scale-up, unpredictable cellular responses may hinder reproducibility [8]. Hence, overcoming the challenges of scaling up intensified mammalian process necessitates a comprehensive understanding of shear stress dynamics and its impact on the cellular behavior [8–11].

Furthermore, the remarkable surge in cell and gene therapies has

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propelled the medical field into a new era of innovative treatments, promising hope for patients afflicted with previously incurable diseases [12,13]. As this field expands rapidly, a notable shift in bioprocess development away from the conventional Chinese Hamster Ovary (CHO) production processes towards Human Embryonic Kidney (HEK) for viral vector production and other lesser-studied mammalian cell lines has taken place. The concept of personalized medicine through autologous cell therapies, wherein a patient's own cells are genetically modified and re-infused, presents an enticing prospect for tailoring treatments to individual needs, minimizing adverse reactions and maximizing therapeutic efficacy [13]. However, the diversity of cellular processes intensifies the aforementioned manufacturing challenges.

The importance of physical process parameters in mammalian cell cultivation cannot be overstated, with shear stress representing just one factor of this complex landscape [11,14,15]. The dynamic environment from production systems introduces shear stress, arising from impeller rotation, sparging or pumping can significantly impact cell viability, proliferation rates, and product quality [16–18]. In turbulent flow conditions, a well-established yet simplified concept is based on the Kolmogorov scale of turbulence. According to this concept, there is a likelihood of cell damage when the size of Kolmogorov eddies, which occurs at the maximum specific energy dissipation rate, is similar to or smaller than the size of the suspended particles [19]. This principle also applies to microcarrier cultures, where potential cell damage can occur if the size of the microcarriers is comparable to or smaller than the turbulent eddies.

There are multiple methods available to analyze and characterize the stress distribution within different cultivation systems. These methods include the measurement of droplet sizes [20], laser doppler anemometry (LDA) [21], particle image velocimetry (PIV) [22] or computational fluid dynamics (CFD) predictions [23]. An alternative experimental technique using shear-sensitive poly(methyl methacrylate) (PMMA) nanoparticles has been applied to various cell cultivation systems to determine the maximum hydrodynamic stress (τ_{max}) [8–11,23–26]. The advantages of this system include multiphase flow measurements, scalability, cost-effectiveness, and comparability to a wide range of different biotechnological equipment such as pumps and bioreactors [25].

Investigating robustness for perfusion processes at laboratory scale bioreactor is possible, yet resource and time intensive [9,11,24]. The Mobius® Breez Microbioreactor system has been recently used as a promising system for an optimization study of perfusion processes, greatly reducing the experimental effort [27]. Moreover, the low volumes required for perfusion processes makes it an ideal tool to develop intensified processes for cellular therapies [28]. Numerous small-scale shear stress measurement systems have been developed, but none have been specifically tailored to a milliliter format, capable of performing perfusion processes, and possessing high-throughput capacity [29,30].

Therefore, this study aims to investigate the maximum shear stress in Mobius® Breez Microbioreactor systems at various conditions, utilizing a shear-sensitive PMMA aggregate system. By screening from gentle to harsh conditions, the measured maximum shear stress obtained via shear sensitive aggregates can be compared to microbioreactors, benchtop and large-scale bioreactors. The comprehensive overview from traditional bioprocess for recombinant protein production enable to relate process conditions from microscale to many larger bioprocess systems. As a result, the Mobius® Breez Microbioreactor system, with its perfusion capabilities and ability to explore harsh shear stress conditions, emerges as a valuable tool for probing for robustness and thus effective scale up for intensified mammalian cell culture processes.

2. Materials and methods

2.1. Microbioreactor mixing system

The microfluidic bioreactor consists of two plastic layers separated by a thin silicone membrane. The silicone membrane separates the liquid and air chambers in the plastic layers and can inflate towards either layer by sending pressurized gas or vacuum into any of the air chambers. These structures enable the integration of valves and pumps into the device as shown in Fig. 1. For more details on the operation of the microbioreactor, see reference [27].

The cell culture growth chamber consists of three membrane chambers with an air side and a liquid side separated by a silicone membrane. Each membrane chamber has a total volume of approximately 1 mL and the fluid sides are connected in a ring with short connecting channels. Fluid flow is driven by independently pressurizing the air side of each chamber in sequence while venting the two other chambers. This displaces the fluid away from the pressurized chamber and into the vented chambers, generating mixing. The mixing sequence is parameterized by setting the percentage (%) of the total mixing cycle that a section is pressurized, and the frequency (Hz) that the pressurization states change.

2.2. Shear generation in mixing system

Shear stress is generated through two primary mechanisms in the reactor as shown in Fig. 2A. Channel shear refers to the bulk fluid flow velocity gradients through the interconnecting channels and squeeze film shear refers to the stress from squeezing flow when the membrane reaches the bottom of the chamber. The short connecting channels between the large chambers are trapezoidal in shape, where at the smallest cross section has approximate dimensions of 1.22 mm. Both mechanisms are affected by the speed of membrane deflection, which is determined by the pressurization/depressurization rate of the air side. The pressurization rate of the air side (pressure chamber) is determined by a pressure source and a combination of two tunable flow resistors labelled as the back resistor (BR) and the front resistor (FR) in relation to their physical location in the controller as shown in Fig. 2B. Each pressure chamber has a tunable back resistor to compensate for differences in resistance between the independent flow paths. The front resistor provides a global tuning resistor that allows easier adjustment of the common pressurization rate of the chambers. The inflation speed of the silicone membrane in the air chamber is constrained by both the back and front resistors. These resistors limit the ability for gas to enter and exit the pressure chamber and pressurization of one chamber is coupled to depressurization of the other chambers through the liquid.

2.3. Mobius® Breez perfusion microbioreactor resistor tuning

Front and back resistors were tuned using a resistor divider setup to convert a change in system resistance to a corresponding change in pressure (SI Fig. 1). The system resistance refers to the total resistance of the flow path from the mixer chamber through the pneumatic assembly including the front and back resistors and out the common vent output to atmosphere. For resistor tuning, the microbioreactor cassette was replaced with a barb connection at each mixer chamber output and the mixer chamber outputs were used as the inputs for source pressure. When tuning each mixer chamber, the unused input barbs were blocked to prevent crosstalk via the common vent. Source pressure for the flow circuit was maintained at 29.3 kPa and a reference resistor was placed in series between the source pressure and the system resistance. The pressure between the constant reference resistor and the variable system resistance was used as a measure of the system resistance. These pressure values were adjusted by turning the screw on the tunable resistors until the desired value was obtained. Back resistors (BR) were tuned with the front resistor (FR) removed, while front resistors (FR) were

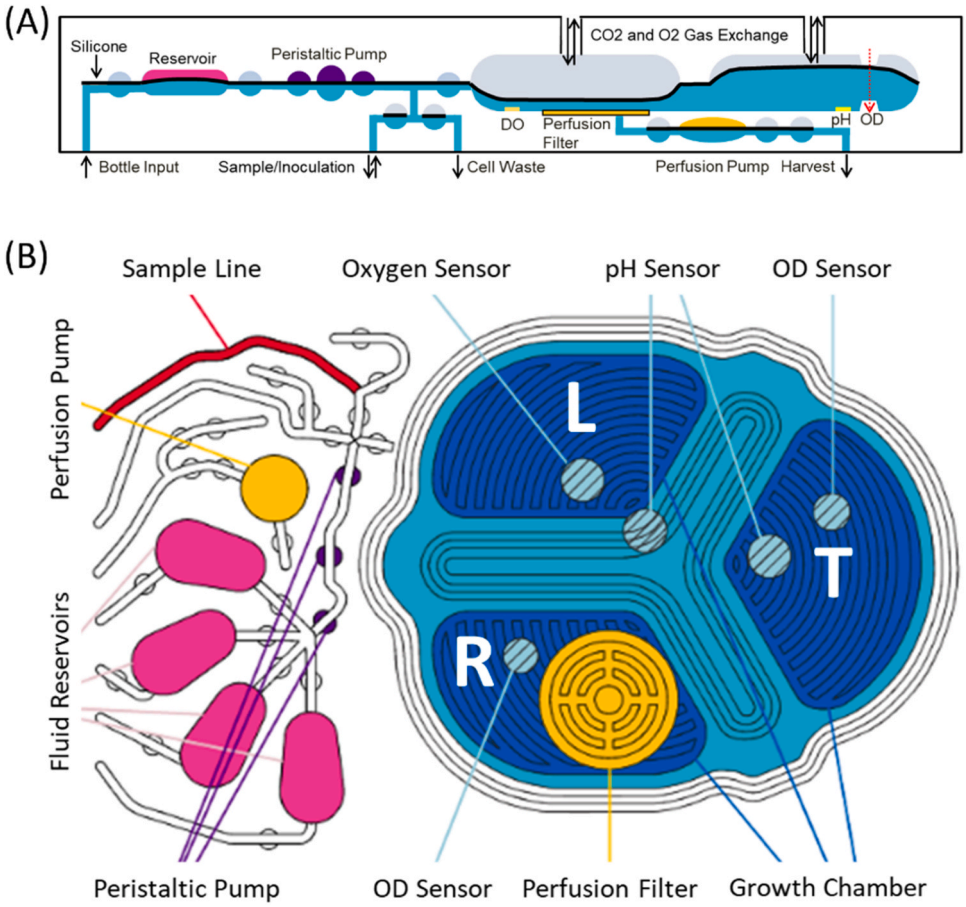

Fig. 1. Schematic representation of microfluidic device of the Mobius® Breez perfusion microbioreactor. Side view (A) and top view (B).

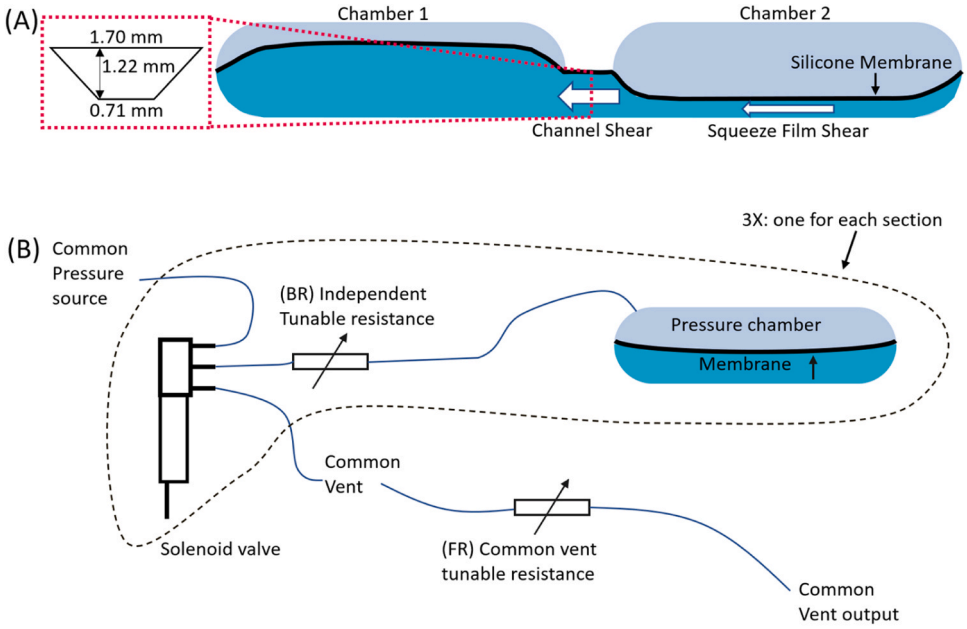

Fig. 2. Schematic representation of mixing system and shear generation (A) and schematic representation of pressure control system within each pressure chamber (B). BR: Back resistor; FR: Front resistor.

tuned after the back resistors were tuned and include the back resistance in their measured value.

Mixing conditions were chosen iteratively with initial conditions tested at the lowest and highest frequencies of 1 Hz and 10 Hz available for the system and the resistor tuned to the lowest system setting. After observing viability differences at the extreme conditions, additional

conditions were chosen to sweep frequency between the boundary cases. Additionally, resistor settings were tested between the lowest setting and the manufacturer default setting of 19.79 kPa back resistance and 20.68 kPa front resistance to explore dependency on resistor settings in visually observed non-membrane contact conditions. An overview of all tested conditions is given in Table 1.

2.4. Overall resistance calculation based on FR and BR

To convert the pressure values to resistances, the resistor divider setup was replaced with a mass flow controller directly connected to the system resistance after each tuning configuration was prepared. The mass flow controller was set to a fixed source pressure of 18.6 kPa and the resulting flow rate through the mass flow controller was measured. The resistor value was calculated as the pressure divided by the volumetric flow rate, in units of Pascals (Pa) over standard cubic centimeters per minute (SCCM) (see SI Fig. 2). The mass flow controller was also used to directly measure the resistance of the reference resistor, which resulted in a resistance value of 61 Pa/SCCM.

2.5. Mixing frequency visualization

The Mobius® Breez Microbioreactor cassette was filled with a red dye solution containing a proprietary mixture of Allura Red AC (Red 40) and Erythrosine (Red 3). Videos of cassettes were recorded with a camera at 120 fps (GoPro Hero11). Mixing percentages were set to the default (34.7 % L, 33.3 % R, 32.0 % T) (see Fig. 1C) with an initial minimal frequency of 1.5 Hz, representing the "Full Membrane Contact Case." Frequencies were increased in 0.1 Hz increments, up to 3 Hz with 4 Hz and 5 Hz also recorded (where it was obvious that there was no lamination of the silicone membrane against the bottom of the growth chamber). The degree of contact between membrane and bottom surface of the chamber was determined by analyzing videos of the left mixer chamber in each condition to determine the maximum difference in dye containing area of the chamber during a mixing cycle (see SI Fig. 3)

2.6. Chemicals and Reagents

Poly(methyl methacrylate) (PMMA) particles were synthesized using methyl methacrylate as monomer, potassium persulfate as polymerization initiator, and sodium dodecyl sulphate as surfactant. All substances were obtained from Sigma-Aldrich. Sodium chloride for particle destabilization and aggregate formation was purchased from PENTA, Czech Republic. High purity deionized water (specific conductivity 1.56 μS/cm, Aqual, Czech Republic) was used for the particle's synthesis, aggregation preparation, and breakup experiments.

Table 1
Summary of conditions used in the microbioreactor for stress and testing mixing conditions for shear stress characterization. An overall resistance based on front and back resistance was calculated according to Section 2.4.

Parameter	Frequency [Hz]	Back resistance [kPa]	Front resistance [kPa]	Overall resistance [Pa/SCCM]
C1	1	15.86	15.86	61
C2	1	19.79	20.68	104
C3	5	19.79	20.68	104
C4	8	15.86	15.86	61
C5	8	15.86	15.86	61
C6	10	15.86	15.86	61
C7	6	17.93	18.62	81
C8	5	19.79	20.68	104
C9	6	17.93	17.93	75
C10	5	19.79	22.06	125
C11	6	19.31	19.31	87
C12	5	19.79	20.68	104
C13	6	17.93	17.93	75
C14	5	19.79	20.68	104
C15	4	19.79	20.68	104
C16	3	19.79	20.68	104
C17	2	19.79	20.68	104
C18	2.3	19.79	20.68	104
C19	2.7	19.79	20.68	104
C20	5	19.79	20.68	104

2.7. Synthesis of primary particles, aggregation procedure and their breakage

The synthesis of monodisperse PMMA nanoparticles (NPs) was carried out using emulsion polymerization inside a 500 mL three-neck round bottom flask [31]. The amount of 0.61 g of sodium dodecyl sulphate was dissolved in 4.5 mL of deionized water followed by dilution to 450 mL of deionized water. The mixture was mixed at 400 RPM using a magnetic stirrer and heated to 80 °C under a nitrogen atmosphere for at least 2 hours. In parallel, 0.36 g of potassium persulphate, the polymerization initiator, was dissolved in 18 mL water under a nitrogen atmosphere. A programmable syringe pump (VIT-FIT LAMBDA, Switzerland) was used to add 48 mL of monomer methyl methacrylate into the reaction flask at a flow rate of 2 mL/min. Polymerization was then continued for two additional hours at constant temperature and stirring speed. At the end of the synthesis, PMMA NPs were purged with an additional amount of nitrogen to remove the non-reacted monomer. The synthesized NPs have an average size of 58 nm in diameter with polydispersity index (PDI) of 0.1 and a mass fraction of 11.2 %.

The aggregation process was initiated by the addition of sodium chloride solution into the PMMA NPs suspension, with the salt concentration being above the critical coagulation concentration (CCC). Sodium chloride solution, with the concentration of 600 mM, was added dropwise into the beaker containing PMMA NPs suspension with a mass concentration equal to 5 x 10^{-2}. A PTFE cross-shaped magnetic bar rotated at 50 RPM was used to promote mixing. The final concentration of salt was equal to 300 mM. To reach a dynamic equilibrium between aggregation and breakup [32,33], the aggregation procedure was continued for the next two hours, resulting in the formation of compact aggregates [32,33]. Once steady state was reached, the solution was gently diluted 50x to reduce the salt concentration well below the CCC to prevent further aggregation. The prepared aggregates were stable for the following three days under gentle mixing (50 RPM) to prevent sedimentation. Such aggregates were consequently used as shear sensitive probes to characterize maximum values of the shear stress since breakage becomes the only mechanism controlling the fragments size [25,32−34].

Breakage studies of the PMMA aggregates were performed inside a Mobius® Breez perfusion microbioreactor with 2 mL working volume. Using a programmable NE-1000 syringe pump (PumpSystems, USA), 2.7 mL of clusters were gently injected into the microbioreactor followed by extra push of 0.3 mL of water to achieve the final overfilled volume of 3 mL. Reducing the excess of liquid to a working volume of 2 mL was carried out according to the manufacturer's guidelines. Afterwards, the mixing continued until the steady-state cluster size was achieved. The sample was gently withdrawn using a programmable syringe pump into the syringe containing 50 mL of water for consequent size analysis.

2.8. Static light scattering for stress determination

The radius of gyration of the aggregates was determined by static light scattering (SLS) using Mastersizer 3000 (Malvern, UK) from measured angularly dependent intensity of scattered light. In particular, the Guinier approximation of structure factor $S(q)$ [35] defined as:

$$\ln(S(q)) = ln\left(\frac{I(q)}{I(0)P(q)}\right) \cong -\frac{q^2}{3}\left\langle R_g^2\right\rangle_{S(q)} \quad (1)$$

Here $I(0)$ is the zero-angle intensity of scattering light, $I(q)$ is the angle dependent intensity of scattering light, $P(q)$ is the form factor of primary particles and $\langle R_g \rangle$ is the mean radius of gyration. The scattering vector q contains information about the detector position and is defined as:

$$q = 4\pi \frac{n}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (2)$$

where n represents a refractive index of the dispersed fluid, λ is the wavelength of the laser in vacuum and θ is the detector location in terms of the scattering light. The radius of gyration was obtained from the linear relationship between $\ln\left(\frac{I(q)}{I(0)}\right)$ and $\frac{q^2}{3}$ via Eq. 1 [36].

The mean steady-state aggregate size $\langle R_g \rangle$ is related to the applied hydrodynamic stress during the mixing period in the perfusion microfluidic reactor. During the mixing period, the aggregates are exposed to hydrodynamic stress caused by membrane deformation, thus their size is related to the applied frequency and back/front resistance of the device. This motion prompts the destabilization process of the aggregates, and they begin to break until reaching a new steady-state size. It means that aggregates have passed through the zone with the harshest conditions and thus the highest value of the hydrodynamic stress in the microfluidic cassette can be determined. Based on the calibration between mean steady-state size $\langle R_g \rangle$ and maximum hydrodynamic stress τ_{max}, it is possible to determine the value of shear stress inside the mixer chamber (SI Fig. 4). Calibration measurement of aggregate strength was investigated by a breakup experiment, where the suspension containing the aggregates was pumping through a contact nozzle with known flow rate, as discussed in our previous work [37]. All measurements for each pod are performed in triplicate.

2.9. Cell culture expansion and inoculation

A proprietary CHO-K1 cell line producing a bispecific monoclonal antibody was expanded in an incubator (Multitron 4, Infors HT, Bottmingen, Switzerland) for 21 days (36.5 °C and 5 % CO_2) using a proprietary chemically defined medium (Merck Serono SA, Corsier-sur-Vevey, Switzerland). On the day of inoculation, cell culture was pelleted for 5 min at 500 g and resuspended in cell culture media at a target viable cell volume (VCV) of 10 %. For inoculation, cell culture was transferred to an inoculation bottle, which was sequentially welded (Terumo SCD IIB, Terumo BCT, Lakewood, Colorado, USA) to all four perfusion bioreactors of the Mobius® Breez Microbioreactor system (EMD Millipore Corporation, Stoneham, MA, USA, an affiliate of Merck KGaA, Darmstadt, Germany). Gentle inoculation was performed by gravity according to the manufacturer guidelines.

2.10. Perfusion cell culture in Mobius® Breez microbioreactor system

Perfusion bioreactors of the Mobius® Breez Microbioreactor system (EMD Millipore Corporation, Stoneham, MA, USA, an affiliate of Merck KGaA, Darmstadt, Germany) were inoculated at target VCV of 10 %. Culture conditions were maintained at 36.5°C with a dissolved oxygen (DO) setpoint of 50 %. The pH was controlled at 7.07 ± 0.2 by CO_2 addition to the headspace above the gas-permeable membrane and a 1.1 M Na_2CO_3 solution. Bioreactors were operated at 2 mL working volume and perfusion was started on day 0 and kept constant at 1.3 reactor volumes per day (RV/day). The built-in optical density sensor (OD) was used to maintain the target VCV of 10 % by bleeding excessive biomass. Several mixing conditions were chosen to characterize the mixing of the Mobius® Breez Microbioreactor system (Table 1). A daily sample of 280 µL was taken from each bioreactor for at-line measurements.

An initial screening was performed to evaluate the cultivation time required to reach stable cell viability as read-out for shear characterization. These conditions covered gentle as well as very harsh shear conditions (C2, C3, C4 and C18 in Table 1). Cultivation duration for harsh conditions was reduced to 3 days due to low viability read-outs leading to higher turbidity which prevented maintaining the steady state at 10 % VCV.

2.11. Analytical methods for cell culture

Cell culture parameters such as cell density, cell viability, cell diameter, and pH were measured using a BioProfile FLEX2 (Nova Biomedical, Waltham, USA). Viable cell volume (VCV) was calculated as follows [38]:

$$\mathrm{VCV} = \frac{4}{3}\pi\left(\frac{D}{2}\right)^3 VCD \quad (3)$$

where D is the average cell diameter and VCD the viable cell density, assuming a spherical shape of the cells.

3. Results

Maximum values of the shear stress were determined using shear sensitive PMMA aggregates. An example of the measured data is presented in Fig. 3A, where structure factor $S(q)$ measured by a static light scattering instrument is plotted as a function of scattering wave vector q. As indicated by the arrow, with time there is a shift of the $S(q)$ curve towards the right. Since bending part of the $S(q)$ corresponds to the mean aggregate radius of gyration, observed shift indicates reduction of the aggregates size to lower values. This is caused by the breakup of initially larger aggregates to smaller fragments in time. As suggested by the data, after approximately 4–6 hours the breakup process is stopped and new steady state is reached, characterized by smaller size of produced fragments. Despite aggregate breakup their internal structure is not affected as supported by similar value of fractal dimension d_f (i.e., slope of the power law region of $S(q)$ vs. q curve). As indicated by the solid dotted lines, during the breakup process d_f is constant and equal to 2.8, confirming compact internal structure of generated aggregates [37]. A summary of the measured aggregates sizes corresponding to three values of the pumping frequency from Table 1 is presented in Fig. 3B. Depending on the frequency used, a different mean radius of gyration of the fragments is achieved. In particular, the size in the steady state is comparable at 2.3 and 5 Hz, while at 1 Hz a drastic reduction in the fragments can be observed, which indicates a much higher breakup and thus a higher stress. In all cases, steady state was reached after approximately 4 h. Therefore, a characterization of the stress in the frequency range from 1 Hz to 10 Hz was carried out in the following in order to cover the conditions specified in Table 1.

While stress determination is crucial for optimization of the hydrodynamic conditions in the bioreactor, it is important to relate hydrodynamic conditions to the cell behavior during cultivation. Hence, in parallel to the stress determination, CHO cells cultivations in the prefusion microreactor were also performed covering a range of frequency conditions (see Table 1). An example of selected cultivation results in terms of cell viability is presented in Fig. 4A. In the case of low frequency of 1 Hz, there is a sharp decrease of the viability during the first 2 days of cultivation, followed by a recovery phase. Similar observations were also reported by other authors for the cultivation of CHO cells in stirred and sparged bioreactors [9,24,26]. On the other hand, high frequency cultivations exhibit only a minor impact of the conditions on the cell viability. Cell diameter and viable cell volume were evaluated as additional parameters (Fig. 4B and Fig. 4C). However, regardless of the conditions applied, these two values showed no significant difference. To demonstrate that the change in cell viability was caused by the hydrodynamic stress and not by the change in pH during cultivation, the time profile of pH calculated as an average value for C2 and C3 cultivations performed is depicted in Fig. 4D. A complete summary of VCV, metabolites and pH for all tested cultivations are presented in SI Fig. 5.

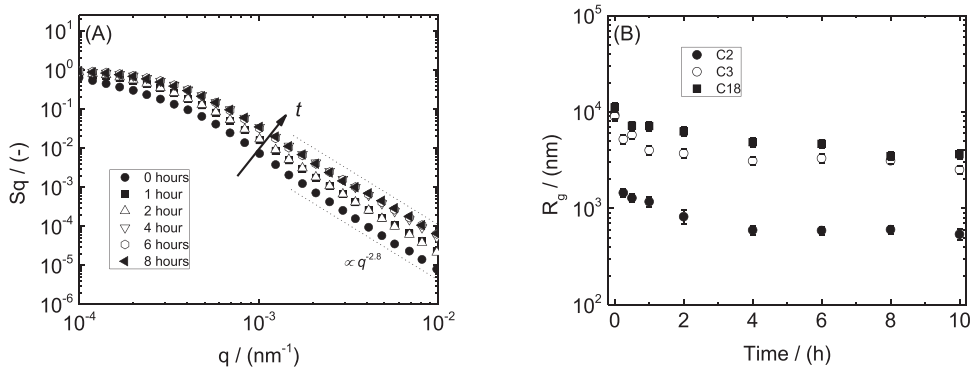

Fig. 3. Log-Log plot of structure factor as a function of scattering vector amplitude at different times measured for frequency of 5 Hz, i.e., C3 experiment in Table 1 (A). Time evolution of mean radius of gyration for clusters during aggregate breakage using different frequencies (B).

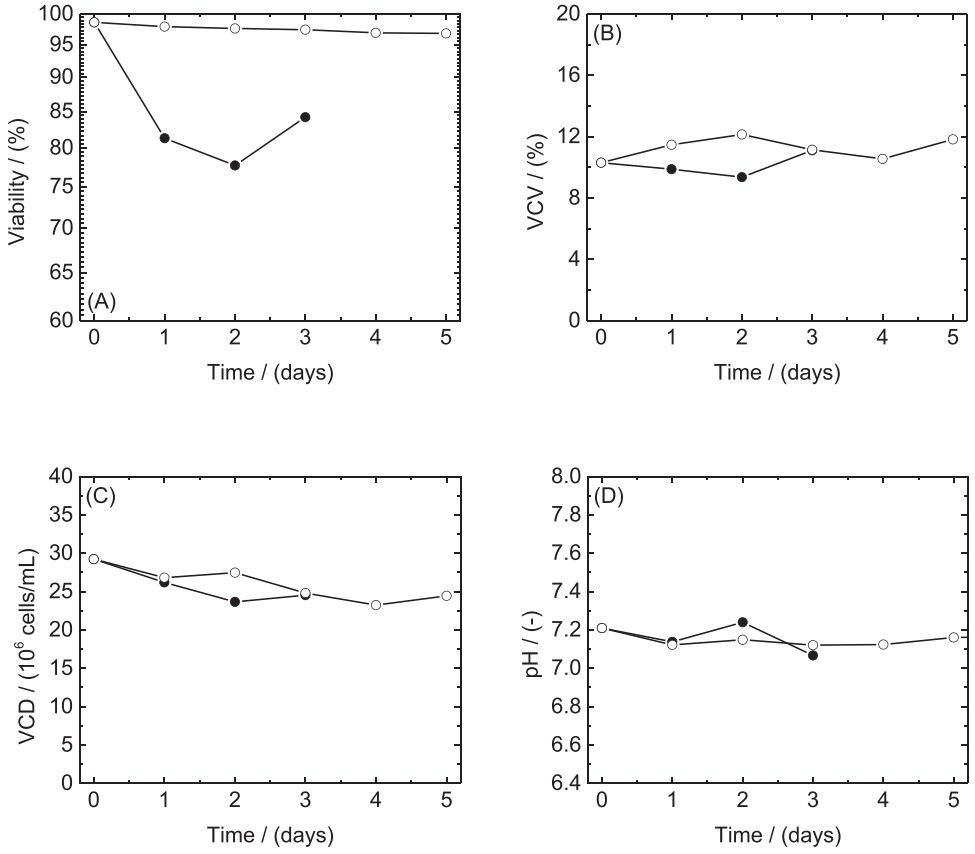

Fig. 4. Perfusion cell culture screening to define necessary time to reach stable viability response (A). VCV trends (B), VCD trends (C) and pH (D) for screening conditions described in Table 1. C2 and C3 conditions correspond to closed and open symbols, respectively.

As can be seen, the pH values do not change during cultivation and fluctuate around the set point values of 7.2. Based on the above presented data, we can conclude that the change of the cell viability during the early stage of the cultivation is a robust indicator for harsh or mild cultivation conditions and will be used in the following analysis.

All the measured results including the determination of shear stress, measurement of the viability drop during early stage of the cultivation, and determination of chamber-membrane contact (i.e., squeeze film shear) are presented in Fig. 5. If the pumping frequency is reduced below 2.3, the maximum shear stress becomes increasingly higher and reaches values of up to 100 Pa when pumping at 1 Hz (see Fig. 5A). Above a frequency of 2.3 Hz, the stress is approximately constant and covers a range between 4 and 6 Pa. Previously determined stress thresholds for mammalian cells are in the range from 25 to 41 Pa [9,10,24,26,39], thus suggesting that cells cultivated in the microbioreactor would experience much lower values. On the other hand, the hydrodynamic stress can be increased to higher values by simply changing the pumping frequency, if required. This observation is consistent with the viability values measured during cell cultivation, where a threshold between mild and harsh conditions of 2.7 Hz was determined (see Fig. 5B). Below this value, the viability drops sharply, while it remains between 92 % and 98 % above the critical frequency determined. The difference between the viability after three days is significantly lower when exposed to harsh conditions (<2.7 Hz) compared to gentle conditions (>2.7 Hz) ($p < 0.01$, see SI Fig. 6). In agreement with these two observations is also the percentage of chamber contact at which the determined critical frequency is equal to 2.7 Hz. (see Fig. 5C). Below this threshold, the membrane comes into contact with the bottom wall of the chamber,

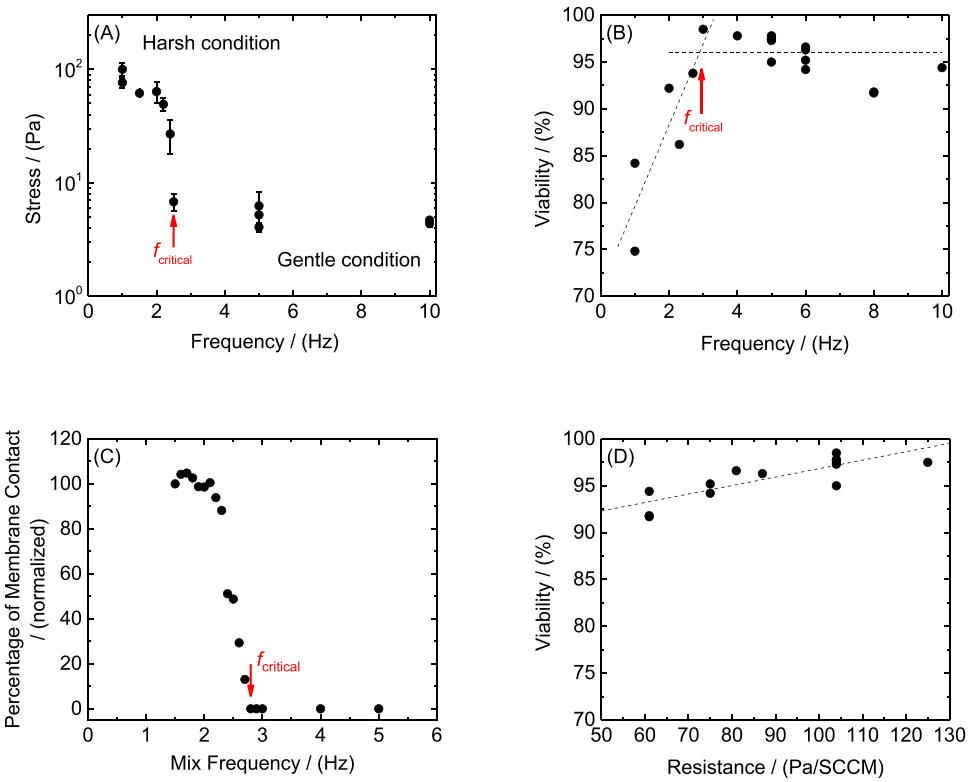

Fig. 5. Hydrodynamic shear stress evaluation to determine critical frequency settings based on: PMMA method (A), viability at day 3 of a perfusion cell culture (B), and mixing dye test (C). Effect of overall resistance, defined by FR and BR settings, on viability for conditions with frequency above $f_{critical}$ of 2.7 Hz (D).

leading to significant cell damage.

All these independent measurements indicate that critical frequency demarcating mild from harsh conditions is equal to 2.7±0.2 Hz. Above this value, the microbioreactors provide mild stress values and high cell viability. To confirm this, the values of the cell viability for various values of the front and back resistance are plotted in Fig. 5D and converted into overall resistance (see Table 1) for cultivation conditions with pumping frequency above 2.7 Hz. Fine tuning of the conditions is possible when higher values of the overall resistance are kept at highest values, characterized with almost no drop of viability. In contrary, even lower values of the overall resistance would not lead to significant drop in cell viability.

In contrast to the widespread use of stirred and sparged bioreactors for cell cultivations, the use of perfusion microbioreactors has just recently started and hydrodynamic comparison between these systems are still scarce. Thus, we aim to compare various cultivation systems employed for mammalian cell cultivations, encompassing various measured stirred bioreactors from 15 mL to 12,000 L. Notably, the maximum shear stress of all these bioreactors was determined using the same shear sensitive PMMA aggregate system. An overview of the experimentally determined shear stresses is presented in Fig. 6. The evaluated critical shear stress is indicated for three different CHO cell lines [9,10,24,26,39]. It is worth noting that only the single-phase measurements are reported, and the results should be treated with caution as the cultivation conditions involve multiphase flow where high shear stresses can occur in the gas-liquid interphase, e.g. at the gas inlet or when bubbles burst. All stirred bioreactor systems cover a continuous shear stress range from single units of Pa up to 100 Pa. However, the Mobius® Breez Microbioreactor (2 mL) operates in two distinguished ranges, the first around 4–6 Pa and a second range at much higher values 30–100 Pa.

In the context of scale-up, determining the maximum shear stress is critical to establishing a safe operational limit, especially when considering geometrically non-similar single-use bioreactor systems.

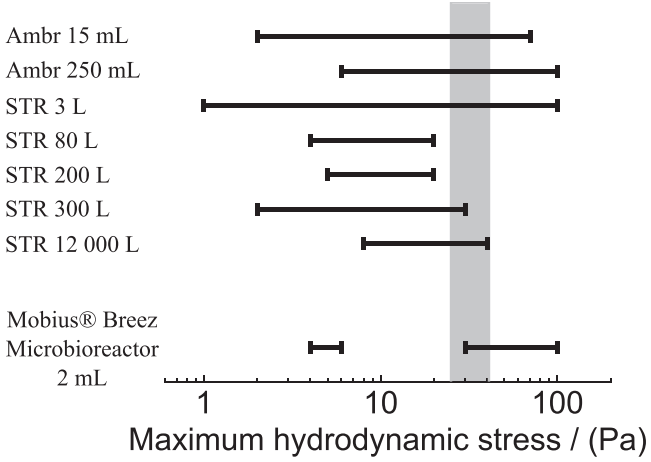

Fig. 6. Comparison of the hydrodynamic stress ranges reachable in various systems characterized by PMMA shear. The grey bar represents measured shear stress thresholds for CHO cells in different bioreactors. Ambr 15 mL [23], Ambr 250 mL [24], Bench top bioreactor (3 L) [42], Pilot scale bioreactor (80 L) [43], Pilot scale bioreactor (200 L) [43], Pilot scale bioreactor (300 L) [10], Production scale bioreactor (12,000 L) [9,43], Mobius® Breez Microbioreactor (2 mL [this work]. It is worth noting that the range of the maximum shear stress depends highly on the operating parameters of the individual equipment (e.g., maximum stirrer speed in a given bioreactor).

Furthermore, diverse cell retention strategies involving different types of pumps require also a test of the maximum allowable shear stress, rather than the average power input or the distribution of the energy dissipation or shear stress. With new insights into shear dynamics of the Mobius® Breez perfusion microbioreactors, it is now possible to rapidly test perfusion processes under harsh conditions, such as at frequencies below 2.3 Hz, where membrane contact generates substantial shear

stress. Successfully withstanding such conditions suggests a high likelihood of robust performance in stirred-tank bioreactors at elevated shear stress. Frequencies between 2.3 Hz and 2.7 Hz provide a shear environment between the extreme condition at 1 Hz and the gentle conditions above 2.7 Hz. Once a threshold has been determined, other parameters such mass transfer coefficients and mixing time can be evaluated without the fear of compromising the cell culture due to shear stress. It is worth noting the limitations of the shear stress measurement system which is providing only the value of the maximum shear stress, yet no information about the abundance of the high shear stress nor the distribution of the shear. Therefore, incorporating a safety margin is advisable to account for the uncertainty as well as the variability between different bioprocess in particular with respect to the size of the biological entity.

Conversely, for highly shear-sensitive processes, the miniaturized bioreactor offers gentle conditions at pumping frequencies above 2.7 Hz, making it an ideal choice for processes requiring low shear stress, such as cell therapy manufacturing involving microcarrier beads [40,41]. The shear stress measured at low frequencies is in line with what would be expected for agitated vessels with low impeller speeds.

4. Conclusions and outlook

A shear stress characterization of the novel bioreactor type Mobius® Breez Microbioreactor designed for perfusion cultivation has been conducted. Due to the novelty of its design, such a bioreactor has not yet been fully characterized in terms of maximum hydrodynamic shear stress, cultivation performance and membrane-wall contact. The results presented show that the different geometry brings new features to the system behavior, in particular the maximum hydrodynamic stress values that can be achieved during the operation of the microbioreactor. It was found that the Mobius® Breez Microbioreactor operated in two distinct conditions, one characterized by no contact of the membrane with the bottom wall, and one characterized with such contact. As a consequence, the maximum hydrodynamic stress is reaching values around 4–6 Pa (mild conditions without membrane contact) and 30–100 Pa (harsh conditions with membrane contact). Such behavior was further confirmed by performing perfusion cultivations of CHO cells as well as by optical analysis of membrane motion. By combining all the results obtained, it was possible to define a critical frequency of 2.7 ± 0.2 Hz of the membrane, which demarcates a range with low and high shear. When comparing the stresses reached in Mobius® Breez Microbioreactor with threshold values of CHO cells found in the literature, mild conditions are representative of typical safe operating conditions in stirred bioreactors [10,26], while harsh conditions would be at or above the CHO cell stress threshold, thus most probably leading to potential cell damage. Comparing the hydrodynamic shear stress behavior as a function of frequency in the Mobius® Breez microbioreactor to stirred tank bioreactor systems across a range of scales provides an opportunity to explore shear stress prior to scale up and therefore enabling a more informed and shear-conscious perfusion process development opportunity.

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CRediT authorship contribution statement

Patrick Romann: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Dan Trunov:** Visualization, Investigation, Formal analysis, Data curation. **Ondřej Šrom:** Visualization, Formal analysis, Data curation. **Harry L.T. Lee:** Writing – review & editing, Resources, Project administration. **Kevin S. Lee:** Writing – review & editing, Resources, Project administration. **Ryan Trocki:** Writing – review & editing, Resources, Project administration. **David Ephraim:** Writing – review & editing, Resources, Project administration. **Jean-Marc Bielser:** Writing – review & editing, Supervision, Project administration. **Jonathan Souquet:** Writing – review & editing. **Miroslav Šoóš:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Thomas Kaspar Villiger:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Harry L.T. Lee, Kevin S. Lee, Ryan Trocki and David Ephraim are employees of EMD Millipore Corporation (an affiliate of Merck KGaA, Darmstadt, Germany), a company that produces and markets the Mobius® Breez perfusion microbioreactor and that provided the funding the present study. They were not involved in the interpretation of data or statistical analysis. The remaining authors declare no conflict of interest. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109556.

Data availability

Data will be made available on request.

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