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An automated laboratory-scale methodology for the generation of sheared mammalian cell culture samples

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Continuous disk-stack centrifugation is typically used for the removal of cells and cellular debris from mammalian cell culture broths at manufacturing-scale. The use of scale-down methods to characterize disk-stack centrifugation performance enables substantial reductions in material requirements and allows a much wider design space to be tested than is currently possible at pilot-scale. The process of scaling down centrifugation has historically been challenging due to the difficulties in mimicking the Energy Dissipation Rates (EDRs) in typical machines. This paper describes an alternative and easy-to-assemble automated capillary-based methodology to generate levels of EDRs consistent with those found in a continuous disk-stack centrifuge. Variations in EDR were achieved through changes in capillary internal diameter and the flow rate of operation through the capillary. The EDRs found to match the levels of shear in the feed zone of a pilot-scale centrifuge using the experimental method developed in this paper (2.4×10^5 W/kg) are consistent with those obtained through previously published computational fluid dynamic studies (2.0×10^5 W/kg). Furthermore, this methodology can be incorporated into existing scale-down methods to model the process performance of continuous disk-stack centrifuges. This was demonstrated through the characterization of culture hold time, culture temperature and EDRs on centrate quality.

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1 Introduction

Understanding a product's key quality attributes and its process of manufacture through the use of design spaces is a regulatory requirement for biopharmaceutical manufacturers [1]. In typical mammalian cell culture purification platforms multiple unit operations each with individual process parameters affect the critical quality attributes of a product. Adoption of a Quality by Design (QbD) approach can help in defining the design space especially when combined with high throughput scale-down techniques to generate considerable quantities of data that mimic commercial-scale unit operations [2]. These extensive experimental datasets enable a thorough understanding of the effect input parameters have on process performance and the product's critical quality attributes [3]. Monoclonal antibody (mAb) manufacturing platforms often begin with the processing of cell culture material for the removal of cells and cellular debris. Disk-stack centrifuges are typically used at manufacturing-scale because of their capacity to handle a wide variety of feedstock [4]. They also enable processing

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Abbreviations: CFD, computational fluid dynamics; **CSD,** capillary shear device; **EDR,** energy dissipation rates; **LDH,** Lactate dehydrogenase; **mAb,** monoclonal antibody; **PSD,** particle size distribution; **QbD,** quality by design; **RSD,** rotating shear device

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in a continuous fashion by utilizing the functionality of a semi-continuous solids discharge [5]. However, most disk-stack centrifuge designs suffer from high levels of shear being present in the feed zones. This results in breakage of shear-sensitive mammalian cells creating sub-micron particles. In order to understand the potential effects of sub-micron particles on subsequent unit operations, pilot-scale studies (>50 L harvest material) are often conducted.

Pilot-scale studies are material, time and resource-intensive. Scale-down methods potentially allow for the reduction of these requirements and enable such studies to be conducted at laboratory-scale. Methods incorporating Sigma Theory [6] facilitate such scaling down and hence enable subsequent comparisons between centrifuges of varying geometries and sizes. These methods however do not consider the effect of shear experienced in the feed zones, typically associated with disk-stack centrifugation [7].

Many studies have shown the exposure of mammalian cells to shear through flow constriction [8, 9] or through the use of rheometers [10–12] can have a detrimental effect on cell viability. A number of devices described in the literature were shown to generate levels of shear comparable to those prevailing in the feed zone of typical disk-stack centrifuges [7, 13–16]. Most devices are based upon either the Rotating disk shear device (RSD) or the Capillary shear device (CSD). The RSD developed at University College London [7, 13–14] mimics the prevailing shear in disk-stack centrifuge feed zones. The capillary shear device developed by Westoby et al. [15], has also shown the ability to generate levels of shear similar to those of a disk-stack centrifuge feed zone. Additionally, the combination of both devices (RSD and CSD) with a laboratory-scale centrifuge have been shown to create centrates with comparable levels of solids remaining to those from a disk-stack centrifuge [7, 13–16]. This publication develops an automated experimental methodology based upon the CSD capable of generating a range of shear levels typically produced in the feed zone of a disk-stack centrifuge in addition to quantifying the back pressure enabling calculation of the EDRs. EDR is the irreversible rate of increase in internal energy within a system. The effect of EDR on cell culture has been used in previous studies to characterize bioprocess-related scenarios such as centrifugation [7, 13–15] and cross flow filtration [17]. The unique feature of the approach described in this paper lies in the ability to use commonly available laboratory equipment to enable these functionalities and facilitate high throughput experimentation. The methodology is based on a simple setup utilizing standard downstream processing laboratory equipment. The method requires a fast protein liquid chromatography (FPLC) instrument to act as the delivery system which enables high flow rates to be reached at high back pressures and has the added utility of generating performance feedback through back pressure readings. This provides a preparative bench top route for the exposure of samples to defined levels of shear ahead of rating studies of typical clarification steps e.g. centrifugation and depth filtration.

2 Materials and methods

2.1 Energy dissipation rate (EDR)

EDR is the irreversible rate of increase in internal energy within a system. It is measured in terms of unit of power per unit volume or mass. Under laminar flow conditions within a capillary, the maximum energy dissipation (ε_{max}) occurs at the capillary wall. This can be described (Eq. 1) as a function of pressure increase (ΔP), where; l and D are the length and diameter of the capillary; μ and ρ are the viscosity and density of the process fluid [15].

$$\varepsilon_{\max} = \frac{D^2 \Delta P^2}{16 l^2 \mu \rho} \quad (1)$$

2.2 Cell culture

The cell culture material was generated using CHO cell lines expressing mAb products. The cultures were produced at bench-scale (5 L) and pilot-scale (100 L) bioreactors and were harvested during the decline phase of growth (days 11–14). Table 1 summarizes the cell culture properties at time of harvest. Cell density and cell viability of the culture were measured through an industrial standard trypan blue dye exclusion method [18]. The ViCell (Beckman Coulter, High Wycombe, UK) was also used to provide particle size distribution (PSD) data based on trypan blue stained images.

2.3 Fast protein liquid chromatography (FPLC) instrument and super loop

An AKTA Explorer (GE Life Sciences, Uppsala, Sweden) was used as the FPLC system. Through the use of accurate piston pump devices this system is capable of delivering flow rates up to 100 mL/min with an accuracy of ±2%. It is also configured with monitors for multi-wavelength detection, conductivity and pressure. Superloops (GE Life Sciences) are often used in a chromatographic setting for sample injection and collection while in this

Table 1. Properties of cell culture materials used in CSD studies

Material	Bio Reactor Size (L)	Cell Density × 10^6 (cells/mL)	Cell Viability (%)
Culture-A	100	23.9	90
Culture-B	100	20.3	96
Culture-C	5	30.8	31

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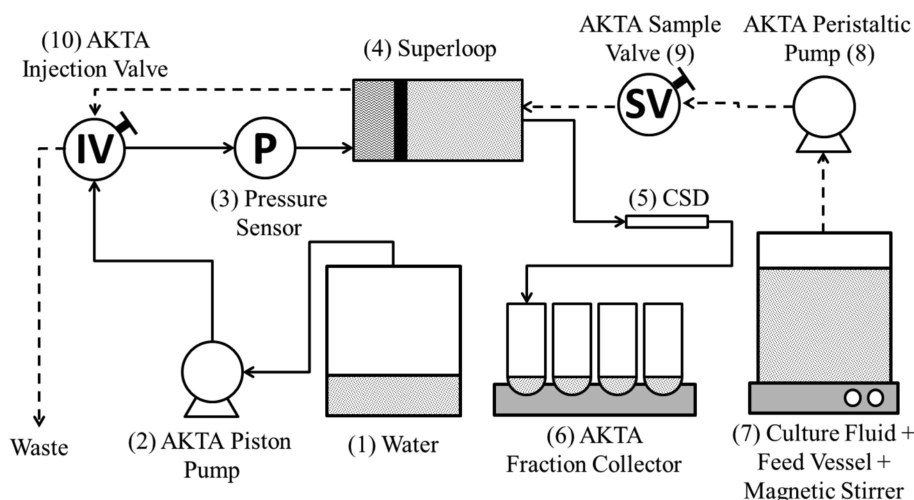

Description	Supplier	ID
AKTA Piston Pump (P-901)	GE Life Sciences (Little Chalfont, UK)	2
Pressure Sensor (P-901)	GE Life Sciences (Little Chalfont, UK)	3
Superloop (18-1113-81)	GE Life Sciences (Little Chalfont, UK)	4
10cm 0.01" ID Steel Capillary (U112)	IDEX Health & Science (Oak Harbor, WA, USA)	5
Fraction Collector (Frac-950)	GE Life Sciences (Little Chalfont, UK)	6
AKTA Peristaltic Pump (P-960)	GE Life Sciences (Little Chalfont, UK)	8
AKTA Sample Valve (INV-907)	GE Life Sciences (Little Chalfont, UK)	9
AKTA Injection Valve (INV-907)	GE Life Sciences (Little Chalfont, UK)	10

Figure 1. Schematic of CSD apparatus, parts description and supplier information. Loading phase of CSD is indicated with dashed lines (__) while shearing phase of the CSD is indicated with the solid lines (__).

paper they were used for the injection of cell culture material through the capillary. A method was developed that comprised of two parts; first the sample is loaded into the Superloop using the peristaltic (low shear) sample pump. Once loaded the piston pumps displace the feed from the Superloop though the capillary and into a collection vessel (Fig. 1). Using the Unicorn control software's scouting functionality and this method, multiple conditions (different feed material, flow rates, capillaries sizes etc.) could be queued and run sequentially without manual intervention.

2.4 Capillary shear device (CSD)

Adjusting the flow rate of the piston pump and altering the stainless steel capillary diameter of the CSD allowed varying levels of shear to be imposed on the cell culture material. Previous studies have established the relationship between shear, flow rate and capillary diameter in detail [15, 19–21].

2.5 Analytical techniques

2.5.1 LDH release

Lactate dehydrogenase (LDH) is an enzyme that is released during cell rupture. In this paper % LDH release (LDH_{REL}) was used as a measure of the shear levels experienced at varying flow rates [17] of the CSD and during operation of the pilot-scale centrifuge. Assay protocols (Abcam, Cambridge, UK) provided by the supplier were followed to identify % LDH release. LDH_{REL} was calculated by dividing the difference between LDH release from the sheared sample (LDH_{SS}) and the no shear sample (LDH_{NS}) by the difference between the total LDH release (LDH_{TR}) sample and the no shear sample. The total LDH release sample was generated through mixing the non-sheared cell culture sample with a lysis solution at 1/10 dilution which was subsequently incubated for 30 min. This ensured all cell membranes were ruptured and provided the total amount of LDH present. The % LDH release (LDH_{REL}) following exposure to shear in the CSD was calculated using Eq. 2.

$$LDH_{REL} = \left(\frac{LDH_{SS} - LDH_{NS}}{LDH_{TR} - LDH_{NS}} \right) \times 100 \quad (2)$$

2.5.2 Turbidity

The performance of the centrifuge for the removal of cell and cellular debris was quantified offline by measuring the turbidity of the centrate in an Orion Thermo Scientific (Waltham, MA, USA) turbidity meter. Centrates were placed in clear 10 mL glass vials and illuminated at a wavelength of 450 nm. The intensity of transmitted light received by the detector was quantified in nephelometric turbidity units (NTU). Centrates with high cell debris content translated to $>$100 NTU and similarly low cell debris levels translated to $<$100 NTU.

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2.6 Pilot-scale and laboratory-scale centrifugation

A continuous GEA Westfalia SO1-06-107 (Oelde, Germany) disk-stack centrifuge was used to generate centrates at pilot-scale. These experiments were conducted at a set bowl speed of 10 000 rpm and flow rates were varied between 0.3–0.9 L/min. A laboratory-scale Beckman J-HC centrifuge with a JS 4.2A rotor (Brea, CA, USA) was used to process material following exposure to shear in the CSD.

2.6.1 Identification of pilot-scale centrifugation shear

The levels of shear corresponding to a range of different EDRs generated in the CSD were estimated by measurement of the LDH concentration of the cell culture measured before and after shear (Eq. 2). The relationship between the levels of LDH release as a function of maximum energy dissipation rate (ε_{max}) was established. The level of shear in the pilot-scale centrifuge was quantified by matching the level of % LDH release in the centrate to the existing relationship between % LDH release and maximum energy dissipation rate (ε_{max}).

3 Results and discussion

3.1 CSD characterization

This section summarizes the performance of the methodology developed to enable a range of EDRs relevant to the feed zone of disk-stack centrifugation to be investigated in an automated and high throughput manner. A key advantage of using a FPLC instrument as a delivery system was that it enabled higher flow rates to be reached when compared to typical syringe pumps [16] which were unable to maintain a set flow rate at high back pressures. Furthermore, the FPLC instrument had the utility of generating performance feedback through back pressure readings which enabled the calculation of EDRs (Eq. 1). With this functionality Fig. 2A examines the effect of changes in capillary diameter and operational flow rates on the back pressures and EDRs developed. The relationship between capillary internal diameter and pressure increase is very well understood [22] and experimentally explored in a previous study [15], as expected it was found that increasing operational flow rate resulted in higher back pressures. The relationship between flow rate and back pressure becomes more pronounced with narrower internal capillary diameters. Furthermore, the pressures found in this experiment were within the range quoted by Westoby et al. [15], when processing mammalian cell culture through a capillary with an internal diameter of 0.01” confirming consistency with earlier published data.

Higher levels of cellular damage are associated with high levels of cellular debris, leading to increased centrate turbidities [15]. Figure 2Bi shows the extent of small cel-

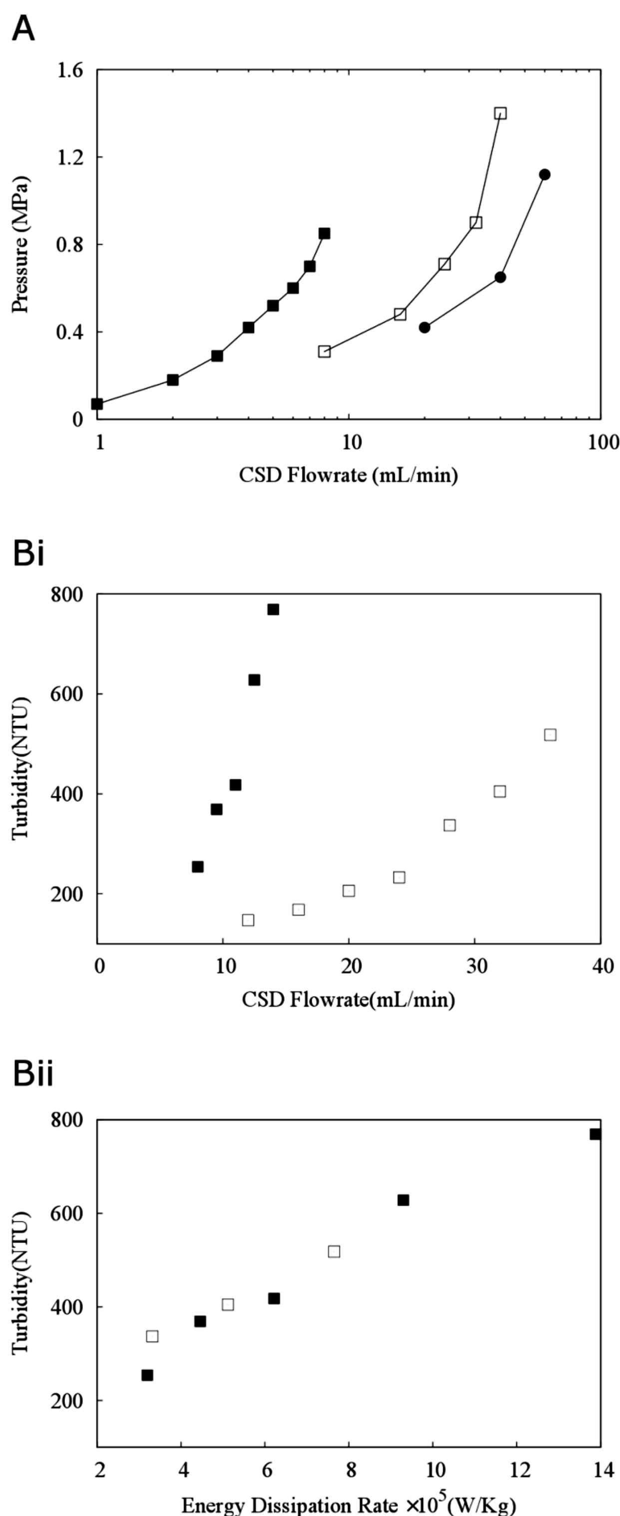

Figure 2. (**A**) Study examining the effect of capillary size over a flow rate range of 1–60 mL/min on the back pressure produced by the CSD. Capillary sizes: 0.007” (■), 0.01” (□) and 0.02” (●). (**B**) Examining the effect of (**Bi**) flow rates and (**Bii**) EDR on centrate turbidity using capillary sizes 0.007” (■) and 0.01” (□). A range of EDRs were generated using the CSD apparatus by processing Culture-C. The sheared material was subsequently centrifuged at $V/t\,\Sigma$ 2.41 × 10^{-8} m/s to quantify centrate turbidity.

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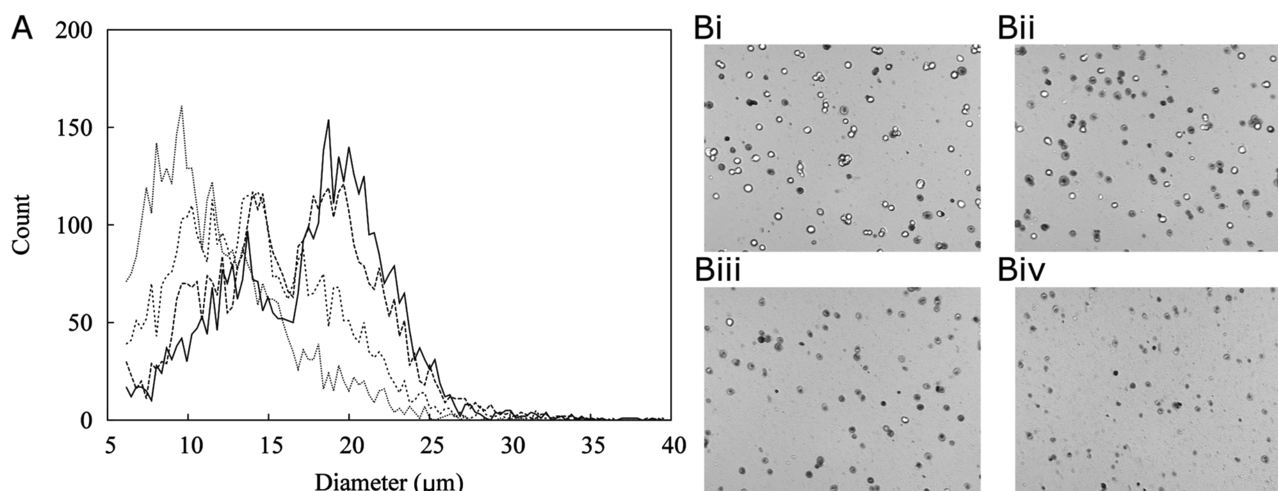

Figure 3. (**A**) Comparison of particle size distribution of Culture-B when passed through the CSD at EDRs of 0 (__), 1.84 (_ _), 11.85 (- - -) and 31.89 (……) × 10^5 W/Kg (**B**) Vi-Cell XR Coulter Counter images of Culture-B sheared using the CSD at EDRs of 0, 1.84, 11.85 and 31.89 × 10^5 W/Kg (**Bi–iv**, respectively).

lular debris created as assessed through centrate turbidity for two capillaries of different diameters. An increase in turbidity was expected with increased CSD flow rates for both capillaries but there was significantly more damage when the material was passed through the 0.007" capillary compared to the 0.01" capillary (Fig. 2Bi). This was due to the higher levels of energy dissipation (Fig. 2Bii) created using the narrower 0.007" capillary which resulted in higher levels of breakage and hence an increase in centrate turbidity post shear. Figure 2Bi also suggests that in order for the 0.01" capillary to generate equivalent amounts of energy dissipation to that of the 0.007" capillary, and hence generate the same levels of damage the operational flow rate through the 0.01" capillary would be required to increase significantly.

Figure 3A shows the effect of increased EDR on the PSD of cell culture material. With increased levels of EDR there was a reduction of counts for particles greater than 20 μm. This suggests the breakup of viable and non-viable cells into finer particulates. Particles below 6 μm are below the detection limit of the ViCell hence the build-up of <6 μm fines could not be quantified but had to be inferred. Trypan blue stained images obtained from the ViCell showed large quantities of small particulates present in cell culture samples exposed to increased EDRs using the CSD (Fig. 3Bi–iv).

A correlation (Fig. 4A) was developed between the levels of LDH release through operation of the CSD at a range of EDRs (1.2 × 10^5 to 10.7 × 10^5 W/kg). Regression analysis of the dataset showed that LDH release from the centrifuge was equivalent to an EDR of 2.4 × 10^5 W/kg created using the CSD with a 0.01" capillary. The values found in this study match closely those from earlier published data [7, 23] where CFD analysis of a similar non-hermetic GEA Westfalia SAOOH disk-stack centrifuge (Oelde, Germany) reported an EDR of 2.0 × 10^5 W/kg. As such these results validate the methodology developed to identify levels of shear prevailing within a centrifuge.

The process performance of a given disk-stack centrifuge can be altered through changes in bowl speed and flow rate. A previous study [16] has shown that increases in bowl speed lead to higher levels of shear, as quantified through LDH release. In the present study (Fig. 4B) the effect of flow rate, at a fixed bowl speed on shear damage was explored. Figure 4B shows there was a decrease in the levels of LDH release with increasing flow rate (0.3–0.9 L/min). The operation of disk-stack centrifuges at low flow rates can lead to prolonged exposure of shear sensitive mammalian cells to the high shear feed-zone region [24, 25]. The decrease in LDH release observed could be a result of the lower residence times which accompany the operation of the centrifuge at an increased flow rate [26]. Furthermore, the levels of LDH release in the centrate from this experiment (10%) was consistent with the values obtained in earlier experiments which quantified the prevailing levels of shear in a GEA Westfalia SO1-06-107 disk-stack machine (Oelde, Germany).

3.2 Industrial case study

Upstream developments in mammalian cell culture have led to increased titers often resulting in high cell density cell culture streams with elevated levels of host-cell impurities (e.g. host-cell proteins, DNA), which increase the burden on subsequent purification operations [27]. To address these levels of impurities, non-chromatographic techniques to alter cell culture broth conditions pre-harvest are increasingly commonplace [28]. These alterations typically involve changes in pH conditions or broth temperature so as to improve the performance of subsequent

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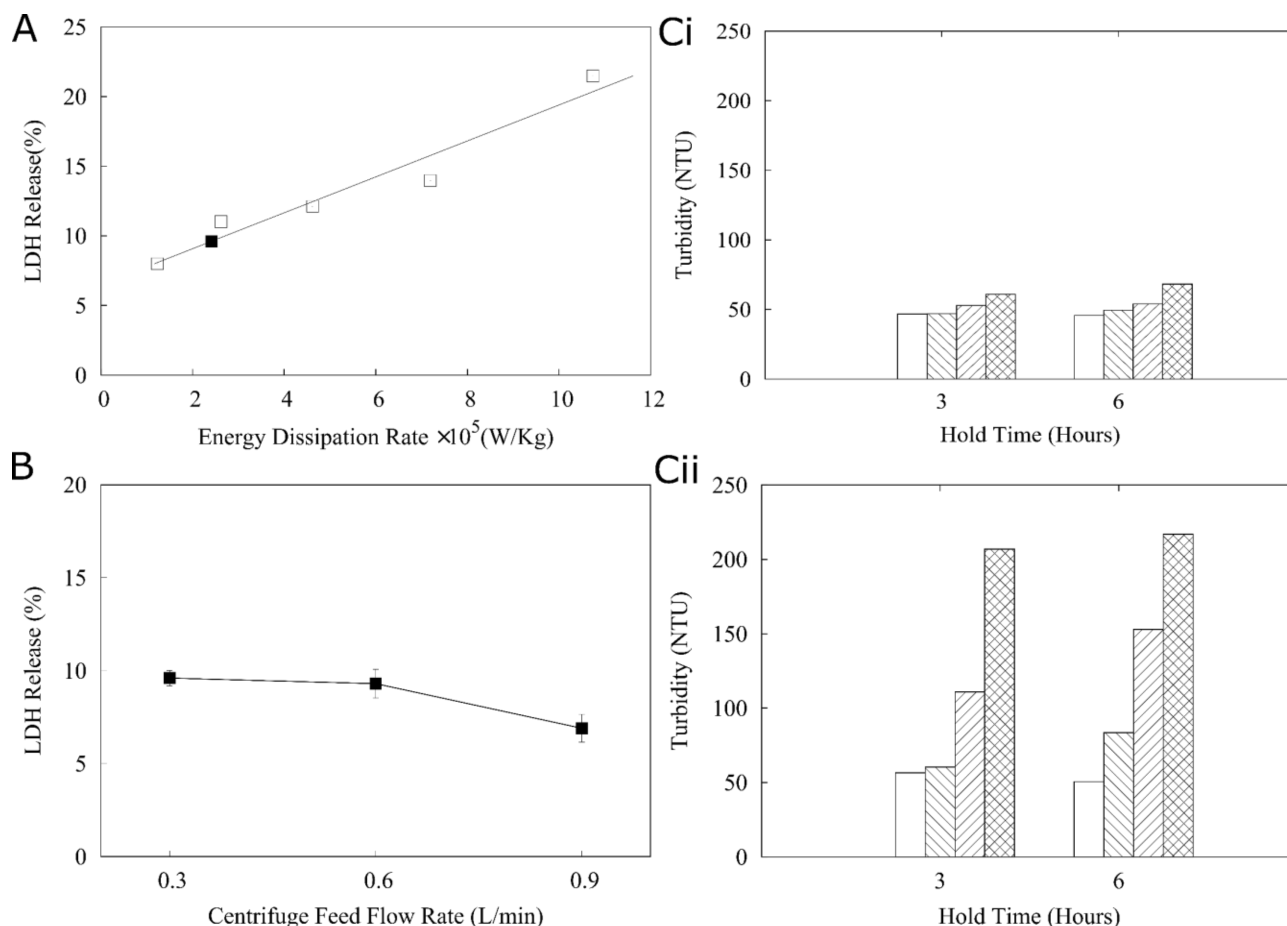

Figure 4. (**A**) Study examining the correlation between the % LDH release (Measure of cell rupture) and EDR of CSD so that cell damage in the feed zone of the GEA Westfalia SO1-06-107 can be quantified. LDH release for the modelling datasets (□) was generated by shearing material (Culture-A, Table 1) using the CSD. Regression analysis (__) was used to determine the following relationship between LDH Release (LDH_{Rel}) and EDR (ε_{max}) to identify the levels of shear generated in the GEA Westfalia SO1-06-107 centrate (■): $LDH_{Rel} = 1.29\ \varepsilon_{max} + 6.49$, $R^2 = 0.94$. (**B**) Study examining the levels of LDH released at a range of feed flow rates (0.3–0.9 L/h) while the bowl speed of the Westfalia SO1-06-107 was maintained at 10 000 rpm. Culture-B (Table 1) was used as feed material during this study. The values plotted are shown as mean ± standard deviation (n=3). (**C**) Examining the effects of cell culture hold at a range of temperatures; (**Ci**) 5 and (**Cii**) 35°C, hold times; 3 and 6 h and EDRs; 0 (□), 1.84 (▧), 11.85 (▨) and 31.89 (▩) × 10^5 W/kg on centrate turbidity. Culture-B was used as source material and centrifuged at $V/t\ \Sigma$ 2.41 × 10^{-8} m/s to generate the centrates for subsequent turbidity measurements.

unit operations [29]. The CSD based method developed in this paper was integrated with existing scale-down centrifugation methods [15, 16] to characterize the influence of hold time, temperature and levels of shear on the performance of the disk-stack centrifugation step. Figure 4C shows there is a clear increase in supernatant turbidity with increasing levels of shear as would be expected. Statistical analysis of the variables hold time, temperature and level of shear revealed shear to be the most influential parameter (P-Value = 0.003) that affected the turbidity of the centrate. This is not surprising as higher levels of shear result in higher levels of cell rupture and subsequent generation of fine particulates which are difficult to remove through centrifugal separation. Figure 4Ci suggests longer hold times at lower temperatures (5°C) had a negative effect on separation performance. This is likely due to the higher viscosities that are expected when operating at lower temperatures (5°C) which hinders centrifugal separation. Conversely higher temperatures (35°C) are expected to yield better levels of clarification with an increased hold time. However, in this experiment higher temperatures and increased hold time resulted in the rapid reduction in viability of the material from 94% at the start of the experiment to 60% at 3 h and subsequently to 17% at 6 h. At lower viabilities there was an increased population of fine particulates which were difficult to remove through centrifugation and hence resulted in high turbidities being present in the centrate potentially challenging latter filtration operations. Typical continuous disk-stack centrifuge processes platforms operate at a fixed bowl speed. Changes in shear as a result of alterations to the bowl speed are therefore unlikely but there is a potential for the cell broth under certain circumstances to be held before processing over an extended period of

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time. This study identified that hold time and temperature can each have a significant influence on the performance of the centrifugation step.

4 Concluding remarks

The purpose of this work was to develop an alternative methodology to those available in the literature in order to characterize the shear generated when utilizing continuous disk-stack centrifugation and enable high-throughput experimentation. Studies were conducted to show that the Capillary shear device (CSD) using the automated methodology developed in this paper had the ability to process cell culture to create multiple levels of shear simply through changes in capillary diameter and operational flow rates. The developed method also had the ability to quantify back pressure hence having the ability to estimate Energy dissipation rates (EDR). Furthermore, the CSD had the ability to generate the levels of shear created in the feed zone of a pilot-scale disk-stack centrifuge. The EDR values obtained in this publication describing non-hermetic centrifuges matched closely those developed in the literature using computational fluid dynamics (CFD) for a pilot-scale centrifuge with a similar design. The methodology was also used in combination with existing scale-down centrifugation protocols to give an insight into the negative influence of high temperatures and lengthy hold times on centrifugation through increases in centrate turbidity which would result in a potential reduction in the ease of filtration for the subsequent depth and sterile filtration steps.

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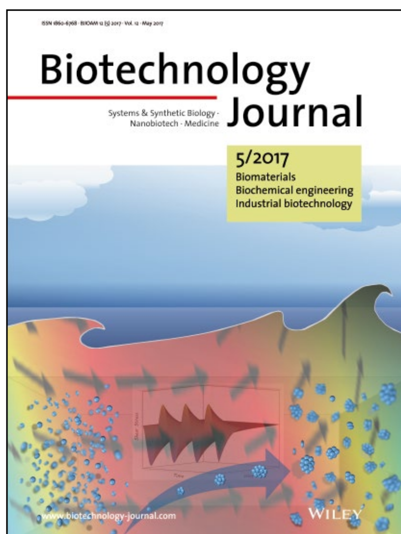

Cover illustration

The cover shows a cartoon of wave motion that provides an ideal hydrodynamic environment to induce cell-cell collision and subsequent aggregation of human mesenchymal stem cells with controlled aggregate size in suspension. The cover is prepared by Ang-Chen Tsai, Yijun Liu, Xuegang Yuan, Ravindran Chella and Teng Ma authors of the article "Aggregation kinetics of human mesenchymal stem cells under wave motion" (https://doi.org/10.1002/biot.201600448).

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Food biotechnology training in developing countries – from genomics to synthetic biology
Ruiyan Wang
https://doi.org/10.1002/biot.201500635

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Research Article
Aggregation kinetics of human mesenchymal stem cells under wave motion
Ang-Chen Tsai, Yijun Liu, Xuegang Yuan, Ravindran Chella and Teng Ma
https://doi.org/10.1002/biot.201600448

Research Article
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Reasearch Article
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Lukas Marsalek, Clemens Gruber, Friedrich Altmann, Markus Aleschko, Diethard Mattanovich, Brigitte Gasser and Verena Puxbaum
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Research Article
Identification of acetylcholinesterase inhibitors using homogenous cell-based assays in quantitative high-throughput screening platforms
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An automated laboratory-scale methodology for the generation of sheared mammalian cell culture samples
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https://doi.org/10.1002/biot.201600730

Biotech Method
Dual display of proteins on the yeast cell surface simplifies quantification of binding interactions and enzymatic bioconjugation reactions
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