

METHOD FOR DETERMINING THE SHEAR STRESS SENSITIVITY OF CELLS DURING A CULTIVATION PROCESS

5 TECHNICAL FIELD

The invention relates to a method for determining the shear stress sensitivity of eukaryotic or prokaryotic cells during a cultivation process.

BACKGROUND OF THE INVENTION

10 It is known that eukaryotic or prokaryotic cells in cell cultures are sensitive to external influences such as hydrodynamic and mechanical stresses, so that the quality and quantity of the cell culture can suffer. These hydrodynamic and mechanical stresses can result, for example, from the vessel or reactor in which the cell culture is grown, from the stirrer used, the liquid media or gases supplied and discharged, and other equipment used during cultivation. A better understanding of the resulting influences and a better
15 assessment or even quantification of their effects on the cells would contribute to a significant increase in the robustness and performance of cell cultivation.

There are already numerous approaches to solving these problems in the prior art. For example, US 2006/0223175 A1⁽¹⁾ discloses a bioreactor capable of applying shear stress to cultivated tissue cells. The
20 shear stress is applied by a movable shaft to which a substrate is fixed on which the tissue cells are attached. The shear stress is required as a mechanical stimulation to increase the efficiency of cultivating tissue cells in vitro.

US 2008/0057571 A1⁽²⁾ describes a system for exposing a three-dimensional cell culture to a fluid shear
25 force imparted by a fluid flow passing through a channel defined by a flow chamber. The aim is to apply mechanical factors to the cell culture to influence and regulate the differentiation of human mesenchymal stem cells (MSCs), while visual inspection due to transparent tubes is possible.

Furthermore, US 2010/0041128 A1⁽³⁾ relates to a microfluidic device for single-cell culture to apply fluid
30 induced stress. It is a non-invasive method for continuously investigating cell behavior while allowing both spatial and temporal control of cell growth conditions. The device includes a channel having a cell culture chamber, which has a cell attachment surface. The cell attachment surface is flanked by vacuum channels. Shear stress can be controlled by controlling the flow rate of the medium.

CN 102559492 A⁽⁴⁾ shows a cell culture device comprising a peristaltic pump, a signal generator, an
35 upstream liquid storage cylinder, and a downstream liquid storage cylinder. The signal generator is connected to a metal electrode on the cover of the tank body of the cell culture device so that the cells are exposed to uniform electrical stimulation. Shear stress and electrical stimulation are applied to adherent cells in a flow chamber by regulating fluid flow.

40 Furthermore CN 102965333 A⁽⁵⁾ is directed to screening of cells suitable for a bioreactor or cell factory, and culturing the screened cells by using the bioreactor or cell factory on large scale. The culturing step comprises selecting cells by the applied small shear stress in the respective devices for cell culture on large

scale while focussing on the physiology of the cells, however, no quantification of the shear stress is made here.

Therefore, existing publications of patent applications in this field mostly seek to apply shear stress as mechanical stimulation to enhance the efficiency of cultivating adherent cells in vitro by using e.g. a movable shaft (US 2006/0223175 A1⁽¹⁾), by the shear stress imparted by the fluid flow passing through a chamber (US 2008/0057571 A1⁽²⁾ and CN 102559492 A⁽⁴⁾) or by microfluidic devices in a single cell analysis (US 2010/0041128 A1⁽³⁾). Others have a more similar intent to apply large scale shear stresses to screen cells suitable for bioreactor (fixed bed) and cell factory cultivation (CN 102965333 A⁽⁵⁾). However, quite different devices as provided in the invention are used to apply shear stress. Yet, methods for the quantification of shear stress as levied upon cultured cells appear to be underdeveloped in the state of the art.

A microfluidic channel to investigate the influence of shear stress was also used in literature. Hereby cells were either harvested from a T-flask (Mollet et al.⁽⁶⁾) or pumped from a stirred bioreactor via external loop (Godoy-Silva et al.⁽⁷⁾). For example, Mollet et al.⁽⁶⁾ describe the establishment and characterization (simulation) of a microfluidic channel, whereby cells are subjected to well-defined hydrodynamic forces. The application of this system to investigate apoptosis and necrosis of CHO cells is described. The cells were harvested from a T-flask culture, and not pumped in a loop. The concept of subjecting cells to hydrodynamic shear stress in a device (called 'torture chamber' or 'TC' by e.g. Godoy-Silva et al.⁽⁷⁾, Mollet et al.⁽⁶⁾ and Sieck et al.⁽⁸⁾) is known from the prior art. According to the invention, such an arrangement, yet in a further elaborated form is referred to as a "shear stress sensitivity determination device" or in brief "shear stress sensitivity device".

Godoy-Silva et al.⁽⁷⁾ disclose the exposure of CHO cells to hydrodynamic forces in a microfluidic channel of Mollet et al.⁽⁶⁾, which is called torture chamber (TC). The channel was connected to a stirred bioreactor via an external loop. The microfluidic device of Godoy-Silva et al.⁽⁷⁾ consists of a contracting channel, where the flow rate determines the maximum shear rate as well as the frequency of exposure characterized by specific levels of the energy dissipation rate (EDR). The flow was enabled by a syringe pump, which pumped continuously in alternate mode. Continuous stress exposure of CHO cells during cultivation is performed, different stress levels on growth, viability, and glucose/lactate are investigated and the effect of maximum values of EDR in the bioreactor is estimated.

Sieck et al.⁽⁸⁾ describe how to develop a scale-down model of a hydrodynamic stress present in large scale production bioreactors to investigate the performance of CHO cells under simulated production bioreactor conditions. Various levels of hydrodynamic stress were generated in 2 L bioreactors mimicking those present in different locations of a large scale stirred tank bioreactor. A lab-scale vessel is described which was characterized regarding power input by current and voltage measurement of the stirrer engine. Large scale power input was applied in small scale to mimic large scale hydrodynamic conditions. Thereby also agitation rates in lab scale were periodically adjusted to mimic repeated exposure to different local hydrodynamic conditions in large scale.

Neunstoecklin et al.⁽⁹⁾ disclose a scale-down model to determine stress threshold values of CHO and Sp2/0 cells until negative influence on performance was measured. The system consists of a 3L bioreactor with

an external loop including a pump and a nozzle. Different levels of maximal shear stress were controlled by various nozzle diameters and flow rates. The pump and nozzle have been characterised in terms of maximal occurring shear stress by computational fluid dynamics (CFD) and by a shear sensitive aggregate system.

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At first sight, Godoy-Silva et al.⁽⁷⁾ and Neunstoecklin et al.⁽⁹⁾ appear to assume a similar approach to the present invention, with shear stress being generated within an external loop. However, this is not the case. The teaching according to the present invention goes far beyond this prior art approach, with not only the intensity of the shear stress and frequency being considered according to the present invention, but also the duration of exposure. Moreover, in these studies, a completely different device was used to generate the shear stress, i.e., a completely different pump and, moreover, a nozzle were used.

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Fries et al.⁽¹⁰⁾ disclose the influence of shear stress, i.e. increasing power input by increasing agitation rate, a biological system was examined, namely CHO cells by determining death rate and a non-biological system, namely an emulsion. Fries et al.⁽¹⁰⁾ used a magnetically levitating centrifugal pump (PuraLev® 200SU) within an external loop where the frequency and duration of exposure was done by controlling the flow rate using a pressure drop through a valve and reducers for the line. They cultivated and monitored cells over 12 hours and recorded the cell death rate for specific settings. The authors characterize the pump by computational fluid dynamics (CFD) and attributed the cell death rate to the average turbulent kinetic dissipation rate, which correlates with average shear stresses. That is, the authors intended to determine an operating range of the pump for their biological system expressed as cell death rate.

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Villiger et al.⁽¹¹⁾ describes how a calibration procedure can be carried out using a shear stress-dependent physical quantity, whereby the aggregate size of poly(methyl methacrylate) (PMMA) nanoparticles is used as the shear stress-dependent physical quantity. The calibration device used is, as in an embodiment of the invention, a contracting nozzle device of various diameters as described and illustrated, for example, in Soos et al.⁽¹⁴⁾. A characterization procedure, as in an embodiment of the invention, is also carried out in Villiger et al.⁽¹¹⁾ (see p. 1741, right-hand column, second paragraph), where the relationship between the maximum stable aggregate size and the applied maximum effective hydrodynamic shear stress, measured in the nozzle device, was used to convert the measured aggregate sizes for a stirred tank.

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However, in contrast to the present invention, a simple stirred tank is used in Villiger et al.⁽¹¹⁾. Furthermore a completely different device to generate shear stress is used in Villiger et al.⁽¹¹⁾. There is no determination of what causes the maximum shear stress in the device (according to the invention the rotational speed of the pump in the shear stress device causes the maximum shear stress), so that the practical benefit of the obtained shear stress values is questionable. The stirred tank in Villiger et al.⁽¹¹⁾ contains only water or a surfactant-containing solution. A cell cultivation process is not present, so that application to a cultivation process in practice is lacking. There is also no control procedure that would make it possible to determine the significance of the shear stress values determined. There is also a lack of a determination of the shear stress sensitivity or shear stress limit in a cultivation process up to which the cells just about tolerate the level of shear stress.

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In addition, the determination of the maximum hydrodynamic shear stress in Villiger et al.⁽¹¹⁾ is used to examine the stress generated by the gas jet occurring during bubble detachment from the sparger, stress induced during bubble rise, and burst, or stress generated by the turbulent flow due to impeller motion. However, in the present invention, it was found that the shear stresses induced by gassing or stirring in a conventional bioreactor have only a rather negligible influence on the hydrodynamic shear stress and never reach a level of >10 Pa, regardless of the conditions applied. Stirring and gassing are only present as a kind of background shear stress and play practically no role in determining the total shear stress (shear stress intensity) that occurs in cell cultivation processes. Only the shear stress parameters (maximum shear stress, duration of shear stress exposure, shear stress frequency) play a role in a cultivation system. The experimental results in Villiger et al.⁽¹¹⁾ are therefore not relevant to the present invention.

For the sake of completeness, US 2007/034014 A1⁽²⁶⁾ and US 2023/103671 A1⁽²⁷⁾ are also referred to. US 2007/034014 A1⁽²⁶⁾ discloses a wall shear stress sensor that measures the shear stress at a test surface. This is only a minimal section of a highly complex system, such as a bioreactor, and does not provide any reliable indication of the conditions in the bioreactor as a whole.

Finally, US 2023/103671 A1⁽²⁷⁾ describes a method of quantifying the impact of shear stress on cells, wherein the method comprises the steps of: (a) exposing immobilised cells to forces that cause shear stress; and (b) nanoindenting the cells from step (a) to determine their mechanical properties at different stress levels. Initially, a nanoindenter is used here on stationary cells to measure the shear stress, for example after shaking in a flask or stirring in a bioreactor. Nanoindentation is a method of materials testing for determining the hardness of materials on small length scales, in particular the hardness determination of thin layers. A nanoindenter is an indenter that is an optical probe that measures in situ. The type of nanoindenter used strongly influences the results obtained and can therefore falsify them. In US 2023/103671 A1⁽²⁷⁾ samples are taken for measurement, so the conditions in a dynamic system such as a bioreactor are not reflected. According to the invention, the determination is carried out directly in the process. In addition, the mechanical shear stress is measured here by the nanoindenter, which has nothing to do with the hydrodynamic shear stress according to the invention. The procedure described has nothing to do with practice.

The object of the present invention is to overcome deficiencies of the prior art and to find out the maximum tolerable shear stress for an organism or a cell line before important performance indicators of a cell culture, such as biomass/viable cell density or productivity as well as quality characteristics, are significantly affected. Furthermore, it was an object to investigate the equipment and/or parameters used in a cultivation process with respect to their contribution to shear stress.

SUMMARY OF THE INVENTION

The object of the invention is solved by a method for determining the shear stress sensitivity of cells in a shear stress sensitivity device during a cultivation process comprising a liquid culture medium, which comprises the following steps:

- (1) providing a shear stress sensitivity device comprising
- a bioreactor,

- a closed loop system connected with and located outside the bioreactor;
 - a pump placed in the loop system, the pump being a kinetic pump and
 - means to control the hydraulic resistance in the loop system;
- 5 (2) selecting cells, liquid culture medium, cultivation mode and cultivation conditions for the cultivation process to be performed;
- (3) selecting one, two or three shear stress parameters to be investigated from the group consisting of:
- 10 - maximum shear stress represented by a rotational speed of the pump;
- duration of shear stress exposure represented by a residence time of the cell culture within the pump; and/or
- shear stress frequency represented by a number of passages of the cell culture comprising cell culture medium and cells through the loop system per time;
- 15 (4) characterizing the shear stress sensitivity device by determining the shear stress values that occur at varying maximum shear stress by using a calibration method;
- (5) performing one, two, three or more cultivation runs of the same cultivation process of step (2) in the shear stress sensitivity device over the same selected period of time, by successively increasing or decreasing one, two or three shear stress parameters in each subsequent cultivation run, and measuring one or more performance characteristics of the cells during each run of the cultivation process and generating a curve for each performance characteristic in each run over time;
- 20 (6) performing a control cultivation process in the shear stress sensitivity device for each performance characteristic using the same cultivation process as in step (5), wherein a lower maximum shear stress than in step (5) is used and generating a control curve for each performance characteristic over time;
- (7) selecting the curve from the set of curves in step (5) for a performance characteristic that is closest to the control curve for said performance characteristic of step (6) which represent the shear stress sensitivity of the cells and constitute the shear stress limit for the cells;
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whereby it is ensured that the maximum shear stress occurring in the closed loop system is higher than the maximum shear stress occurring in the bioreactor.

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The core concept of the invention is that the cells are stressed, in particular by a pump connected to a bioreactor via an external loop. This allows the bioreactor to be operated in various cultivation modes such as batch, fed batch, continuous such as perfusion or other process control strategies, continuously applying shear stress in a defined manner by continuously pumping the cell culture comprising the liquid culture medium and cells in a loop. Through this external loop, the sole effect of shear stress on the culture can be studied without changing the conditions inside the bioreactor. Not only the maximum shear stress that can occur - which will be explained in more detail later - which is adjusted by the rotational speed of the pump, but also the shear stress frequency and duration of shear stress exposure can be controlled and thus studied.

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The shear stress sensitivity device provides insights into cell behavior and hydrodynamic shear stress that allow for accurate conclusions about the level of shear stress applied, contributing to predictions for other cultivation procedures, of equal, larger or smaller scale using similar or different hardware components.

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Because the shear stress of the cells is quantified by the calibration with a mechanic model the result is independent of the specifically used shear stress sensitivity device like the individually applied pump but reflects the conditions of the respective complete cultivation system.

10 The invention is also directed to a process for culturing cells in liquid cell culture, wherein the shear stress sensitivity determined according to the method of the present invention is used in the same cultivation process but performed on another scale as the method for determining the shear stress sensitivity.

The invention is also directed to a process for culturing eukaryotic cells or prokaryotic cells in liquid cell
15 culture in a bioreactor and performing a method for determining the shear stress sensitivity of cells in a shear stress sensitivity device during the cultivation process according to the present invention.

The invention is also directed to a process for producing a recombinant protein, the process comprising the steps of:

20 step I) culturing eukaryotic or prokaryotic cells expressing a recombinant protein in cell culture in a bioreactor;
step II) harvesting the recombinant protein;
step III) purifying the recombinant protein;
wherein performing in step I) a method for determining the shear stress sensitivity of the cells in a shear
25 stress sensitivity device during the cultivation process according to the present invention.

The terms 'method' and 'process' both refer to a procedure in connection with the present invention and are synonymous and interchangeable. The two terms have been used to make it easier to understand and distinguish between the different procedures: 'Method' refers to the method according to the invention,
30 while the term 'process' refers to other procedures used in connection with the method according to the invention or to procedures in which the method according to the invention is used.

The present invention is also directed to the use of a device comprising

- a bioreactor,
35 - a closed loop system connected with and located outside the bioreactor;
- a pump placed in the loop system, the pump being a kinetic pump and
- means to control the hydraulic resistance in the loop system;
as a shear stress sensitivity determining device or a shear stress sensitivity device,
wherein the device has no nozzle,
40 for culturing cells in a liquid culture medium, wherein a defined shear stress is set during the culturing.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the prior art and of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale so

that no assumption of precise geometric values can be made regarding the original size. The figures of the present disclosure are incorporated in and constitute a part of the specification, also illustrating embodiments of the invention without limitation to the specific embodiments described. The drawings together with the summary of the invention and detailed description serve to explain the principles of the present disclosure. The same features are denoted by the same reference signs throughout the figures. In the figures:

- Figure 1A shows a schematic illustration of a set-up of the shear stress sensitivity device for fed-batch or batch cultivation according to an embodiment of the present invention;
- Figure 1B shows a schematic illustration of a set-up of the shear stress sensitivity device for continuous cultivation such as perfusion cultivation according to an embodiment of the present invention;
- Figure 2 shows the results of shear stress measurements for different gassing and agitation rates performed in a simple 3 L bioreactor without external loop;
- Figures 3A and 3B show the flow rate in [mL/min] over various rotational speeds in [rpm] of a pump shown for different set-ups in a 2L water reservoir with an external loop;
- Figure 4A shows the measured pressure drop (dP) in [bar] over the flow rate in [mL/min] for various inner diameters (ID) of the tube in a 2L water reservoir with an external loop;
- Figure 4B shows the measured pressure drop (dP) in [bar] over the flow rate in [mL/min] for different lengths of hollow fiber filter modules in a 2L water reservoir with an external loop;
- Figure 5 shows aggregate sizes of poly(methyl methacrylate) (PMMA) nanoparticles as the radius of gyration (Rg) in [μm] plotted versus the rotational speed of the pump in [rpm] for tubes with different inner diameters (IDs) and two different hollow fiber filter modules;
- Figure 6 shows a schematically simplified sectional view of a main part of a magnetically levitating centrifugal pump;
- Figure 7 shows the visualization of radial flow profile in a cylindrical laminar pipe flow;
- Figure 8A shows a schematic diagram for a known calibration method of the prior art;
- Figure 8B shows a schematic diagram for the characterization of the shear stress sensitivity device with the calibration method of Fig. 8A according to an embodiment of the present invention;
- Figure 9A shows the measured aggregate sizes of poly(methyl methacrylate) (PMMA) nanoparticles as radius of gyration Rg in [μm] plotted against the rotational speed in [rpm] of a kinetic pump, namely a magnetically levitated centrifugal pump in a shear stress sensitivity device;

- 5 Figure 9B shows a calibration curve generated from a known calibration method of the prior art in a replicated device of the prior art to correlate measured aggregate sizes as radius of gyration in μm with known shear stress values in $[\text{Pa}]$ according to an embodiment of the invention;
- 10 Figure 9C shows the shear stress values in $[\text{Pa}]$ plotted against the rotational speed of the pump in $[\text{rpm}]$, wherein the aggregate sizes of poly(methyl methacrylate) (PMMA) nanoparticles as radius of gyration in μm of Fig. 9A measured in a shear stress sensitivity device are converted with the calibration curve of Fig. 9B into shear stress values in $[\text{Pa}]$ according to an embodiment of the invention;
- 15 Figures 10A to 10E show an overview of different operating modes of a shear stress sensitivity device according to Cases 1 to 4 according to embodiments of the invention;
- 20 Figures 11A and 11B show an embodiment of the present invention without characterization of a shear stress sensitivity device, showing exemplary curves of a cultivation process using a CHO cell line performed in a production-scale bioreactor (curve P, selected cultivation process) and in a small-scale bioreactor (curve S, selected control cultivation process) with two performance characteristics measured, namely viable cell density (VCD) in $[10^6 \text{ cells/mL}]$ and the titer in $[\text{g/L}]$ plotted against the time in $[\text{h}]$, respectively;
- 25 Figures 12A and 12B show the curves of Figures 11A and 11B and curves of additional control cultivation runs performed in the shear stress sensitivity device with varying shear stress intensity represented by the rotational speed, shear stress frequency and duration of shear stress exposure according to an embodiment of the invention;
- 30 Figures 13A to 13F show the results for the measured performance characteristics of CHO cell fed-batch cultures over time based on the measured offline data according to an embodiment of the invention, whereby the shear stress parameter studied is the maximum shear stress represented by the rotational speed of the pump;
- 35 Figures 14A and 14B show the measured online data of Figs. 13A to 13F;
- 40 Figures 15A to 15D show the results for the measured performance characteristics of CHO cell perfusion cultures over time based on the measured offline data according to an embodiment of the invention, whereby the shear stress parameter studied is the maximum shear stress represented by the rotational speed of the pump;

Figures 16A

and 16B show the measured online data of Figs. 15A to 15D;

5 Figures 17A

to 17D

show the results for the measured performance characteristics of CHO cell perfusion cultures over time based on the measured offline data according to an embodiment of the invention, whereby 2 shear stress parameters are studied, namely the shear stress frequency and the duration of shear stress exposure, with varying bypass flow;

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Figures 18A

and 18B

show the measured online data of Figs. 17A to 17D;

Figures 19A

15 to 19D

show the results for the measured performance characteristics of other CHO cell perfusion cultures over time based on the measured offline data according to an embodiment of the invention, whereby 3 shear stress parameters are studied, namely the rotational speed of the pump, the shear stress frequency and the duration of shear stress exposure, with varying bypass flow; and

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Figures 20A

and 20B

show the measured online data of Figs. 19A to 19D.

The legends of the figures are provided at the end of the description.

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DETAILED DESCRIPTION OF THE INVENTION

Definition of terms

30 Terms not specifically defined herein should be given the meanings that would be given to them by a person skilled in the art in light of the disclosure and the context.

A "bioreactor" also referred to herein as a fermenter, is a vessel, container, receptacle, apparatus or device in which living organisms, especially certain microorganisms, cells or small plants, are cultivated or fermented under the best possible conditions. A bioreactor may consist of or comprise a biocompatible
35 vessel in which a chemical or biochemical method is carried out which involves organisms and/or biochemically active substances derived from such organisms. A bioreactor uses additional equipment, for example stirrers, baffles, one or more spargers and/or ports, which specifically allows for the cultivation and propagation of the cells. Commonly the bioreactor is in the form of a cylindrical tube, having two end parts, the end parts forming the top and the bottom of the bioreactor. The bioreactor ranges in size from litres to
40 cubic metres and is often made of stainless steel designed for multiple use. Cultivation in a bioreactor is used to obtain the cells or cell components or metabolic products. These are used, for example, as active ingredients in the pharmaceutical industry for the production of drugs, e.g. as antibiotics, antibodies or insulin; or as basic chemicals in the chemical industry, e.g. in wastewater treatment, in the food industry, in pest control or in the biological degradation of waste or pollutants, e.g. in oil spills. The bioreactor according

to the present disclosure may be used from laboratory scale up to large-scale production and is used as a component of a shear stress sensitivity device to determine the shear stress in a cultivation process.

5 The cells cultivated in the bioreactor are not particularly restricted. They are preferably prokaryotic cells or eukaryotic cells, especially eukaryotic cells. These eukaryotic cells are in particular animal cells, such as mammalian cells, insect cells, plant cells, bacterial cells, yeast, fungi and the like. Eukaryotic cells like chinese hamster ovary (CHO) or yeast cells are for example used to produce antibodies such as monoclonal antibodies and/or recombinant proteins such as recombinant proteins for therapeutic use. Alternatively, the cells may produce, for example, peptides, amino acids, fatty acids or other useful
10 biochemical intermediates or metabolites or any other useful substances. The term "eukaryotic cell" as used herein particularly refers to cells that have a nucleus within a nuclear envelope and include animal cells, human cells, plant cells and yeast cells. In the present invention an "eukaryotic cell" particularly encompasses mammalian cell, such as Chinese hamster ovary (CHO) cell or HEK293 cell derived cells, and yeast cells.

15 The term "cell cultivation" or "cell culture" includes cell cultivation and fermentation methods in all scales (e.g. from sub mL-scale to > 10000 L scale), in all different method modes, e.g. batch, fed-batch, continuous cultivation (e.g. perfusion), in all method control modes (e.g. non-controlled, fully automated and controlled systems with control of e.g. pH, temperature, oxygen content), in all kind of fermentation systems (e.g.
20 single-use systems, stainless steel systems, glass ware systems). In exemplary embodiments the cell culture is a cell culture in a volume of ≥ 10 mL, ≥ 15 mL, ≥ 250 mL, ≥ 500 mL, ≥ 1 L, ≥ 2 L, ≥ 10 L, ≥ 1000 L, ≥ 5000 L or ≥ 10000 L.

The term "liquid culture medium" or "cell culture medium" as used herein is a medium to culture cells, e.g.
25 mammalian cells, comprising a minimum of essential nutrients and components such as vitamins, trace elements, salts, bulk salts, amino acids, lipids, carbohydrates in a preferably buffered medium. Typically a cell culture medium for mammalian cells has an about neutral pH, such as a pH of about 6.5 to about 7.5, preferably about 6.8 to about 7.3, more preferably about 7. Non limiting examples for such cell culture media include commercially available media like Ham's F12 (Sigma, Deisenhofen, Germany), RPMI-1640
30 (Sigma), Dulbecco's Modified Eagle's Medium (DMEM; Sigma), Minimal Essential Medium (MEM; Sigma), Iscove's Modified Dulbecco's Medium (IMDM; Sigma), CD-CHO (Invitrogen, Carlsbad, CA), CHO-S-Invitrogen), serum-free CHO Medium (Sigma), and protein-free CHO Medium (Sigma) etc. as well as proprietary media from various sources. The cell culture medium may be a basal cell culture medium. The cell culture medium may also be a basal cell culture medium to which the feed medium and/or additives
35 have been added. The cell culture medium may also be referred to as fermentation broth.

The term "basal medium" or "basal cell culture medium" as used herein is a liquid culture medium or cell culture medium to culture mammalian cells. It refers to the medium in which the cells are cultured from the start of a cell culture run and is typically not used as an additive to another medium, although various
40 components may be added to the basal medium. The basal medium serves as the base to which optionally further additives (or supplements) and/or a feed medium may be added during cultivation, i.e., a cell culture run resulting in a cell culture medium. The basal cell culture medium is provided from the beginning of a cell cultivation process. In general, the basal cell culture medium provides nutrients such as carbon sources, amino acids, vitamins, bulk salts (e.g. sodium chloride or potassium chloride), various trace elements (e.g.

manganese sulfate), pH buffer, lipids and glucose. Major bulk salts are usually provided only in the basal medium and should not exceed a final osmolarity in the cell culture of about 280-350 mOsm/L, so that the cell culture is able to grow and proliferate at a reasonable osmotic stress.

- 5 The term “feed” or “feed medium” as used herein relates to a concentrate of nutrients/a concentrated nutrient composition, particularly used as a feed in a culture of mammalian cells. Thus, it is provided as a concentrate that is added into the cell culture. It is provided as a “concentrated feed medium” to minimize dilution of the cell culture, typically a feed medium is provided at 10-50 ml/L/day, preferably at 15-45 ml/L/day, more preferably at 20-40 ml/L/day and even more preferably at 30 ml/L/day based on the culture
- 10 starting volume (CSV, meaning the start volume on day 0) in the vessel. This corresponds to a daily addition of about 1-5%, preferably about 1.5-4.5%, more preferably about 2-4% and even more preferably about 3% of the culture starting volume. For cultures using high density seeding or ultra-high density seeding higher feeding rates may be beneficial such as 10-50 ml/L/day, 15-45 ml/L/day or 25-45 ml/L/day. This corresponds to a daily addition of about 1-5%, about 1.5-4.5%, or about 2.5-4.5 % of the culture starting
- 15 volume. The feeding rate is to be understood as an average feeding rate over the feeding period. A feed medium typically has higher concentrations of most, but not all, components of the basal cell culture medium. Generally, the feed medium substitutes nutrients that are consumed during cell culture, such as amino acids and carbohydrates, while salts and buffers are of less importance and are commonly provided with the basal medium. The feed medium is typically added to the (basal) cell culture medium/ fermentation
- 20 broth in fed-batch mode. The feed medium added (repeatedly or continuously) to the basal medium results in the cell culture medium. The feed may be added in different modes like continuous or bolus addition or via perfusion related techniques (chemostat or hybrid-perfused system). Preferably, the feed medium is added daily, but may also be added more frequently, such as twice daily or less frequently, such as every second day. More preferably the feed medium is added continuously. The addition of nutrients is commonly
- 25 performed during cultivation (i.e., after day 0). In contrast to the basal medium, the feed medium typically consists of a highly concentrated nutrient solution (e.g. > 6x) that provides all the components similar to the basal medium except for ‘high-osmolarity-active compounds’ such as major bulk salts (e.g., NaCl, KCl, NaHCO₃, MgSO₄, Ca(NO₃)₂). Typically a 6x-fold concentrate or higher of the basal medium without or with reduced bulk salts maintains good solubility of compounds and sufficiently low osmolarity (e.g. 270-1500
- 30 mosmol/kg, preferably 310-800 mosmol/kg) in order to maintain osmolarity in the cell culture at about 270-550 mosmol/kg, preferably at about 280-450 mosmol/kg, more preferably at about 280-350 mosmol/kg. The feed medium may be added as one complete feed medium or may comprise one or more feed supplements for separate addition to the cell culture. The use of one or more feed supplements may be necessary due to different feeding schedules, such as regular feeding and feeding on demand as often
- 35 performed for glucose addition, which is therefore typically at least also provided as a separate feed. The use of one or more feed supplements may also be necessary due to low solubility of certain compounds, solubility at different pH of certain compounds and/or interactions of compounds in the feed medium at high concentrations. The feed medium is preferably chemically defined (optionally comprising a recombinant protein, such as insulin or IGF). It does not contain cells, has not been in contact with cells in culture or does not contain cell derived metabolic waste products. Thus, as used herein, the term “feed medium”
- 40 excludes a pre-conditioned medium derived from a cell culture or a culture medium in cell culture, i.e., in the presence of cells (also referred to as liquid culture medium or cell culture medium herein).

The term "feed supplement" as used herein relates to a concentrate of a nutrient, which might be added to the feed medium before use or may be added separately from the feed medium to the basal medium and/or the cell culture medium. Thus, a compound may be provided with the feed medium or the feed supplement or a compound may be provided with the feed medium and the feed supplement. For example, cysteine
5 may be added in a two-feed strategy with the feed medium and the feed supplement. As the feed medium, the "feed supplement" is provided as a concentrate in order to avoid dilution of the cell culture.

The liquid culture medium or cell culture medium, both basal medium and feed medium is preferably serum-free and chemically defined. The basal medium and/or the feed medium may further be protein-free. A
10 "serum-free medium" as used herein refers to a cell culture medium for in vitro cell culture, which does not contain serum from animal origin. This is preferred as serum may contain contaminants from said animal, such as viruses, and because serum is ill-defined and varies from batch to batch. The basal medium and the feed medium according to the invention are serum-free.

15 A "chemically defined medium" as used herein refers to a cell culture medium suitable for in vitro cell culture, in which all components are known. More specifically it does not comprise any supplements such as animal serum or plant, yeast or animal hydrolysates. A chemically defined medium is therefore also serum-free. The basal medium and the feed medium according to the invention are preferably chemically defined. In one embodiment the basal medium and/or the feed medium are serum-free and chemically-defined and
20 optionally comprises a recombinant growth factor such as insulin or insulin-like growth factor (IGF). The basal medium and/or the feed medium as referred to herein comprise no further proteins, except for, once in cell culture to provide the cell culture medium, proteins produced by the mammalian cell to be cultured.

A "protein-free medium" as used herein refers to a liquid culture medium or cell culture medium for in vitro
25 cell culture comprising no proteins (except for proteins produced by the cell to be cultured in cell culture), wherein protein refers to polypeptides of any length, but excludes single amino acids, dipeptides or tripeptides. Specifically, growth factors such as insulin and insulin-like growth factor (IGF) are not present in the medium. Preferably, the basal medium and feed medium according to the present invention are chemically defined and protein-free.

30 The term "viability" as used herein refers to the % viable cells in a cell culture as determined by methods known in the art, e.g., trypan blue exclusion with a Cedex device based on an automated-microscopic cell count (Roche Diagnostics; Rotkreuz). However, there exist a number of other methods for the determination of the viability such as fluorometric (such as based on propidium iodide), calorimetric or enzymatic methods
35 that are used to reflect the energy metabolism of a living cell e.g. methods that use LDH (lactate dehydrogenase) or certain tetrazolium salts such as alamar blue, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or TTC (tetrazolium chloride).

The expression "batch" or "batch culture" stands for a culture in which a certain volume of medium is
40 inoculated with cells. The cells grow in the medium and are exposed to changing conditions, as nutrients are depleted and waste products accumulate. The system can be considered as a closed one, with no additions or removals during the period of incubation, but oxygen is regularly added to the system by gassing.

The term "fed-batch" as used herein relates to a cell culture in which the cells are fed continuously or periodically with a feed medium containing nutrients. The feeding may start shortly after starting the cell culture on day 0 or more typically one, two or three days after starting the culture. Feeding may follow a preset schedule, such as every day, every two days, every three days etc. Alternatively, the culture may be monitored for cell growth, nutrients or toxic by-products and feeding may be adjusted accordingly. In general, the following performance characteristics are often determined on a daily basis and cover the viable cell concentration, product concentration (titer), pH value, osmolarity and several metabolites such as glucose, lactate (a measure for salt content), and ammonium (growth inhibitor that negatively affects the growth rate and reduces viable biomass). Compared to batch cultures (cultures without feeding), higher product titers can be achieved in the fed-batch mode. Typically, a fed-batch culture is stopped at some point and the cells and/or the medium is harvested and the product of interest, such as a heterologous protein or a recombinant virus is isolated and/or purified. A fed-batch process is typically maintained about 2-3 weeks, e.g., about 10-24 days, about 12 to 21 days, about 12 to 18 days, preferably about 12 to 16 days. Particularly, a fed-batch process for the production of a heterologous protein is typically maintained about 2-3 weeks, e.g., about 10-24 days, about 12 to 21 days, about 12 to 18 days, preferably about 12 to 16 days.

"Perfusion" or "a perfusion culture" is understood here as a continuous process. It is a special process method for cultivating cells, in which nutrients in the form of fresh media are added to the cell culture and cell waste products and media depleted of nutrients are continuously removed from the cell culture. The cells are retained by a filter (hollow fiber filter module) according to the present disclosure. Usually, fresh media is provided to the cells at the same rate as the spent media is removed thus keeping the bioreactor volume constant.

The term "laboratory scale" refers to an experimental set-up in which work is carried out on a small scale. These are, for example, approaches in which a volume of several mL to several litres is used.

The expressions "industrial scale" or "large-scale" are used interchangeably and synonymously and relate to a product which is obtained in a large production amount whereby there is often a cost advantage with costs per unit of output decreasing with increasing scale. A large manufacturing unit is to be expected to have a lower cost per unit of output than a smaller unit, all other factors being equal. An industrial scale may be understood in connection with the cultivation of cells to have a volume of the bioreactor used which is equal or greater than about 100 L. According to a further embodiment the volume of the bioreactor used in industrial scale may be equal or greater than 600, 800, 1000, 1200, 1500 L or even more.

As background information for the cultivation of cells, type of cells, media used therefore and methods, reference is made to WO 2021/165302 A1, the contents of which be incorporated by reference in their entirety in the present disclosure. In particular, reference is made to paragraphs [0054] to [0088].

The expression "loop" or "loop system" refers to the circuit tubing system outside the bioreactor that is part of the shear stress sensitivity device. The term "loop", "external loop" and "loop system" are used synonymously and interchangeably.

The term "closed loop system" means that the bioreactor is connected to the loop on both sides and the loop has no other outflow. The bioreactor is connected in between in such a way that the liquid flow of cultivation medium and cells enters the bioreactor on one side and leaves it on the other side. A closed loop system is schematically shown in Figures 1A and 1B. The closed loop system is used for circulating the cell culture as a whole comprising cells and liquid culture medium in the shear stress sensitivity device. "Circulating" is understood here to mean that the cell culture as a whole, comprising the cells and liquid culture medium, is circulated from the bioreactor into the closed loop system and back to the bioreactor and again through the closed loop system and so on. The circulation is performed by a pump placed in the closed loop system, whereby the pump continuously pumps to circulate the cell culture, which comprises cells and the liquid culture medium, in the closed loop system. The pump used is a kinetic pump.

The term "shear stress" refers to the force divided by the area of the surface on which it is acting. In engineering mechanics, shear stress is the load on a body resulting from the action of counter-parallel forces on parallel surfaces. This means that opposing transverse forces act. These forces then cause the so-called shear load. As in earthquakes, when two adjacent layers of earth slide over each other, the stress created between them is called shear stress. Generally, the unit of the shear stress τ (tau) is Pascal [Pa]. Shear stresses for cells are external influences resulting from the type and procedure of cell cultivation.

The "shear stress sensitivity determination device" herein also referred to briefly as "shear stress sensitivity device" is a device in which the shear stress acting on the cells during cultivation is determined. The shear stress sensitivity device comprises a bioreactor, a loop located outside the bioreactor, a kinetic pump and means to control the hydrodynamic shear stress in the loop such as a variable tube, and optionally cell retaining means such as a hollow fiber filter module. In the shear stress sensitivity device, the cell culture comprising a liquid culture medium used to cultivate the cells and the cells are continuously circulated by the pumping action of the pump to determine the shear stress in each case by varying certain parameters.

"Hydrodynamic shear stress" is shear stress resulting from hydrodynamic effects during a cultivation process, in particular caused by any form of movement, e.g. by the liquid medium in the cell culture, by stirring, by the supply of gases and the like. The "shear stress" and the "hydrodynamic shear stress" are intended to be synonymous here and are used interchangeably.

Like living organisms single living cells like e.g. bacteria and yeasts and especially those with no cell wall like living cells derived - in principle - from multicellular organisms like animals are sensitive to shear stress affecting the integrity of their shape, overall physical stability and biological functions in general. The "shear stress sensitivity" of cells therefore represents a measure of to what extent cells can withstand shear stress and thus, in particular, the resilience of cells to shear stress intensity, which is directly reflected in their properties, especially the product-related properties referred to here as "performance characteristics".

The "shear stress limit" of cells is considered here as the shear stress or shear stress intensity that cells can withstand without significantly adversely affecting the cell cultivation process. This affects the performance characteristics in cell cultivation of cells, such as live cell density, productivity of cells, e.g. in view of production of proteins or other molecules of interest (qualitatively and/or quantitatively), and the like. The term "limit" or "threshold" means a limit above which adverse deterioration occurs in the cultivation

of cells. A quantitative statement about the shear stress sensitivity of cells can be made with the help of the shear stress limit.

5 The "shear stress intensity" is the strength or magnitude of the overall shear stress acting on the cells, for example, in the shear stress sensitivity device. The shear stress intensity represents the combination or totality (or sum) of all shear stress parameters. The shear stress parameters are the maximum shear stress, the shear stress frequency and the duration of shear stress exposure. It is part of the expert's knowledge that a parameter is a quantifiable variable or quantity that has a specific value depending on the chosen conditions, so that a parameter is specified using values. Therefore, a shear stress parameter is a
10 quantifiable variable or quantity that has a specific value depending on the selected conditions, i.e. a shear stress parameter is given in the form of values that quantify it. 'Shear stress parameter(s)' and "shear stress values" are both technical terms whose meaning is part of the skilled person's knowledge. Furthermore, the mathematical equations to describe the individual shear stress parameters are given in the paragraph 'Relationships between the shear stress parameters and the influence of the bypass flow'.

15 The "maximum shear stress" is a shear stress parameter which is determined and represented by the rotational speed of the pump used, i.e. a kinetic pump present in the shear stress sensitivity device. The values given for the shear stress parameter 'maximum shear stress' $T_{\max}(\tau)$ also have the unit Pascal [Pa].

20 The "duration of shear stress exposure" is a shear stress parameter which is determined and represented by the residence time of the cell culture comprising liquid culture medium and cells within the pump used i.e. a kinetic pump present in the shear stress sensitivity device.

25 The "shear stress frequency" is a shear stress parameter which is determined and represented by the number of passages of the cell culture comprising liquid culture medium and cells through the loop system of the shear stress sensitivity device per time.

30 The expression "bypass flow" represents the flow of the culture medium together with the cells in the loop system of the shear stress sensitivity device. The loop system represents an external loop or external circuit to the bioreactor. The flow may be measured, for example, using a flowmeter.

The term "characteristic" represents any measurable variable that changes over time during the cultivation of cells and can be determined. A "performance characteristic" represents any measurable variable that
35 changes over time during the cultivation of cells and can be determined, which is a parameter that is characteristic for a certain cultivation process for the cultivated cells and characterizes them. Performance characteristics of a cell culture as understood in the present invention are especially so-called product-related quality parameters. Examples of performance characteristics are growth and productivity of the cell culture, in particular relative living cell density, viability, glucose concentration, lactate concentration, LDH
40 concentration, relative titer (product concentration), glycosylation of the product and many more. The term 'performance characteristics' is known to those skilled in the art (e.g. mentioned in the reference book of Kompala et al.⁽²⁴⁾), whereby the numerous performance characteristics influenced by shear stress in the cultivation of cells are also known to the person skilled in the art (Sieck et al.⁽⁸⁾, Neunstoecklin et al.⁽⁹⁾, and Gaugler et al.⁽²⁵⁾).

The "parameters" and "conditions" that could be set and changed in the shear stress sensitivity device are very numerous. These are all variable parameters and conditions with which cell cultivation can be performed. These are known to the skilled person in the prior art. For example, these are the setting of the gassing, the stirring in the bioreactor, the temperature, the feed rate, the dissolved oxygen tension, the pH and many more. When investigating the shear stress level in the shear stress sensitivity device, the parameters and conditions in the shear stress sensitivity device are selected and set once and are then kept as constant as possible throughout the procedure so as not to interfere with the investigations.

The expressions "comprising", "comprise", "comprised", "containing", "contain" or "contained" shall also encompass the more specific term "consisting of" unless otherwise stated or apparent from the context.

In addition, it should be noted that in this disclosure, the singular and plural forms are not used in a restrictive manner. As used herein, the singular forms "a", "an", "one" and "the" therefore refer to both the singular and the plural, unless otherwise stated or apparent from the context.

The expression "about" or "approximately" means within 10 %, particularly within 5 % and more particularly within 1 % or within 0.1 % of a value specified or an upper or lower range value as indicated.

EMBODIMENTS OF THE INVENTION

The invention will be explained below with reference to various exemplary embodiments.

According to the method of the present invention in this embodiment, the method comprises or consists of steps (1), (2), (3), (4), (5), (6) and (7). According to an embodiment, these steps are performed in the order indicated. According to a further embodiment, there are no intermediate steps performed between these steps.

The method according to the invention for determining the shear stress sensitivity of cells during a cultivation process is carried out in a shear stress sensitivity device. This is a device that makes it possible to better assess the effects of the shear stress acting on the cells during a cultivation process and even to measure the shear stress sensitivity in different modes of operation of the device. The focus here is on the specific influence on the cells, whereby changing parameters of the shear stress sensitivity device and changing conditions of the cultivation process lead to different influences and thus results. Of importance in this context is the shear stress intensity to which the cells can be subjected/exposed, which is crucial for the performance of the cells. The shear stress intensity has a direct effect on the so-called product-related quality parameters of cell cultivation, here referred to as 'performance characteristics', such as cell growth.

General set-up of the shear stress sensitivity device

A general set-up of a device commonly used for culturing cells has been taken as the basis of the present invention as a "shear stress sensitivity device". In this device, the shear stress, in particular the shear stress sensitivity or limit to which the cells can be subjected, is determined herein.

A shear stress sensitivity device according to the present invention comprises:

- a bioreactor,

- a closed loop system which is used for circulating the cell culture as a whole comprising cells and liquid culture medium, the closed loop system is connected at both ends with the bioreactor and is located outside the bioreactor;
- a pump placed in the loop system particularly downstream from the bioreactor, whereby the pump continuously pumps to circulate the cell culture comprising cells and the liquid culture medium in the loop system, the pump used being a kinetic pump, and
- means to control the hydraulic resistance that is placed in the loop system, especially downstream of the pump.

10 According to step (1) of the method according to the invention, such a shear stress sensitivity device is provided.

The shear stress sensitivity device of the invention will be described in detail for better illustration with reference to the schematic embodiments of the present invention shown in Figures 1A and 1B. The shear stress sensitivity device can be adapted according to the operating mode of cultivation to be investigated, i.e. batch, fed-batch or continuous (e.g. perfusion). Two different set-ups of the shear stress sensitivity device are therefore depicted in Figures 1A and 1B. In Figure 1A the set-up for fed-batch cultivation is shown. This set-up is also suitable for batch processes. In Figure 1B the set-up for continuous cultivation, particularly perfusion cultivation, is shown.

20 First, an embodiment of the shear stress sensitivity device for fed-batch or batch cultivation according to the invention as shown in Fig. 1A will be explained. In Fig. 1A, shear stress sensitivity device 100 has a bioreactor 110 connected to a loop system. To circulate cell culture comprising the cells with the culture medium 115, a closed loop system is provided which is connected with the bioreactor 110 and the loop system is arranged outside the bioreactor 110. In the present embodiment an outlet in the lower part of the bioreactor is connected to an inlet in the head part of the bioreactor. Other configurations of how the loop system is connected to the bioreactor are also possible. In Fig. 1A, the bioreactor 110 contains liquid culture medium 115 together with the cells to be cultured (not shown). In the embodiment shown the outlet 114 in the lower part of the bioreactor 110 is connected to the inlet 116 in the head part of the bioreactor 110 via tubes 125a, 125b, 125c, 125d1 and 125d2. The type of tube is not further limited and includes any type of fluid conduits known to those skilled in the art, such as lines, hoses, pipes, and the like. The tubes may be selected from polymers, such as rubber, or metal.

35 The loop system is used to circulate the entire cell culture comprising the liquid culture medium 115 and cells, and is located outside the bioreactor 110. The flow of the cell culture comprising liquid culture medium 115 with the cells is in the present embodiment in the direction of the arrows A and B as shown. An agitator 118 can additionally be provided in the bioreactor 110. Other embodiments for the loop system and other positions for the connections of the loop system to the bioreactor - than those shown in Fig. 1A - are also possible according to the invention. For example, a dip tube extending into the bioreactor from above could be used to pump the cell culture from the bioreactor into the loop system.

40 In the loop system a pump is present, which continuously circulates the cell culture comprising the liquid culture medium and the cells. In Fig. 1A, the bioreactor 110 is connected to a tube 125a. The tube 125a is then connected to the suction side of the pump 130. The pump 130 is a kinetic pump, particular a centrifugal

pump, especially a magnetically levitating centrifugal pump. This type of pump has proven to be particularly advantageous for performing shear stress tests. On the discharge side, the pump 130 is again connected to a tube 125b. This tube 125b is then connected with means 125c1, 125c, 125c2 to control the hydrodynamic resistance. "Means to control the hydrodynamic resistance" are one, two or more devices, aids or tools to create a pressure drop, particularly a higher or lower pressure drop, in the loop system. This plays a role when the increase of one shear stress parameter causes an undesired increase of another parameter that should be kept constant. This will be explained in more detail later.

In the embodiment of Fig. 1A means to control the hydrodynamic resistance is a tube 125c of variable length and/or variable diameter, which is also referred to here as "variable tube". Hereby the length and/or diameter can be adjusted depending on the operating conditions. This can be achieved, for example, by replacing the tube 125c with another tube having a different length and/or a different diameter. To vary the diameter of the variable tube 125c, two reducers can also be provided, for example, at one end of the tube 125c reducer 125c1 and at the other end of the tube 125c reducer 125c2. For example, increasing the length or decreasing the diameter of the variable tube 125c increases the pressure drop. Other means to control the hydrodynamic resistance are also possible. Exemplarily mentioned are valves, e.g. pinch valves. Also other practical implementations of this means are possible, such as variable winding of the variable tube 125c, for example to increase the pressure drop.

In Fig. 1A, the exemplarily shown means to control the hydrodynamic resistance 125c1, 125c, 125c2 are connected to tube 125d1 which is connected to the bioreactor 110 via tube 125d2. A flowmeter 140 may be installed between the bioreactor 110 and the means to control the hydrodynamic resistance 125c1, 125c, 125c2. Additionally, pressure gauges or pressure sensors (not shown) may be used to determine the pressure drop over the means to control the hydrodynamic resistance 125c1, 125c, 125c2, if required. In Fig. 1A, the means to control the hydrodynamic resistance 125c1, 125c, 125c2 are located downstream of the pump 130. Other configurations are conceivable.

A cell culture is cultivated in a liquid in the bioreactor and continuously pumped out of the bioreactor by a kinetic pump such as a magnetic driving force centrifugal pump. As was found in experiments, the highest shear stress is applied with the pump which is controlled via the rotational speed [rpm] of the pump. There are some parameters for setting the shear stress levels in the shear stress sensitivity device: these are, in addition to the maximum shear stress represented by the rotational speed of the pump, the residence time of the cell culture within the pump (herein referred to as duration of shear stress exposure) and the number of passages of the cell culture comprising liquid culture medium and cells through the loop system per time (herein referred to as shear stress frequency). These will be explained in more detail later.

In continuous mode, such as perfusion mode as shown in Figure 1B, the set-up of Fig. 1A is extended by a cell retaining means, e.g. a hollow fiber filter module. In Figure 1B the set-up of the shear stress sensitivity device 100 for continuous mode, especially perfusion mode, is shown, including a hollow fiber filter module 150 with permeate flow (arrow C). The cells are retained by the hollow fiber filter 150. In Figure 1B the tube 125c is connected via tube 125d1 to a hollow fiber filter module 150 as depicted in Figure 1B. Also in Figure 1B, a flowmeter 140 may be installed between bioreactor 110 and hollow fiber filter module 150. Pressure gauges or sensors (not shown) may be used to determine the pressure drop over the hollow fiber filter module 150. The hollow fiber filter module is not further restricted according to the invention. Any hollow

fiber filter module and any cell retention method/device suitable for culturing cells in continuous mode such as perfusion can be used.

5 In step (2) of the method according to the invention, the cells, the liquid culture medium, the cultivation mode and the cultivation conditions are selected. The selections are not further limited within the scope of the invention, so that any cultivation method with any cells, any liquid culture medium, any cultivation mode and any cultivation conditions can be used for the method according to the invention. The only prerequisite is that the cultivation must take place in a liquid medium so that it can be circulated in the shear sensitivity device. The purpose of the invention is that there are various cultivation methods (e.g. large-scale methods
10 for producing New Biological Entities (NBEs)) in which increased shear stress leads or could lead to a loss of performance. The method according to the invention makes it possible to simulate cultivation methods in the shear stress sensitivity device and thus to characterise/examine the shear stress under the corresponding conditions.

15 These selection steps according to step (2) can determine which of the two set-ups of the shear stress sensitivity device, i.e., the set-up according to Fig. 1A or the set-up according to Fig. 1B, is used. If the shear stress sensitivity device is to be operated in continuous mode such as perfusion, the set-up also includes a cell retention device that may be placed between the means to control the hydrodynamic resistance such as a variable tube and the bioreactor. Other configurations are possible.

20 The cells are not further limited. Any type of cells may be used, in particular eukaryotic cells or prokaryotic cells. Eukaryotic cells are in particular animal cells, for example mammalian cells, insect cells, plant cells, bacterial cells, yeast, fungi and the like. Eukaryotic cells include in particular mammalian cells, such as cells from the ovary of the Chinese hamster (CHO) or HEK293 cells, and yeast cells.

25 The liquid culture medium is also not particularly limited; any type of liquid culture medium used for the cultivation of cells can be used. Liquid culture medium includes, for example, basal medium, chemically defined medium, protein-free medium as explained in detail in the definitions of terms.

30 The cultivation conditions include all conditions that can be selected in connection with the cultivation of cells. These include, for example, the seeding cell density, the oxygen concentration supplied, the cultivation scale selected, the type of feed medium, the feeding rate, the pH range used, the temperature set during cultivation, the glucose supply, the type of hollow fiber module selected during perfusion, the perfusion rates, aeration rate, specific power input and many others more.

35 The cultivation mode for the cultivation process to be performed comprises batch, fed-batch and continuous (e.g. perfusion) processes.

Shear stress characterizations

40 In the following, the shear stress characterizations as a basis for the development of the shear stress sensitivity device described above and the results found for this are explained in general. Here, all parts of the previously described device were characterized with respect to shear stress. Furthermore, the source at which maximum shear stresses occur was identified, which plays a central role for the present invention.

First, experiments were conducted to determine whether shear stresses triggered by gassing or agitation within an usual bioreactor are really lower compared to shear stresses within the loop used in the shear stress sensitivity device. Only if this is clear, meaningful results about the shear stress can be obtained. For this, shear stresses in a 3L bioreactor were determined in separate experiments. The shear stress values were calculated as in the method according to the invention. This is explained in detail in the paragraph "Methods for determining the maximum hydrodynamic shear stress" and the following paragraphs. This is done by using a shear stress-dependent quantity which is clearly related with occurring maximum shear stress values. In the present experiments the shear stress-dependent quantity is the aggregates size, more precisely the radius of gyration of particle aggregates selected from poly(methyl methacrylate) nanoparticles and subjecting these poly(methyl methacrylate) nanoparticle aggregates to shear stress in a device, here a 3 L bioreactor. The shear stress here is gassing and agitation. Poly(methyl methacrylate) nanoparticle aggregates are known to be shear stress sensitive, with the aggregate decomposing in a defined manner upon shear stress, and the resulting aggregate size is adjusted as a result of the shear stress occurring.

The correlation between aggregate size and shear stress value known from the prior art (e.g. Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾) serves as a calibration curve to convert the obtained aggregate sizes to the maximum shear stress values that must have been present in the device (here: 3L bioreactor) to obtain these aggregate sizes. Examples of how this is accomplished in detail will be explained in the chapter: "Methods for determining the maximum hydrodynamic shear stress", and particularly under "An exemplary calibration method" and "An exemplary characterization method".

Figure 2 shows the results of shear stress measurements for different gassing and agitation rates performed in a simple 3L bioreactor without external loop. The shear stress $\tau_{\max}$ in [Pa] is plotted against the impeller agitation speed in [rpm]. The shear stress values for $\tau_{\max}$ in [Pa] were determined using poly(methyl methacrylate) nanoparticle aggregates, as will be explained later. The gas flow was set to a constant value in each case, as indicated in Figure 2, and is given in [vvm]. The unit "vvm" means volume of air per volume of culture medium per minute.

In the experiments, 1 g/L surfactant (Pluronic F-68) was added to prevent bubble-aggregate attachment between the bubbles of a sparger used for gassing and the poly(methyl methacrylate) nanoparticle aggregates. In the same manner cells are prevented to attach to the bubbles.

Figure 2 shows that the shear stress increases with increasing impeller agitation rate in the 3L bioreactor. Nevertheless, the effect of gassing rates and agitation speeds on the hydrodynamic shear stress is rather negligible. In particular, the shear stresses never reach a level of >10 Pa regardless of the applied conditions. Therefore, the actual shear stress factors are found elsewhere in the culture system. The stirring and the gassing are therefore only present as a kind of background shear stress and play practically no role according to the invention.

In order to be able to verify the shear stress intensity to which cells are subjected during cultivation, further experiments were carried out, i.e. further shear stresses occurring within an external loop were characterized. Of particular interest in this context is the maximum hydrodynamic shear stress that occurs, which is of importance according to the present invention.

Maximum hydrodynamic shear stress

The shear stress sensitivity device used is characterized with the maximum shear stress values that occur in the shear stress sensitivity device. This is performed using a calibration method, particularly a calibration method known from prior art. In this connection it must be ensured that only the maximum shear stress source is used for characterization. "Characterizing" or "characterization" of the shear stress sensitivity device is a calibration method used to determine maximum shear stress values under various conditions. This calibration method of the shear stress sensitivity device is referred to here as "characterization" in order to clearly distinguish it from the used calibration method known from prior art. The maximum shear stress is used in order to be able to apply known calibration methods from the prior art for its determination. This will be explained in detail later. The maximum shear stress source in the shear stress sensitivity device of the present invention is found to be the rotational speed of the pump as demonstrated in detail below:

The characterizing of a shear stress sensitivity device of Fig. 1B using a known calibration method is performed in an experiment. In this experiment the known calibration method is selected from a method which is based on determining a shear stress-dependent quantity, which in the present case is the radius of gyration of particle aggregates selected from poly(methyl methacrylate) nanoparticles. It is known from prior art (e.g., Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾) that the break-up of the aggregates of these particles is a function of the maximum hydrodynamic shear stress, so that it is possible to extrapolate from the size of the aggregates to the maximum shear stress prevailing in a device. These non-biologic aggregates, such as PMMA aggregates, have an irregular size expressed by the (average) radius of gyration (Rg). The radius of gyration (Rg) is a known quantity for characterizing the spatial extent or size of irregularly shaped aggregates and is comparable to the average particle size. This quantity is known to those skilled in the art.

Furthermore, the influence of the respective flow rates and pressures in the shear stress sensitivity device was investigated and the results are explained below in connection with Figs. 3A, 3B, 4A and 4B.

A simplified experimental set-up was used by replacing the previously used 3L bioreactor with a 2L water reservoir that was only gently stirred to prevent high occurring stresses. To find out whether the highest stresses occurred in tubes of variable length or in different types of hollow fiber filter modules, the experimental set-up was adjusted accordingly. Either a tube with a specific inner diameter (ID) or a hollow fiber filter module with a specific length was used in the experiment. In other words, a simplified set-up similar to Figures 1A and 1B was chosen, but a 2L water reservoir was used instead of the bioreactor.

In Figures 3A and 3B the flow rate in [mL/min] over various rotational speeds in [rpm] of a pump is shown for different set-ups. The pump used was a magnetically levitating centrifugal pump. For the characterization set-up the tube length was fixed to 245 mm.

Fig. 3A shows the influence of the three investigated inner diameters (ID) on the flow rate in [mL/min] at different rotational speeds in [rpm] of the pump. The flow rate in [mL/min] increases with increasing rotational speeds in [rpm]. For an inner diameter (ID) of 6.4 mm a flow rate of > 6500 mL/min were reached with $\approx$ 13000 rpm, whereas the tubes with the smallest inner diameter (ID) of 1.6 mm only resulted in a flow rate < 1000 mL/min at the same rotational speed in [rpm]. In a similar way the flow is reduced for a long

hollow fiber filter module (HF long) compared to a short hollow fiber filter module (HF short) for identical rotational speeds in [rpm] (cf. Figure 3B).

Furthermore, Figure 4A shows the measured pressure drop (dP) in [bar] over the flow rate in [mL/min] for various inner diameters (ID) of the tube. Figure 4B shows the measured pressure drop (dP) in [bar] over the flow rate in [mL/min] for different lengths of hollow fiber filter modules. As already discussed and displayed in Figures 3A and 3B, smaller inner diameters (IDs) and longer hollow fiber filter modules lead to a reduction of the flow rate for identical rotational speeds in [rpm], which is due to the increased pressure drop as depicted in Figures 4A and 4B.

Considering the above-mentioned flow and pressure studies it was demonstrated that the shear stress sensitivity device can be operated at different flow rates while keeping rotational speed in [rpm] of a pump constant by using different tube inner diameters (IDs) or different lengths of hollow fiber filter modules and thereby varying the pressure drop. Alternatively, the rotational speed in [rpm] of the pump can be increased, while keeping the flow constant due to an increased pressure drop.

In Fig. 5, the measured average size of poly(methyl methacrylate) nanoparticle aggregates are plotted as radius of gyration (Rg) in [μm] against the respective set rotational speeds of a pump in [rpm]. The pump used in this case was a kinetic pump, namely a magnetically levitating centrifugal pump.

In detail, Fig. 5 shows the aggregate radius of gyration (Rg) in [μm] versus centrifugal pump rotational speed in [rpm] whereby the means to control the hydrodynamic resistance are selected from variable tubes with different inner diameters (IDs) and the cell retaining means are selected from two different hollow fiber filter modules: a long hollow fiber filter module (HF long) and short hollow fiber filter module (HF short). The two black dotted lines in Fig. 5 indicate the 95% prediction interval of the fit. Remarkably the aggregate size (Rg) decreases within the same manner with increasing rotational speed of the pump independent of the tubing or hollow fiber filter modules used. The trend follows a power law function with a R^2 of 0.92 as depicted in Fig. 5. The fitted curve in Fig. 5 follows the function $y = a * x^b$, where $a = 5.91e+04$, $b = -1.34$, $R^2 = 0.92$, and adjusted $R^2 = 0.92$.

Since the aggregate sizes (Rg) can be attributed to maximum hydrodynamic shear stresses in the system (Villiger et al.⁽¹¹⁾), this experiment shows that highest shear stresses within this system are not originating from the tubing or the hollow fiber filter modules (used in this set-up) but are to a large extent dependent on the rotational speed in [rpm] of the pump.

Furthermore, the rotational speed of the pump can be considered as the maximum shear stress occurring in the shear stress sensitivity device. This means that the characterization of the shear stress sensitivity device is performed based on the maximum shear stress resulting from the rotational speed of the pump. Furthermore, the maximum shear stress serves as an orientation to ensure that the maximum shear stress in the loop system of the shear stress sensitivity device is higher than the maximum shear stress in the bioreactor. In this case it is guaranteed that the calibration method of the prior art lead to correct results.

Shear stress parameters

According to the invention, the shear stress acting on the cells is investigated on the basis of 3 parameters. These are the maximum shear stress, the shear stress frequency and the duration of shear stress exposure. The shear stress parameters are also simply referred to here as stress parameters.

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Accordingly, in step (3) of the method according to the invention, at least one stress parameter is selected, on the basis of which the influence of the shear stress on the cells is investigated. The influence of the shear stress parameter(s) on the cells is(are) determined by varying the selected shear stress parameter(s) in several cultivation runs of a cultivation process, which is always carried out in the same way, so that in particular the change in the shear stress parameter(s) has an effect on the data that can be obtained from the cell culture and knowledge is thus gained about the shear stress load on the cells.

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The shear stress parameters that can be varied are selected from the following group, consisting of:

- maximum shear stress represented by a rotational speed of the pump;
- 15 - duration of shear stress exposure represented by a residence time of the cell culture comprising liquid culture medium and cells within the pump; and/or
- shear stress frequency represented by a number of passages of the cell culture comprising liquid culture medium and cells through the loop system per time.

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The individual shear stress parameters are realized in the shear stress sensitivity device of the invention by varying certain physical parameters:

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The shear stress intensity represents the strength or magnitude of the overall shear stress acting on the cells. Since - as already shown - the shear stress intensity represents the combination or totality of all shear stress parameters.

According to the invention, the maximum shear stress is controlled by the use of the pump, whereby different values of maximum shear stress acting on the cells can be set by varying the rotational speed of the pump. The unit of the rotational speed of the pump is [rpm]. The higher the rotational speed of the pump is set, the higher the maximum shear stress acting on the cells. The pump can therefore be operated at different rotational speeds [rpm], in which case the pump causes the actual shear stress. The pump used is a kinetic pump such as centrifugal pump, particularly a magnetically levitating centrifugal pump. Also other kinetic pumps may be used.

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35 The shear stress in the pump is therefore determined by the rotational speed, where the rotational speed could be assigned to an individual flow in the loop system. If only one selected stress parameter is varied in the shear stress sensitivity device, all other parameters should be kept constant – as far as possible - to determine only the influence of this one shear stress parameter. If, for example, the rotational speed [rpm] of the pump is varied, a constant bypass flow is set in the loop system in each case. This is obtained by a pressure drop in the loop system in the form of a hydrodynamic resistance, namely the means to control the hydrodynamic resistance. This are for example a variable tube with varying length and/or diameter, the presence of one or more valves, e.g. pinch valves, and/or providing a variable winding of the variable tube. It is then convenient if the pressure drop is measured by providing one or more pressure gauges in the shear stress sensitivity device.

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Our experiments have shown that the rotational speed or rotational frequency of the pump corresponds to the maximum shear stress occurring in the shear stress sensitivity device. The maximum shear stress is therefore an important shear stress parameter. Furthermore, it should be ensured that the maximum hydrodynamic shear stress is caused by the pump so that the maximum hydrodynamic shear stress is occurring in the closed loop and not in the bioreactor to achieve meaningful results.

The duration of shear stress exposure is realized by the residence time of the cell culture within the pump. A longer residence time of the cell culture within the pump means a longer duration of the shear stress exposure. A shorter residence time of the cell culture within the pump means a shorter duration of the shear stress exposure. A longer residence time of the cell culture within the pump can be achieved, for example, by having several pumps connected in series in the shear stress sensitivity device in the loop at the same time. This increases the residence time of the cells in the pumps. In other words, the duration of exposure means how long the cells remain in the pump. In other words the dead volume in the pump head is virtually assigned to a certain residence time of the cells in the pump.

To better illustrate the duration of shear stress exposure or the residence time of the cell culture within a pump, reference is made to Fig. 6, which shows a schematically simplified sectional view of a main part of an embodiment of a magnetically levitating centrifugal pump. The magnetically levitating centrifugal pump 130 shown is comprised of a single motor/bearing unit that provides both drive and magnetic bearing functions simultaneously. In essence, the pump 130 comprises three parts: an impeller 133 that comprises a rotating magnet such as an over-moulded magnet ring 134, a bottom casing and a top casing, which form the entire pump housing 131. The pump housing 131 is the housing of the whole pump head 132. The impeller 133 is levitated, contact free, inside the pump housing 131 and is driven by the magnetic field of the motor (motor/bearing winding 135). The rotational speed of the impeller 130 can be precisely controlled by electronical regulation. The pump head 132 can be easily detached from the motor/bearing stator 136 and cleaned without difficulty. In certain applications, the pump head 132 is a disposable unit.

The two arrows in Fig. 6 show the path that a liquid containing cell culture comprising liquid culture medium and cells take through the pump 130, with the entry at arrow D into the pump 130 and the exit at arrow E from the pump 130. The cell culture comprising liquid culture medium and the cells is therefore sucked in centrally by a suction that forms and expels laterally. If the cells are in the pump 130 together with the liquid culture medium, they are subjected to shear stress as long as they are in the pump 130. According to the invention, this is referred to as the residence time in the pump which is referred to as the duration of shear stress exposure. For example, if 2 pumps are connected in series in the shear stress sensitivity device, the residence time of the cells in the pumps is higher and likely doubles; if 3 pumps are connected, the residence time is even higher and likely triples.

According to the invention, a kinetic pump such as a magnetically levitating centrifugal pump is used. However, any type of kinetic pump can be used. It is particularly advantageous if the pump head of the pump is a single-use pump head, i.e., one can simply replace the pump head of the pump. For example, the pump head can be used for only one application (e.g., several runs of the same cultivation process) and then the pump head may be replaced. The advantage of this is that the measurements are then extraordinarily reproducible.

The shear stress frequency is the frequency with which the cells are exposed to shear stress. According to the invention, this is equated with the number of passages through the external loop in the shear stress sensitivity devices, respectively, according to Fig. 1A or 1B. In Fig. 1A, the loop outside the bioreactor 110 is represented by tubes 125a + 125b + 125c + 125d1 + 125d2. In Fig. 1B, the loop is represented by tubes 125a + 125b + 125c + 125d1 + 125d2 + 125d3. The continuous recirculation of the cell culture comprising liquid culture medium with the cells in the loop is done from the bioreactor into the tubes of the external loop and into the bioreactor, from the bioreactor again through the tubes of the external loop and again into the bioreactor, etc. The shear stress frequency can be increased, for example, by providing additional loops through which the cell culture comprising liquid culture medium and the cells is passed. Another possibility to adjust the frequency is to adjust the filling volume of the bioreactor (cf. equations 1-4 which will be explained under the chapter "Relationships between the shear stress parameters and the influence of the bypass flow").

In this context, it should be noted that shear stress frequency in realiter rather represents a distribution. For simplicity, idealized conditions for shear stress frequency are assumed here, but this is acceptable for the method according to the invention.

According to the invention, not only one stress parameter can be varied and its influence tested in the shear stress sensitivity device, but also several stress parameters can be varied simultaneously and their influence investigated. This will be explained in detail later.

Relationships between the shear stress parameters and the influence of the bypass flow

In the means to control the hydrodynamic resistance, besides increasing the length or decreasing the diameter of the tubing to increase the pressure drop, the usage of valves (e.g. pinch valves) or other approaches, such as winding of the tubing, can be applied to increase the pressure drop as long as it is ensured that the maximal hydrodynamic shear stress is caused by the pump. In addition, it must be ensured according to an embodiment of the invention that the maximum shear stress occurring in the closed loop system is higher than the maximum shear stress occurring in the bioreactor. For a better understanding of the relationships between the parameters bypass flow ($\dot{V}_{bypass}$) [L/min], shear stress frequency (freq.) [1/min] and duration of shear stress exposure (residence time/length of exposure) (t_{res}) [min] the following equations are given:

Equation 1 $freq. = \frac{\dot{V}_{bypass}}{V_{reactor}} [1/min]$

where $V_{reactor}$ is the filling volume [L] of the bioreactor and

Equation 2 $t_{res} = \frac{V_{pump}}{\dot{V}_{bypass}} [min]$

where V_{pump} is the wet pump volume [L]. Combining Equation 1 and Equation 2 explains the relationship between freq. and t_{res} with

Equation 3 $freq. = \frac{V_{pump}}{t_{res} \times V_{reactor}} [1/min]$

and

Equation 4 $t_{res} = \frac{V_{pump}}{freq. \times V_{reactor}}$ [min].

Therefore, the invention is suitable for several process control strategies including perfusion. It is worth mentioning that the variation of the other shear stress parameters, besides the maximum shear stress can also lead to meaningful results. Not only the maximum hydrodynamic stress but also the other shear stress parameters contribute to the performance of a cultivation process.

Methods for determining the maximum hydrodynamic shear stress

It is not possible to measure the hydrodynamic shear stress simply by using a measuring device which then measures the occurring shear stress level. Therefore, for the determination of shear stress sensitivity of living cells methods are used which are per se known from the prior art but have to be developed further to solve this specific problem. These methods always determine the maximum shear stress, so that this is also one element of the invention.

In step (4) the characterizing of the shear stress sensitivity device is performed by using a calibration method or by using computer calculations and simulations.

A calibration method in step (4) is, for example, a known calibration method selected from a method wherein values of a shear stress-dependent quantity are determined and correlated with the known occurring maximum shear stress values. A shear stress-dependent (physical) quantity is a physical value or physical variable or physical parameter that changes its value or magnitude depending on the applied shear stress, in the present case depending on the applied maximum shear stress.

In the present invention, calibration is a procedure in which the values of a shear stress-dependent (physical) quantity are assigned to maximum shear stress values. A direct measurement of the maximum shear stress values is as said not possible. However, for the implementation of the method according to the invention the values of a shear stress-dependent (physical) quantity are determined. The values of a shear stress-dependent (physical) quantity can in principle either be measured and determined in an experimental procedure by reproducing a procedure from the state of the art, or the values of a shear stress-dependent (physical) quantity can also be taken directly from the state of the art, where these are specified. The maximum shear stress values could be determined by using computer calculations or the maximum shear stress values can also be taken directly from the state of the art, where these are indicated.

Such a shear stress-dependent quantity is, for example, the aggregate size of shear sensitive aggregates. For example, these are shear sensitive aggregates of poly(methyl methacrylate) (PMMA) nanoparticles (e.g. Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾). When the shear sensitive aggregates of PMMA nanoparticle aggregates are subjected to defined conditions of shear stress, the PMMA nanoparticle aggregates change their size depending on the shear stress, so that a certain aggregate size means a certain shear stress value. The aggregate sizes and associated shear stress values are known from the prior art. This method is referred to herein as the PMMA method.

Other known shear stress sensitive aggregates have become known as the so-called Blauton polymer floc system (see e.g. DE 202010018640 U1⁽¹⁵⁾ and Stintzing, A. et al.⁽¹⁶⁾). The Blauton polymer floc system consists of a cationic polymer (Praestol BC 650) and a clay mineral (Blauton). By adding the cationic polymer to the clay mineral, a flocculation reaction is started, which produces flocs of defined size. The aggregate size change rates determined are a measure of the shear stress prevailing in a system.

The characterization of the shear sensitivity device is a procedure in which the values of a shear stress-dependent (physical) quantity obtained by/in the shear stress device are assigned to maximum shear stress values. The values of a shear stress-dependent quantity are measured in the shear stress sensitivity device and converted into the maximum shear stress values using the selected calibration procedure. According to the calibration procedure, the maximum shear stress values are known when the shear stress-dependent quantity has a certain value. When certain (defined) conditions are set in the shear stress sensitivity device, the value obtained for the shear stress-dependent quantity can be used to deduce the maximum shear stress value that reflects the conditions.

A general method to determine maximum shear stress values in a device to be examined may be performed as follows:

Calibration method:
If, for example, the aggregate size of shear sensitive aggregates is selected as shear stress-dependent quantity a known calibration method using a known calibration device is selected which uses aggregate sizes and correlates the aggregate sizes with known maximum shear stress values. For example, a known calibration device described in prior art is reconstructed and the known calibration method using such calibration device is reworked as described in prior art. That is, the selected known calibration method is reworked and the aggregate sizes of the aggregates are measured. Based on the measured aggregate size values a calibration curve is generated in which the measured aggregate size values are plotted against the known maximum shear stress values taken from prior art.

Characterization method:
In the characterization method it is convenient to use the same shear stress-dependent quantity as used in the calibration method. Therefore, particularly the shear sensitive aggregates as used in the calibration method are also used in the characterization method. Thus, the same known calibration method as already used is reworked, in particular with the same aggregates used in the calibration method, but using a device used according to the invention instead of the calibration device. The device used according to the invention is the shear sensitivity device.

In the calibration and characterization methods, for example, the characteristic aggregate size is selected to be the radius of gyration of aggregates and the aggregates are selected from poly(methyl methacrylate) nanoparticle aggregates or Blauton polymer floc system.

According to step (4) of the present invention the shear stress sensitivity device is used, which is characterized for the maximum shear stress occurring in the shear stress sensitivity device.

Computer calculations and simulations:

Another method for determining the maximum hydrodynamic shear stress is the use of computer calculations and computer simulations. In the present invention, this relates in particular to the characterization of the maximum hydrodynamic shear stress occurring in a pump. Numerical flow simulation, like computational fluid dynamic (CFD), is a widely used tool to characterize process engineering equipment⁽¹⁷⁾. If it is used correctly, it can provide useful information cheaply and quickly. These simulations involve solving mathematical equations that describe the conservation of mass, momentum, and energy in a fluid. The primary goal of numerical flow simulations is to obtain detailed information about the fluid's velocity, pressure, temperature, and other relevant properties throughout the system. Furthermore, the use of numerical methods to characterize the hydrodynamic shear stress in pumps can be widely found in literature⁽¹⁸⁾⁽¹⁹⁾⁽²⁰⁾. To characterize the hydrodynamic shear stress in a pump the following steps should be performed, by means of commercially available software like, but not limited to, Ansys Fluent, STAR-CCM+, M-Star CFD, COMSOL Multiphysics, OpenFOAM:

1. create a detailed three-dimensional geometry including the moving and static parts
2. selecting a numerical method to solve the governing equations
 - a. finite volume
 - b. Lattice Boltzmann
 - c. etc....
3. selecting a turbulence model
 - a. Reynolds-averaged Navier–Stokes (RANS)
 - b. Large eddy simulation (LES)
 - c. Direct numerical simulation (DNS)
4. performing grid / mesh study to ensure that the numerical solution is independent of the mesh size and that discretization errors are minimized
5. running the shear stress characterization simulations for different operation parameters, like:
 - a. flow rate
 - b. rotation frequency of the pump head
 - c. pressure drop
 - d. fluid properties (viscosity).
6. Analyze the simulation results to characterize the hydrodynamic shear depending on the operation parameters. This can include visualizing the flow field, calculating shear stress and strain rate, and identifying regions of maximum shear stress.

The hydrodynamic shear stress is determined by calculation using mathematical formulas with numerical methods. The simplest type of shear stress occurs in a laminar pipe flow, which can be described by the Hagen–Poiseuille equation. The radial flow profile of a laminar pipe flow is visualized in Figure 7. Figure 7 shows the visualization of radial flow profile in a cylindrical laminar pipe flow⁽²¹⁾. The legend for Figure 7 is given separately at the end of the description.

In a laminar pipe flow the shear stress τ can be directly derived from the flow profile. Under the assumption of a steady, fully developed, incompressible, Newtonian and radial symmetric (2D) flow, the shear stress

$$\tau = \eta \frac{du}{dr}$$

depends only on the viscosity and the velocity gradient in radial direction.

T shear stress [Pa]
H viscosity [Pa s]
du/dr velocity gradient in radial direction [1/s]

- 5 For a three-dimensional transient flow, the shear stress can be calculated using the strain tensors S . The three-dimensional transient flow field can be determined either experimentally using thomo-PIV, 4D PTV or numerically.

$$\tau = \sqrt{2 S_{i,j} S_{i,j}} \eta^{(20)}$$

T shear stress [Pa]
10 $S_{i,j}$ strain tensors [1/s]
H viscosity [Pa s]

An exemplary calibration method

- 15 In Figs. 8A and 8B an exemplary calibrating method of the prior art as well as an characterization method of a shear stress sensitivity device carried out on the basis of this calibration method (step 4), according to an embodiment of the present invention, are explained in detail below.

20 The calibration method explained here is the so-called PMMA method, which is based on the publications by Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾. This prior art calibration procedure is shown schematically in Fig. 8A:

25 As shown in Fig. 8A under step a), poly(methyl methacrylate) nanoparticle aggregates are provided in an (average) aggregate size of 60 nm $\pm$ 20%, especially 60 nm $\pm$ 10%, in a suspension in water, for example a 10 wt.% water suspension. The nanoparticles are either commercially available or can be prepared, for example, as explained in Šrom et al.⁽¹²⁾, p. 2, under "2.1. *Synthesis of primary nanoparticles*". For example, the aggregate size can be measured using static light scattering (SLS), dynamic light scattering (DLS), a focused beam reflectance measurement, or other measurement methods known in the art.

30 The aggregate size of the PMMA agglomerates represents the (average) radius of gyration (R_g) as already explained. The (average) radius of gyration (also referred to as scattering mass radius) is a known quantity used for characterizing the spatial extent or size of irregularly shaped aggregates. Therefore, in the context of the present invention, the "aggregate size" of PMMA nanoparticle aggregates is always understood to mean the (average) radius of gyration (R_g).

35 As can be seen from Fig. 8A, step a), at least 5 mL of the monodisperse nanoparticle suspension was used. A larger amount could also be used. The indication is only to be understood as an example. The suspension had a nanoparticle weight fraction of 10% (w/w).

40 The nanoparticles were then aggregated according to step b) of Fig. 8A as described in Šrom et al.⁽¹²⁾ on p. 2, under "2.2. *Preparation of aggregates and their breakage*" using a NaCl solution by mixing the poly(methyl methacrylate) nanoparticles and a NaCl solution resulting in a final concentration in the suspension equal to 300 mM NaCl. During the aggregation procedure, the system is stirred. A 1% (w/w) suspension of the poly(methyl methacrylate) nanoparticle aggregates is obtained. If 5 mL of the

nanoparticles are used, about 50 mL of the suspension will then be present after addition of the NaCl solution.

The subsequent dilution of the suspension by factor 200 with water in step c) of Fig. 8A leads to the fact that no further re-aggregation of nanoparticles occurs under such conditions and breakage becomes the only mechanism controlling the aggregates size. From the initial 5 mL diluted 200 times, 10 L of diluted suspension is obtained. The steady-state aggregates are then transferred into the device to be examined. In the prior art the calibration was performed in a device which continuously pumps the aggregates through the contracting nozzle connected with two syringe pumps⁽¹⁴⁾ as schematically illustrated in step d) of Fig. 8A. The given nozzle configuration was characterized in terms of shear stress via calculation by computational fluid dynamics (CFD). This means that for each nozzle or the given conditions used, the maximum shear stress $\tau_{\max}$ is known as a numerical value in [Pa] by computer calculation. The explicit prior art apparatus, the detailed measurement procedure and the theoretical calculations can be taken from the state of the art as already cited.

In step e) of Fig. 8A, the break-up of the PMMA aggregates as a function of the maximum hydrodynamic shear stress in the device of step d) is shown schematically.

In step f), the aggregate sizes of the shear sensitive poly(methyl methacrylate) nanoparticle aggregates obtained in step e) are measured, for example, by static light scattering (SLS). Also dynamic light scattering (DLS), focused beam reflectance measurement (FBRM) or another method can be used for the measurement. A Mastersizer 2000 or 3000 (Malvern Instruments, UK), for example, can be used as a measuring instrument for SLS/DLS, which should result in the same values for the measurement. For further details, reference is made to the aggregate size measurement according to Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾, in particular reference is made to Šrom et al.⁽¹²⁾, p. 2 ff., under "2.2. Preparation of aggregates and their breakage".

In Fig. 8A, "-> Fig. 9B" means that the values from the measurement performed in step f) are plotted in a graph (step g)) shown in Fig. 9B.

Therefore, in step g), a calibration curve is generated wherein the maximum shear stress values in [Pa] obtained by CFD (computational fluid dynamics) calculation are plotted against the measured aggregate sizes of the PMMA agglomerates in the form of the radius of gyration (R_g) in [μm]. The calibration curve of step g) is shown in Fig. 9B. According to an embodiment the prior art procedure was reworked, i.e. the device of the prior art was reconstructed and the values for the PMMA agglomerates were measured in this reconstructed device and expressed as radius of gyration (R_g) in [μm]. Then, a calibration curve was generated using the maximum shear stress values in [Pa] given for the aggregate sizes in the prior art. The values for the radius of gyration (R_g) in [μm] as measured in the reconstructed device and the calculated values for maximum shear stress values $\tau_{\max}$ (tau) in [Pa] taken from the prior art are listed in Table 1 below:

Table 1:

(Rg) in [μm]	maximum shear stress values T _{max} in [Pa]
0.4	157
0.6	61
1.2	28

The whole procedure and the device used are described in detail by Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾, the disclosure content of which is incorporated herein by reference in its entirety.

An exemplary characterization method

The purpose of characterizing the shear stress sensitivity device, the set-up of which has already been selected in step (2) of the method according to the invention, is to determine the maximum shear stress present in the shear stress sensitivity device. As determined in experiments, the source of maximum shear stress in the shear stress sensitivity device of the present invention is the pump as already explained. Therefore, the maximum shear stress represented by the rotational speed of the pump is used for characterization of the shear stress sensitivity device.

The characterization of the shear stress sensitivity device means the quantification of the occurring maximum shear stress. A known calibration method from the prior art is used for this purpose.

As already explained, if a shear sensitivity-dependent quantity is selected and used in the calibration method, in particular the same shear sensitivity-dependent quantity is also used in the characterization of the shear stress sensitivity device. For example, if the aggregate size of certain aggregates is used as shear sensitivity-dependent quantity in the calibration method, the aggregates size of the same aggregates is also used in the characterization. In particular, even the same batch of purchased or manufactured aggregates is used for both the calibration and the characterization method. The aggregates are for example poly(methyl methacrylate) (PMMA) nanoparticle aggregates. In particular, the aggregates size is the radius of gyration.

The characterization of the shear stress sensitivity device may be performed according to an embodiment as follows:

The calibration method known from the prior art is reworked with the same aggregates used in the calibration method, in particular poly(methyl methacrylate) (PMMA) nanoparticle aggregates, but using the shear stress sensitivity device instead of the prior art device. In the shear stress sensitivity device, continuous recirculating runs of the aggregates in a liquid medium such as water are performed by the pump through the shear stress sensitivity device until the aggregates no longer change their size. The liquid medium is the same as used in the calibration method. The rotational speed of the pump is varied in each subsequent run, and in particular the rotational speed of the pump is increased from run to run. The aggregates size, in particular the radius of gyration of particle aggregates, is measured after each run and a curve is generated, plotting the measured aggregates size against the rotational speed of the pump. Then the measured aggregate size values of the shear stress sensitivity device are converted to maximum shear stress values using the calibration curve based on prior art, so that the shear stress sensitivity device can

now be assigned a shear stress value to each rotational speed of the pump. This represents the characterization of the shear stress sensitivity device.

5 This characterization is explained in more detail below using an example, namely the PMMA method known from the prior art. Another known calibration method could also be used. The characterization is illustrated schematically in Fig. 8B, based on the PMMA method.

10 Fig. 8B shows that steps a) to f) are carried out identically as in Fig. 8A, but in step d1) an embodiment of the shear stress sensitivity device of the present invention (abbreviated as SSD in Fig. 8B) is used instead of the prior art nozzle device in step d) of Fig. 8A. In the prior art device according to Fig. 8A, step d), the use of the nozzle leads to the maximum shear stress, whereas according to the invention in the shear stress sensitivity device according to Fig. 8B, step d1), this is generated by the maximum shear stress represented by the rotational speed of a pump. It should be mentioned in passing that according to the invention, no nozzle is used in the shear stress sensitivity device of the present invention.

15 For the characterization of the shear stress sensitivity device the poly(methyl methacrylate) nanoparticle aggregates are continuously circulated by the pump in the loop system (and the bioreactor), whereby the pump generates the maximum shear stress in the shear stress sensitivity device. Different rotational speeds of the pump mean different maximum shear stresses. The poly(methyl methacrylate) nanoparticle
20 aggregates are exposed to the maximum shear stress in the loop and - as already explained for the PMMA method - change their aggregate size depending on this (step e)).

In the shown embodiment the poly(methyl methacrylate) nanoparticle aggregates are placed in aqueous suspension in the shear stress sensitivity device, the rotational speed of the pump is set to the desired
25 value, and the poly(methyl methacrylate) nanoparticle aggregates are continuously recirculated by the pump through the shear stress sensitivity device until the aggregates do not further change their aggregate size. This is also referred to as "steady state" in the prior art.

30 For example, a certain rotational speed of the pump is set, the nanoparticle aggregates are circulated in the loop system, and this is done until the size of the agglomerates no longer changes. Then a sample is taken and the size of the agglomerates is measured in step f). Then, for example, a higher value than before is set for the rotational speed of the pump and the aggregates still present in the shear stress sensitivity device are circulated again until the size of the aggregates no longer changes and again a sample is taken and the size of the aggregates is determined. This procedure is repeated several times until a
35 curve can be generated from the measured values.

According to an embodiment, the characterization is started with a low value for the rotational speed of the pump, and this value is increased from time to time, whereby the aggregate size decreases from run to run. This simplifies the procedure, since only the rotational speed of the pump is turned up and then the
40 aggregate size is determined after the steady state is reached.

In step f) of Fig. 8B, the aggregate sizes of the shear sensitive poly(methyl methacrylate) nanoparticle aggregates obtained in step e) are measured in terms of the radius of gyration (R_g) - as already explained

for Fig. 8A - for example by static light scattering (SLS), dynamic light scattering (DLS), a focused beam reflectance measurement or also by another method.

5 For each pump rotational speed set in the shear sensitive device, a certain aggregate size of the shear sensitive poly(methyl methacrylate) nanoparticle aggregates obtained in step e) is obtained expressed as radius of gyration (R_g).

In Fig. 8B, "-> Fig. 9A" means that the values from the measurement performed in step f) are plotted in a graph (step g1)) shown in Fig. 9A.

10

In Fig. 8B, "-> Fig. 9A + Fig. 9B -> Fig. 9C" means that the values from Fig. 9A (obtained in step g1)) are transformed into the values of Fig. 9C (step g2)) using the values or curve function from Fig. 9B (obtained in step g)). That is, the sign "+" means that the combination of Fig. 9A and Fig. 9B results in Fig. 9C.

15 Therefore, in step g1) of Fig. 8B, the measured aggregate sizes of the PMMA agglomerates in the form of the radius of gyration (R_g) in [μm] are plotted against the set values for the rotational speed of the pump in [rpm]. The graph of step g1) is shown in Fig. 9A.

20 In step g), the calibration curve has already been obtained using the prior art calibration method (see Fig. 9B).

25 In step g2) the graph of Fig. 9C is obtained from the combination of step g1) (Fig. 9A) and step g) (Fig. 9B). That is, the aggregate sizes of the PMMA nanoparticles measured at the set rotational speeds of the pump in the shear stress sensitivity device (step g1): Fig. 9A) are converted to maximum shear stress values (step g2): Fig. 9C) by using a prior art calibration curve correlating maximum shear stress value and aggregate size (step g): Fig. 9B). The characterization of the shear stress sensitivity device therefore serves, via the detour of the aggregate size of shear sensitive aggregates, to determine the shear stress value for the adjusted rotational speed of the pump.

30 According to an embodiment of the invention, if the aggregate size as shear stress-dependent quantity is used for the calibration and characterization methods in step (4) then it is convenient if the same batch of aggregates is used for both the calibration and the characterization. It can be assumed that aggregates can always behave slightly differently from batch to batch. It therefore makes sense to use the same batch for both methods.

35

In order to better understand this calibration according to steps a) to g) in Figure 8A and the characterization according to steps a) to g2) in Figure 8B, the practical implementation is explained in detail below using an exemplary embodiment of the present invention:

40 In the exemplary embodiment, the maximum shear stress represented by the rotational speed of a pump is used as the maximum shear stress source to characterize the shear stress sensitivity device for the maximum shear stress values that occur. In this example a magnetically levitated centrifugal pump is used as pump, namely a PuraLev i30 SU pump, Levitronix, Switzerland. Another kinetic pump could also be used. The set-up of Fig. 1B is used as the shear stress sensitivity device in this embodiment, i.e. the

continuous mode such as perfusion mode, is taken as a basis. The device of Fig. 1A could also be used, so that the batch or fed-batch mode of cultivation would be investigated.

5 The maximum shear stress values which occur within the shear stress sensitivity device are determined as already described (see Fig. 8B). In this example the PMMA method is used as the known calibration method. The already described generated aggregates of PMMA nanoparticles (steps a) to c) of Fig. 8B) are transferred into the shear stress sensitivity device (SSD) of Fig. 1B (step d1) of Fig. 8B), to be characterized for the maximum shear stress that occur. A suspension of the PMMA aggregates is recirculated in the loop system and after a certain number of cycles when the size of the aggregates no
10 longer changes, a sample is taken. This procedure is then repeated several times, each time with an increased value of the rotational speed of the pump (steps d1) and e)). The resulting aggregate sizes of the PMMA aggregates, expressed as radius of gyration (R_g), are measured for example, via static light scattering, dynamic light scattering or another method (step f) of Fig. 8B). The measured values for the aggregate sizes (R_g in μm) obtained are plotted in Fig. 9A over the respective set rotational speed of the
15 pump in [rpm].

Figure 9A shows the results of the characterization of the shear stress sensitivity device: The measured aggregate sizes ((average) radius of gyration) R_g in μm in the shear stress sensitivity device which are plotted against the rotational speeds in [rpm] of the magnetically levitated centrifugal pump used in the
20 example experiment. The values on the x- and y-axis in Fig. 9A are plotted logarithmically. The two black dotted lines indicate the 95% prediction interval of the fit. The curve follows a power law function with an R^2 of 0.98, as shown in Fig. 9A. The radius of gyration of the aggregates decreases with increasing rotational speed of the pump in [rpm]. The fitted curve follows the function $y = a * x^b$, where $a = 3.42e+04$, $b = -1.33$, $R^2 = 0.99$ and adjusted $R^2 = 0.98$. Fig. 9A has already been shown in Fig. 8B in smaller format
25 as graph g1) for explanatory purposes.

Figure 9B shows the calibration curve generated with a known calibration method of the prior art, in the present experiment the PMMA method is used: The calibration curve is generated with the values given in Table 1, whereby the values were obtained by reproducing the process as described in the prior art using
30 the device replicated from the prior art. (see Villiger et al.⁽¹¹⁾, Šrom et al.⁽¹²⁾, Harshe & Lattuada⁽¹³⁾ and Soos et al.⁽¹⁴⁾). The values on the x- and y-axis in Fig. 9B are plotted logarithmically. The fitted curve in Fig. 9B follows the function $y = a * x^b$, where $a = 24.69$, $b = -2$, $R^2 = 0.98$ and adjusted $R^2 = 0.96$. Fig. 9B has already been referred to in Figs. 8A and 8B, step g).

35 The calibration curve generated according to prior art as shown in Figure 9B is used to convert the measured aggregate size R_g in μm for a given rotational speed of the pump shown in Figure 9A into a hydrodynamic shear stress value, as shown in Figure 9C. Figure 9C therefore gives the resulting shear stress value in [Pa] versus the rotational speed of the pump derived from the measured data in Fig. 9A. The values on the x- and y-axis in Fig. 9C are also plotted logarithmically. The two dotted lines in Figure
40 9C indicate the 95% prediction interval of the fitted model. The fitted curve in Fig. 9C follows the function $y = a * x^b$, where $a = 6.412e-07$, $b = 2.21$, $R^2 = 0.99$, and adjusted $R^2 = 0.99$. That is, a model was fitted that yields a power law correlation with an $R^2 = 0.99$. R^2 is the coefficient of determination, which describes how well the measured values fit the model function. The coefficient here is close to 1, so that the function describes the measured data very well. As can be seen from Figure 9C, the maximum hydrodynamic shear

stresses increase as the rotational speed of the pump increases and are found to be 2 Pa for 1000 rpm up to 147 Pa for 6000 rpm. The values are also given in Table 2 below.

Table 2: Maximum shear stresses for different rotational speeds of a magnetically levitating centrifugal pump (PuraLev i30 SU centrifugal pump) determined by the PMMA method

[rpm]	$T_{\max}$ [Pa]
1000	2
1500	7
2000	10
3000	39
4000	55
6000	147

The shear stress measurement method of the present invention therefore succeeds in converting process parameters of the equipment used for the shear stress sensitivity device into maximum shear stress values in [Pa].

The maximum shear stress is to be higher in the closed loop system than in the bioreactor

In order to obtain meaningful results in the inventive method it is ensured that the maximum shear stress occurring in the closed loop system is higher than the maximum shear stress occurring in the bioreactor (the shear stress sensitivity device without closed loop system). This is especially applicable in case a calibration and characterization of the shear sensitivity device is performed. This is appropriate because maximum shear stress is usually the largest contributor to shear stress intensity.

However, according to an embodiment, it may be convenient to ensure that the shear stress intensity occurring in the closed loop system is higher than the shear stress intensity occurring in the bioreactor (the shear stress sensitivity device without a closed loop system). This may be used in case no calibration and no characterization of the shear stress sensitivity device is performed. However, in a simplified manner, it could also be assumed in this case that it is ensured that the maximum shear stress occurring in the closed loop system is higher than the maximum shear stress occurring in the bioreactor (the shear stress sensitivity device without a closed loop system).

In any case, the maximum shear stress is used to characterize the shear stress sensitivity device and the bioreactor, respectively, particularly by using a calibration method or by using computer calculations and simulations. This can be done as already explained in detail.

Effect of hydrodynamic shear stress during cultivation

To determine the effect of maximum hydrodynamic shear stress during cultivation, the cultivation of cells is performed. For this purpose, as already explained, the type of cells to be used, the composition and type of liquid culture medium, the cultivation conditions, such as the type and amount of added liquid culture media and the like, and the cultivation mode in the form of batch, fed-batch or continuous such as perfusion are selected in each case according to step (2).

Then - as already explained - in step (3) one, two or three of the shear stress parameters is/are selected to be investigated. The shear stress parameters are selected from the group consisting of:

- maximum shear stress represented by a rotational speed of the pump;
- duration of shear stress exposure represented by a residence time of the cell culture within the pump; and/or
- shear stress frequency represented by a number of passages of the liquid culture medium and cells through the loop system.

The shear stress intensity results from the combination of these 3 shear stress parameters.

- 10 After selecting the shear stress parameter(s) to be evaluated, the shear stress sensitivity device used is characterized in (step 4) by determining the maximum shear stress values, for example, by using a known calibration method, the maximum shear stress is represented by the rotational speed of the pump used. This has already been described in detail.

- 15 In step (5) a process for cultivating cells is performed several times, namely the same cultivation process is performed in one, two, three or more cultivation runs in the shear stress sensitivity device, each over the same selected period of time, by varying or by only varying one, two or three shear stress parameters each time from run to run, and measuring one or more performance characteristics of the cells during each run of the cultivation process and generating a curve for each performance characteristic in each run over time.

- 20 The cultivation process in step (5) is for example carried out as follows:

For example, only one cultivation run can be performed or only 2 cultivation runs can be performed in which, for example, only 1 shear stress parameter is changed in each case. Then conclusions can be drawn about the change in the behaviour of the cells/stress parameter(s) only for said run(s).

25

In a further embodiment a 1st cultivation process in the form of a 1st cultivation run over a 1st period of time is performed. Then a 2nd cultivation process in the form of a 2nd cultivation run over a 2nd period of time is performed. Subsequently, a 3rd cultivation process in the form of a 3rd cultivation run over a 3rd period of time is performed. Optionally, further cultivation processes in the form of further cultivation runs over further periods of time are performed. Always the same cultivation process is performed in all runs, i.e. the 1st, 2nd, 3rd and optionally further cultivation runs, the same cells as starting product, the same liquid culture medium, the same cultivation conditions and the same cultivation mode previously selected in step (2) are used. All periods of time in all cultivation runs are selected to be of equal length.

- 35 In each cultivation run liquid culture medium and cells in the shear stress sensitivity device are continuously circulated through the loop system and the bioreactor and all parameters of the shear stress sensitivity device and all conditions of the cultivation process are kept the same during all cultivation runs. Continuous circulation is provided by the kinetic pump.

- 40 The term "circulation" means circulating through the shear stress sensitivity device, i.e. from the bioreactor into the external loop system and back to the bioreactor and again through the external loop system and so on. The term "recirculation" means the same as "circulation" but for the sake of clarity and better understanding the term "recirculation" is used in connection with the characterization of the shear stress

sensitivity device and the term "circulation" is used in connection with circulating of the liquid containing cell culture comprising a liquid culture medium and cells during a cultivation process or run.

5 In each cultivation run one, two or three shear stress parameters are varied each time from run to run. For example, only one of the 3 shear stress parameters can be varied from run to run, or 2 of the shear stress parameters can be varied from run to run, or 3 shear stress parameters can be varied from run to run.

10 If only one of the shear stress parameters selected in step (3) is to be varied, it is set to a predetermined value in the 1st cultivation run and kept constant and is set to a higher value for each subsequent cultivation run, which is set higher from cultivation run to cultivation run, and the set value for the shear stress parameter is held constant during each cultivation run. Alternatively, the shear stress parameter selected in step (3) is set to a predetermined value in the 1st cultivation run and held constant and is set to a lower value for each subsequent cultivation run, which is set lower from cultivation run to cultivation run, and the set value for the shear stress parameter is held constant during each cultivation run.

15 If 2 or 3 shear stress parameters are to be varied from cultivation run to cultivation run, these can be increased or decreased independently of each other from run to run. This depends on what is to be investigated.

20 In step (5) of the present invention, therefore, a cultivation process is performed multiple times with the selected cells in the shear stress sensitivity device while continuously cycling the entire cell culture comprising liquid medium and cells for a selected period of time, wherein, for example, only the selected stress parameter(s) is(are) varied in each cultivation run of the cultivation process and all other parameters - as far as possible - including the not to be varied stress parameter(s) and all other conditions in the shear stress sensitivity device are left the same.

25 Therefore, one, two or three of the described shear stress parameters is/are selected and evaluated for the runs of a cultivation procedure according to step (5). This one, two or three parameters is/are then examined for the cultivation process with its/their effects on the selected cells. The other shear stress parameter(s) not selected are kept constant during the cultivation process. All other culture conditions and parameters selected prior to the start of the cell cultivation process are also kept constant during the cultivation process. Only if all conditions and parameters are kept constant during the cultivation process, the change of the one, two or three shear stress parameters can be examined and lead to meaningful results. In this context, however, it should be noted that the bypass flow, which is not a shear stress parameter but another parameter, has a special status because it is related to the other shear stress parameters. Therefore, it may be that in order to vary the shear stress parameters, all other parameters except the bypass flow are kept constant, but the bypass flow is increased or decreased accordingly. This will be explained in detail later.

30 A "cultivation run" therefore means performing a cultivation process over a selected period of time, for example several days, with the selected cells, cell culture conditions, and parameters, while continuously cycling the liquid containing cell culture comprising liquid culture medium and cells in the shear stress sensitivity device. It is convenient, if all cultivation runs are performed over the same selected time period.

The term "continuously cycling" and "continuously circulating" implies a constantly ongoing movement from the bioreactor through the loop system back into the bioreactor and repeating these procedures. Both terms should be understood synonymously and are interchangeable.

5 The selected period of time in step (5) depends on many factors, such as the type of cells selected, the size of the batch, etc. The skilled person can easily determine this in each individual case. For example, the period of time may be several days. Exemplarily, the selected time span for the time course of one of the cultivation methods may be, in particular, at least 1 day or up to 30 days or also longer.

10 The same cultivation process from the 1st cultivation run is therefore repeated several times in further cultivation runs, whereby the liquid containing cell culture comprising liquid culture medium and cells in the shear stress sensitivity device is continuously circulated by the pump. In an embodiment only the selected shear stress parameter(s) is(are) changed in each case. This(these) can be increased or decreased. In another embodiment, the selected shear stress parameter(s) and additionally the bypass flow are varied.

15 It goes without saying that a new stock of starting cells, new liquid culture medium and the like are used in each new cultivation run. The cells and liquid culture medium used in the 1st cultivation run cannot be used further. This would falsify the analysis and statements from the procedure.

Therefore, the cultivation is performed in a 1st cultivation run keeping the conditions selected in the shear stress sensitivity device, whereby (a) 1st value(s) is(are) set for the selected stress parameter(s). For example, only one shear stress parameter is varied and the selected shear stress parameter is the maximum shear stress corresponding to the rotational speed [rpm] of the pump. Then, for example, in the 1st cultivation run of the cultivation process, a rotational speed of 2000 rpm is set and the cultivation is performed for 5 days, for example. In the 2nd cultivation run, for example, a rotational speed of 3000 rpm is set and the cultivation is carried out again for 5 days. The other conditions and parameters are identical to those selected for the 1st cultivation run. In the 3rd cultivation run, a rotation speed of 4000 rpm is then set, for example, with all other conditions and parameters for the cultivation process being identical to the cultivation process from the 1st cultivation run. The cultivation process is again performed for 5 days as in the 1st cultivation run. In the 4th cultivation run, for example, a rotational speed of 5000 rpm is then set and the cultivation process is again carried out for 5 days, and in the 5th cultivation run, for example, a rotational speed of 6000 rpm is then set and the cultivation process is again carried out for 5 days.

The number of repetitions or cultivation runs of the cultivation process can be determined by the skilled person in each individual case. It may be appropriate if at least 2 or at least 3 cultivation runs or at least 4 cultivation runs or at least 5 cultivation runs are performed.

Therefore, while performing the cultivation process in one or more runs according to step (5), samples are taken from the cell culture in each run and their performance characteristic values are determined. Several samples may be taken during each cultivation run of the cell cultivation, usually at certain time intervals, for example once a day over several days. For example, at least 5 samples or at least 6 samples or at least 7 samples or more are taken per cultivation run. In each cultivation run, the same performance characteristic is then determined, especially on a regularly basis, in particular in the same time interval(s), as in the 1st cultivation run. If several performance characteristics are to be determined, these are usually determined in all cultivation runs. The values of the performance characteristic(s) may be determined as online or offline

data. I.e. the data are measured inside the shear stress sensitivity device or samples are taken, which are examined and measured outside the shear stress sensitivity device. These characteristic parameters for the cell culture are, for example, growth and productivity of the cell culture, in particular relative living cell density, viability, glucose concentration, lactate concentration, LDH concentration, titer (product concentration), product quality and many more.

As an example, the relative living cell density of the cells is selected as the performance characteristic for the cell culture. For example, 10 days are selected as the period of time, with the relative living cell density in a sample being determined once every day: In the 1st cultivation run, the cultivation process is then carried out for 10 days in the shear stress sensitivity device, with, for example, the rotational speed of the pump selected as the shear stress parameter, all other parameters and conditions being chosen to be the same. For example, the rotational speed of the pump is set to 5000 rpm in the 1st cultivation run. During the 10 days, the relative living cell density is determined regularly, e.g. daily. In the 2nd cultivation run, the cultivation process is then carried out again for 10 days in the shear stress sensitivity device, whereby the rotational speed of the pump is set to 6000 rpm, for example. During the 10 days, the relative living cell density is again determined regularly, e.g. daily. In the 3rd cultivation run, the cultivation process is then carried out again for 10 days in the shear stress sensitivity device, whereby the rotational speed of the pump is set to 7000 rpm, for example. During the 10 days, the relative living cell density is again determined regularly, e.g. daily.

According to step (5) curves for one or more performance characteristics of the cells measured during the individual runs of the cultivation process are generated. That is a curve for the values of the performance characteristic measured are plotted over the selected period of time for each cultivation run. Each curve stands for the set value(s) of the shear stress parameter(s) selected with which the individual cultivation run was performed, which can be converted into a shear stress intensity as already explained. The data obtained show the performance of the selected cells under the prevailing stress conditions. Therefore, a set of curves is obtained for all cultivation runs.

For example, the rotational speed of the pump is selected as the shear stress parameter, which should be 3000 rpm in the 1st cultivation run, 4000 rpm in the 2nd cultivation run and 6000 rpm in the 3rd cultivation run, and the product titer of the cell culture is selected as the performance characteristic, and 3 cultivation runs are carried out, each over 5 days, with a sample of the cells being taken and examined each day in each run. Thus, for each cultivation run of the cultivation process, 5 values are obtained for the product titer in each of the 3 cultivation runs and therefore 3 curves can be generated. In each curve, the value for the product titer is plotted against the days, i.e., the time course of the performance characteristic is recorded. The 1st curve is from the 1st cultivation run where 3000 rpm was set for the rotational speed of the pump. Using the PMMA method, for the rotational speed of the pump a shear stress value of 39 Pa can be measured for the shear stress sensitivity device (see Table 2). For the 2nd cultivation run, the rotational speed of the pump of 4000 rpm results in a measured shear stress value of 55 Pa and for the 3rd cultivation run with a rotational speed of the pump of 6000 rpm a shear stress value of 147 Pa. In other words, the cells are subjected to a maximum shear stress of 39 Pa in the 1st cultivation run, a maximum shear stress of 55 Pa in the 2nd cultivation run and a maximum shear stress of 147 Pa in the 3rd cultivation run. The obtained 3 curves are considered as a set of curves over all cultivation runs. The set of curves therefore

represents the behavior of the cells based on the performance characteristic (here: the product titer) in the shear stress sensitivity device with increasing shear stress intensity.

5 With the obtained model function indicated in Fig. 9C, the above values can also be calculated. Therefore, the model function allows to check the plausibility of the measured data by calculation: The calculation with the model function gives the following results: 3000 rpm -> 32 Pa, 4000 rpm -> 60 Pa and 6000 rpm -> 146 Pa. As already explained, the coefficient of determination $R^2 = 0.99$, which describes how well the measured values fit the model function. Since the coefficient here is close to 1, the function describes the measured data very well. Thus, the calculated data are relatively close to the measured values, so that the measurement is confirmed by the model function which supports the reliability of the results.

10 It should be noted that in the above example, another prior art calibration method can be used instead of the PMMA method used. Alternatively, computer calculations or simulations could be used to determine the shear stress values.

15 In order to assess the influence of the maximum shear stress, a control must be present in which there is low or virtually no shear stress or only negligible shear stress. Therefore, a control cultivation process is performed in step (6) in the shear stress sensitivity device for each performance characteristic using the same cultivation process as in step (5), i.e. the same cells, the same liquid culture medium, the same cultivation mode, the same shear stress sensitivity device. In the control cultivation process a lower maximum shear stress is used than in step (5) and a control curve for each performance characteristic over time is generated. The control cultivation process is e.g. performed in the same way as the 1st cultivation run of the cultivation process of step (5). The same cells, same liquid culture medium, same cultivation mode, same cultivation process conditions, and same shear stress sensitivity device are used for the same period of time as in step (5).

25 A distinction must be made in the control cultivation process to be selected between batch and fed-batch on the one hand and continuous process (e.g. perfusion) on the other. For example, for a batch or fed-batch process, a control cultivation process is carried out such that the shear stress sensitivity device is without a loop system and without a pump. That is the shear stress provided by the loop system and the pump is not present. Therefore, such a device used for the control cultivation process is not actually a shear sensitive device, but just a simple bioreactor. All parameters in the device and all conditions of the cultivation process are kept the same throughout the control cultivation process, and the same performance characteristic(s) for the cell culture is (are) measured over the selected period of time as in step (5), and the measured values of the performance characteristic(s) for the cell culture are plotted over the selected period of time to obtain a control curve for each performance characteristic examined.

30 In a continuous cultivation process (e.g. perfusion), the shear stress parameter that causes the maximum shear stress can be set to a lower value as used in step (5), e.g. to the smallest possible value. In the context of the present invention, the shear stress parameter that contributes most to the shear stress intensity is the maximum shear stress represented by the rotational speed of the pump. In other words, the rotational speed of the pump is set lower than in step (5), for example, as low as possible to fulfil this requirement. Optimally, the control cultivation process is performed with the minimum shear stress level with which perfusion runs by default.

A comparison of the curve array of step (5) with the control curve of step (6) of a performance characteristic leads to the selection of the curve from the curve array of step (5) which is closest to the control curve (step (7)). It is clear to the skilled person that it is a matter of the operating conditions under which the curves were determined. In step (7), therefore, the curve is selected that is closest to the control curve and thus has the operating conditions that most closely match the operating conditions of the control curve. This shear stress value at which the selected curve was measured stands for the shear stress sensitivity of the cells and represents the shear stress limit for the cultured cells. If curves and control curves have been generated for several performance characteristics of the cell culture, the shear stress limit is usually the same or nearly the same value for all performance characteristics selected. If the value is not equal, the average value can be taken.

If, for example, a shear stress limit of 60 Pa is determined, i.e. shear stress values above 60 Pa result in a significantly deteriorated performance in a cell cultivation process, this can be correlated with the shear stress parameter, e.g. a specific rotational speed value of a pump in [rpm] that was used. For example, this could be 4000 rpm for the pump used. That is, this reference value could be used for a cultivation process performed in the same way with a larger scale, so that one would already know the conditions for a suitable cultivation process.

Therefore, the conversion of process parameters of the equipment used for the shear stress sensitivity device into shear stress values in [Pa] allows the shear stress sensitivity, or shear stress limit of the cells to be determined or to be estimated. This shear stress limit for the cells allows a very precise assessment of the behavior of the cells under the cultivation conditions. This allows both a scale-up and a scale-down of a cultivation process. The shear stress can be adjusted in a targeted manner, so that it is possible to calculate back from a larger to a smaller system (scale-down) or from a smaller to a larger system (scale-up). For example, a large scale system can be a larger pump, a larger bioreactor or other equipment.

Alternatively, in step (7), instead of or in addition to the determination of the shear stress sensitivity or shear stress limit for the cells, an analysis of the shear stress behavior of the cells may be performed with the obtained curves for one or more performance characteristics, in particular in comparison to the control curve. This is evident from the fact that the curves each represent defined operating conditions that have been set. The shear stress behavior of the cells directly affects the performance characteristics, so that the curves obtained directly reflect how the cells cope with a given shear stress intensity and also respond in each case to the level of shear stress intensity exerted.

In particular, this allows reliable predictions to be made as to how a cultivation process can best be carried out. The investigation of the cells sensitivity towards shear stress intensity at small scale may allow to accurately predict potential variations in process performance and product quality during scale-up or scale-down and/or thereby enables to pick clones during cell line development based on small or large scale shear stresses. This makes it possible to select at a very early stage which clones are suitable for production purposes and which mechanical stress resistance they exhibit. This is a selection procedure, for example, how to distinguish good-producing clones from poor-producing clones.

Operating modes of the shear stress sensitivity device

As has already been described, it is also possible to vary one, two or three shear stress parameters simultaneously in the shear stress sensitivity device, while keeping all other parameters constant as far as possible. According to another embodiment it is also possible to vary one, two or three shear stress parameters simultaneously in the shear stress sensitivity device, while keeping all other cultivation parameters constant. The shear stress sensitivity device and the numerous shear stress parameters that can be modified provide an extraordinary amount of variance and flexibility for various process control strategies. These are explained below by way of example.

10 The shear stress sensitive device to be used for cultivation of cells can be operated in different ways, i.e. in different modes. Each mode keeps different shear stress parameters constant showing a broad field of application of the shear stress sensitivity device. By varying the individual shear stress parameters, the shear stress intensity is also varied.

15 For example, the variation of one or more shear stress parameters may be performed as follows:

- Keeping the maximum shear stress constant in the form of the rotational speed by keeping the rotational speed of the pump at the same value;
- Increasing or decreasing the maximum shear stress in the form of the rotational speed by setting the rotational speed of the pump higher or lower;
- 20 - Keeping the duration of shear stress exposure constant if the rotational speed of the pump is increased or decreased by using the means to control hydrodynamic resistance, e.g. by varying the length and/or diameter of the variable tube, in the loop system of the shear stress sensitivity device;
- Increasing the duration of shear stress exposure if the rotational speed of the pump is kept constant by adding additional pumps with the same rotational speed in [rpm] in series in the loop system;
- 25 - Increasing or decreasing the duration of shear stress exposure if the rotational speed of the pump is kept constant by using the means to control hydrodynamic resistance, e.g. by varying the length and/or diameter of the variable tube, in the loop system of the shear stress sensitivity device;
- Keeping the shear stress frequency constant if the rotational speed of the pump is increased or decreased by using the means to control hydrodynamic resistance, e.g. by varying the tube length and/or the diameter of the variable tube in the loop system of the shear stress sensitivity device;
- 30 - Increasing the shear stress frequency if the rotational speed of the pump is kept constant by adding one or more external loops to the bioreactor or by adjusting the working volume of the bioreactor; or
- Increasing the shear stress frequency if the rotational speed of the pump is kept constant by providing one or more additional pumps, which are connected in parallel and all pumps operate with the same value of the rotational speed.
- 35

An important parameter, which according to the invention is not a shear stress parameter, is the bypass flow. This is related to the shear stress parameters so that it is useful to know how to influence the bypass flow. For example, the bypass flow increases when the maximum shear stress represented by the rotational speed of the pump increases (provided that the hydraulic resistance is not changed). To change the bypass flow or to keep it constant, the means to control the hydrodynamic resistance can be used. In the following some correlations of bypass flow with other shear stress parameters and also with the means to control the hydrodynamic resistance are given. For the means to control the hydrodynamic resistance, a variable tube

with variable length and/or variable inner diameter is used as an exemplary embodiment. Other means to control the hydrodynamic resistance are possible and known by the skilled person.

Possible measures or parameters to change the bypass flow are, for example, as follows:

- 5 - Keeping the bypass flow constant by keeping the rotational speed of the pump constant;
- Keeping the bypass flow constant if the rotational speed of the pump is increased by increasing the length and/or decreasing the diameter of the variable tube in the loop system of the shear stress sensitivity device;
- Keeping the bypass flow constant if the rotational speed of the pump is decreased by decreasing the length and/or increasing the diameter of the variable tube in the loop system of the shear stress sensitivity device;
- 10 - Increasing or decreasing the bypass flow by increasing or decreasing the rotational speed of the pump;
- Increasing the bypass flow if the rotational speed of the pump is constant by decreasing the length and/or increasing the diameter of the variable tube in the loop system of the shear stress sensitivity device;
- 15 - Decreasing the bypass flow if the rotational speed of the pump is constant by increasing the length and/or decreasing the diameter of the variable tube in the loop system of the shear stress sensitivity device.

20 The shear stress sensitivity device can be operated in various ways. An overview of different exemplary operating modes of the shear stress sensitivity device is as follows:

For example, one of the following cases, namely case 1 to case 4 may be selected, in each of which one or more shear stress parameters are varied, so that the shear stress intensity is also varied:

- 25 case 1 increasing the maximum shear stress from cultivation run to cultivation run while keeping constant the duration of shear stress exposure and shear stress frequency in all cultivation runs
- or
- case 2 increasing the duration of shear stress exposure from cultivation run to cultivation run while keeping constant the maximum shear stress and shear stress frequency in all cultivation runs;
- 30 or
- case 3 increasing the shear stress frequency from cultivation run to cultivation run while keeping constant the maximum shear stress and the duration of shear stress exposure in all cultivation runs;
- 35 or
- case 4 increasing the duration of shear stress exposure from cultivation run to cultivation run while keeping the maximum shear stress constant and decreasing the shear stress frequency in all cultivation.

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Case 1 to 3 are of special interest because only one selected parameter is changed from cultivation run to cultivation run i.e. either increased or decreased from run to run, while the other shear stress parameters are kept constant over all runs.

Case 4 is an example case where 2 parameters are changed simultaneously (one parameter is increased, the other parameter is decreased). Of course there are many more possibilities to vary the shear stress parameters, e.g. all 3 shear stress parameters can be changed at the same time. The variation of the shear stress parameter(s) during the cultivation runs depends in detail on what is to be investigated.

5

In detail:

Case 1:

10 In case 1 the maximum shear stress represented by the rotational speed of the pump in the shear stress sensitivity device is selected and investigated as shear stress parameter. The maximum shear stress in case 1 is increased from cultivation run to cultivation run, i.e. each cultivation run is performed with a higher value for the rotational speed of the pump than the previous cultivation run.

15 The maximum shear stress represented by the rotational speed of the pump in the shear stress sensitivity device is increased by setting the rotational speed of the pump higher.

At the same time, the duration of shear stress exposure and shear stress frequency are kept constant in each cultivation run.

20 One possibility to achieve this is to keep the bypass flow constant. If the bypass flow is kept constant, it follows that the duration of shear stress exposure and shear stress frequency are also equal (in this connection it is referred to equations 1-4 which are already explained). The bypass flow, which represents the flow through the loop system, is kept constant, for example, by keeping the rotational speed of the pump constant. However, if the rotational speed of the pump is increased, as in case 1, the bypass flow also
25 increases, so that it can be kept constant, for example, by using the means to control the hydrodynamic resistance, particularly increasing the length and/or decreasing the diameter of a variable tube, in the loop system of the shear stress sensitivity device via a resulting pressure drop.

30 In case 1, therefore, the influence of the maximum shear stress can be investigated, whereas the other shear stress parameters are kept constant.

Case 2:

Increasing the duration of shear stress exposure while keeping the rotational speed of the pump constant is achieved, for example, by adding additional pumps with the same rotational speed in [rpm] in series.

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The maximum shear stress is kept constant by keeping the rotational speed of the pump at the same value.

Keeping the bypass flow and thus the shear stress frequency constant can be done, for example, as in Case 1.

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In case 2, therefore, the influence of the duration of the shear stress exposure can be investigated, whereas the other shear stress parameters are kept constant.

Case 3:

Increasing the shear stress frequency can be achieved, for example, by providing a second loop or multiple further loops, where each loop has a pump with the same value for the rotational speed. The shear stress frequency can also be controlled by adjusting the working volume of the bioreactor. When operating a bioreactor, there is always some flexibility with regard to the volume with which the bioreactor can be operated. As can be seen from equations 1 - 4 explained below, the bioreactor volume has a corresponding effect on the shear stress frequency, so that it can be influenced accordingly.

Increasing the shear stress frequency can be alternatively achieved by connecting 2 or more pumps in the loop system in parallel, operating at the same value for the rotational speed. This allows to increase the overall bypass flow, increasing the frequency, however, the bypass flow per pump remains similar.

The maximum shear stress can be kept constant, for example, as mentioned in Case 2.

In case 3, therefore, the influence of the shear stress frequency can be studied while keeping the other shear stress parameters constant.

Case 4:

A decrease in the bypass flow can be achieved by using the means to control the hydrodynamic resistance, particularly by increasing the length and/or decreasing the diameter of the variable line, in the loop system of the shear stress sensitivity device via an increasing pressure drop while keeping the rotational speed of the pump constant, thus keeping the maximum shear stress constant.

A decreasing bypass flow results in a decrease of the shear stress frequency, while the duration of shear stress exposure is increased.

In case 4, therefore, the influence of the duration of shear stress exposure and the shear stress frequency can be investigated at the same time, whereas the maximum shear stress is kept constant. This works because the two shear stress parameters always behave in opposite directions: If the bypass flow is increased, the shear stress frequency goes up and the duration of shear stress exposure decreases and vice versa. This makes it possible to classify which of the two parameters (duration of shear stress exposure or shear stress frequency) has a greater influence.

An overview of the exemplary different operating modes of the shear stress sensitivity device according to Cases 1 to 4 is provided in Figures 10A to 10E.

It is understood that the given cases 1 to 4 are only exemplary, there are of course other operation modes to operate the shear stress sensitivity device.

In Figures 10A to 10E, explained below, it is exemplarily shown how one or more shear stress parameters could be changed from the left to the right set-up of the shear stress sensitivity device, which only show possible embodiments without limiting the invention thereto. Although the bypass flow is not a shear stress parameter, it is also indicated in the individual figures for ease of understanding.

Figure 10A shows Case 1 which describes 3 different set-ups of the shear stress sensitivity device with an increasing rotational speed of the pump from the left to the right set-up. Thus, the maximum shear stress applied on the cell culture is increased. As exemplary means to control the hydrodynamic resistance there are shown a tube of variable length and/or variable diameter having two reducers at the ends, and different windings of the variable tube having two reducers at the ends. The means to control the hydrodynamic resistance causes a pressure drop, so that the bypass flow, the shear stress frequency, and duration of shear stress exposure (the residence time within the pump) of cells can be kept constant. A pressure drop could also be generated by using one or more valves.

The illustration in Fig. 10A means that 3 different set-ups of the shear stress sensitivity device can be used to set the stress parameters accordingly - as desired. Of course, many more set-ups can be provided to achieve the desired variations of the shear stress parameters. The 3 different set-ups are only a simplification for explanation. Each of the 3 set-ups is to be considered separately from the others. The sequence of the 3 set-ups in Case 1 illustrates how the shear stress sensitivity device can be modified, for example, if the rotational speed of the pump is to be increased and the other shear stress parameters shall be kept constant. When cultivating the cells, starting for example with the left set-up of Fig. 10A (Case 1) and thus obtaining a low maximum shear stress in the form of a low rotational speed of the pump. To increase the rotational speed of the pump, for example to a medium rotational speed of the pump, it is then switched to the set up of the middle device in Fig. 10A and for a high rotational speed of the pump to the set-up of the right device in Fig. 10A. In all 3 set-ups of Fig. 10A the other shear stress parameters are set constant, namely a constant shear stress frequency and a constant duration of shear stress exposure by the modifications of the set-ups of the shear stress device, respectively, as shown.

Figure 10B shows Case 2, in which the duration of shear stress exposure (residence time within a pump) can be increased if instead of one pump additional pumps with the same rotational speed in [rpm] are added in series. This modification is shown from the left to the right set-up in Fig. 10B. Besides it should be noted that when the pumps are connected in series, it is assumed that the cells are exposed to the stress for a longer time due to the "immediate" connection in series. In reality, the cells leave the pumps and thus the place of shear stress, but this happens only for a short time, with hardly any volume between pump 1 and 2, so that this assumption is acceptable for the method of the invention. In the right set-up of the shear stress sensitivity device exemplary shown are 2 pumps in serial connection in the loop system. If pumps with the same rotational speed in [rpm] are connected in series, the bypass flow will also be increased. However, comparable to case 1, the means to control the hydrodynamic resistance are used in the loop system which are introduced to cause a pressure drop and thus reduce the bypass flow as illustrated in the right set-up of the shear stress sensitivity device. Thereby the applied maximum shear stress, the bypass flow as well as shear stress frequency can be kept constant while having the possibility to investigate the effect of the duration (length) of shear stress exposure.

Figure 10C shows Case 3A in which the maximum shear stress (rotational speed in [rpm]), the bypass flow as well as the duration of shear stress exposure (residence time of the cells in the pump) are kept constant from the left to the right set-up, while the shear stress frequency can be increased by a second loop or multiple further loops. The shear stress frequency can be also controlled by adjusting the working volume of the bioreactor.

Figure 10D shows Case 3B in which the maximum shear stress (rotational speed in [rpm]), the bypass flow (per pump) as well as the duration of shear stress exposure (residence time of the cells in the pump) are kept constant from the left to the right set-up, while the shear stress frequency can be increased by providing additional pumps, which are connected in parallel and operate with the same value of the rotational speed. As an example, 2 pumps are shown in parallel connection in the loop system. The bypass flow is approximately twice as large with a total of 2 pumps as with one pump, so that the shear stress frequency is also doubled. Per pump, however, the bypass flow is the same, so that the duration of shear stress exposure remains the same.

Figure 10E shows Case 4, in which several stress parameters at the same time are changed. While the maximum shear stress (rotational speed of the pump in [rpm]) is kept constant from the left to the right set-up by keeping the [rpm] constant, the duration of shear stress exposure (residence time of the cells within the pump) is increasing. By contrast the bypass flow and shear stress frequency are decreasing from the left to the right set-up due to a pressure drop caused by the means to control the hydrodynamic resistance, e.g. by a longer tubing or tubing of smaller diameter.

According to Figure 10E (Case 4) and equation 1 to 2, the bypass flow influences the shear stress frequency ($freq.$) as well as the duration of shear stress exposure (residence time/length of exposure) (t_{res}). The bypass flow correlates positively with shear stress frequency and negatively with duration of shear stress exposure. Since the cultivation with a low bypass flow shows the lowest and the cultivation with a high bypass flow shows the highest performance in terms of growth, it could be concluded in experiments that the duration of shear stress exposure has a stronger influence on the performance when compared to the shear stress frequency. Overall, it was found that a high duration of shear stress exposure is weighted more heavily than a low shear stress frequency. Based on the set-ups described in Figures 10B, 10C and 10D, Case 2, Case 3A and Case 3B, these parameters can therefore also be investigated independently.

Although Figures 10A through 10E illustrate only the continuous mode such as perfusion mode, it should be noted that the set-up is shown only by way of example. However, the cases shown also apply in the same way to a batch or fed-batch set-up, as illustrated in Figure 1A.

Embodiments of the present invention omitting step (4) (the calibration and characterization methods)

An embodiment of the invention is directed to a method for determining the shear stress sensitivity of cells wherein the characterization of the shear stress sensitivity device according to step (4) is omitted. In simplified terms, a cultivation process is selected and a control process (reference) of the cultivation process with a low or the lowest possible shear stress level is selected, and then the control process is reworked in the shear stress sensitivity device by varying the shear stress parameter(s) in the direction of the cultivation process, so that from the variation of the shear stress parameter(s) the relevant information can be derived. That is, only the relative shear stress levels are compared, the absolute values do not have to be determined.

The method according to this embodiment is therefore directed to a method for determining the shear stress sensitivity of cells in a shear stress sensitivity device during a cultivation process comprising a liquid culture

medium, particularly determining the effect of the shear stress on the cells, which comprises the following steps:

- (1) providing a shear stress sensitivity device comprising

 - a bioreactor,
 - a closed loop system connected with and located outside the bioreactor;
 - a pump placed in the loop system, the pump being a kinetic pump and
 - means to control the hydraulic resistance in the loop system;
- (2') performing a cultivation process or using a cultivation process already performed and providing one or more cultivation process curves of the cultivation process, wherein one or more performance characteristics of the cells are plotted over a selected period of time;
- (3) selecting one, two or three shear stress parameters to be investigated from the group consisting of:

 - maximum shear stress represented by a rotational speed of the pump;
 - duration of shear stress exposure represented by a residence time of the cell culture within the pump; and/or
 - shear stress frequency represented by a number of passages of the cell culture comprising the liquid culture medium and cells through the loop system per time,

whereby the shear stress intensity represents a combination of all 3 shear stress parameters;
- (4') performing a control cultivation process or using a control cultivation process already performed and providing one or more cultivation process curves of the control cultivation process, wherein one or more performance characteristics of the cells are plotted over a selected period of time, wherein the control cultivation process is the same cultivation process as in step (2'), but wherein a lower shear stress intensity is used than in the cultivation process and the same performance characteristics of the cells and the same period of time is used as in step (2');
- (5') performing one, two, three or more control cultivation runs of the control cultivation process of step (4') in the shear stress sensitivity device over the same selected period of time, by successively increasing or decreasing one, two or three shear stress parameters in each subsequent control cultivation run, wherein each subsequent control cultivation run is modified by varying the shear stress parameter(s) toward the selected cultivation process of step (2') and measuring one or more performance characteristics of the cells during each run of the control cultivation process and generating a curve for each performance characteristic in each run over time;
- (6') selecting the curve from the set of curves in step (5') for a performance characteristic that is closest to the cultivation process curve for said performance characteristic;
- (7') determining how the shear stress intensity needs to be changed to move from the control cultivation process to the cultivation process by comparing the obtained curves;

whereby it is ensured that the shear stress intensity occurring in the cultivation process is higher than the shear stress intensity occurring in the control cultivation process.

Therefore, according to this embodiment, the method comprises or consists of steps (1), (2'), (3), (4'), (5'),
5 (6') and (7'). According to an embodiment, these steps are performed in the order indicated. According to a further embodiment, there are no intermediate steps performed between these steps.

According to this embodiment, step (1) is performed as already described.

10 Step (2) is replaced by step (2') wherein a cultivation process is performed or a cultivation process already performed is used and one or more cultivation process curves of the cultivation process are provided, wherein one or more performance characteristics of the cells are plotted over a selected period of time. Unlike in step (2), not only are the cells, liquid culture medium, cultivation mode and cultivation conditions
15 for a cultivation process to be performed selected, but a cultivation process per se is selected that has already been performed or will be performed in step (2'). The cultivation process curves of the cultivation process, in which one or more performance characteristics of the cells are plotted over a selected period of time, are then either picked from known data material or these are measured and generated in a cultivation process performed in step (2').

20 Step (3) is performed as already described. Step (4) is omitted, i.e. the calibration and characterization of the shear stress sensitivity device is not performed and is not necessary.

According to this embodiment step (4') may be performed in the same way as step (6) as already described, wherein a control cultivation process is conducted. This control cultivation process is performed in the same
25 manner as the cultivation process of step (2'), but using a lower shear stress intensity than in the cultivation process, for example, by using a maximum shear stress which is as low as possible by using the lowest possible rotational speed of the pump. It may be proceeded as already described in step (6). The control cultivation process may be performed in the shear stress sensitivity device but this is not absolutely necessary because it must be only ensured that the shear stress intensity is lower than in the selected
30 cultivation process.

In case the shear stress sensitivity device is used and the cultivation mode of the cultivation process is batch or fed-batch, for example, a lower maximum shear stress is achieved by a modified shear stress sensitivity device which is without a loop system and without a pump. In case the shear stress sensitivity
35 device is used and the cultivation mode of the cultivation process is continuous mode, especially perfusion, the shear stress sensitivity device is used in such a manner that the shear stress intensity is adjusted lower as in the cultivation process, particularly as low as possible.

According to this embodiment step (5') is performed in the same way as step (5) as already described, but
40 wherein the control cultivation process of step (4') is performed in the shear stress sensitivity device and is modified by varying the shear stress parameter(s) toward the cultivation process of step (2'). Therefore, one, two, three or more control cultivation runs of the control cultivation process of step (4') are performed in the shear stress sensitivity device over the same selected period of time as in step (2') and step (4'), by successively increasing or decreasing one, two or three shear stress parameters in each subsequent

control cultivation run. Each subsequent control cultivation run is modified by varying the shear stress parameter(s) toward the selected cultivation process of step (2') and measuring one or more performance characteristics of the cells during each run of the control cultivation process. Based on the measured values a curve is plotted over time for each performance characteristic and each run. Curves are therefore again
5 generated for the measured performance characteristics over time, but the shear stress parameter(s) is/are varied in the runs such that the curve(s) obtained in the control runs converge against the curve(s) of the cultivation process.

Therefore, the further embodiment according to the invention is based on a method wherein no calibration
10 and no characterization of the shear stress sensitivity device takes place, i.e. a determination of the maximum shear stress source and a quantification of the shear stress values which occur in the shear stress sensitivity device is not performed.

Thus, it is proceeded by systematically performing the control cultivation process in one, two, three or more
15 control cultivation runs by varying one, two or three shear stress parameters in each subsequent control cultivation run, and one or more performance characteristics of the cells are measured during each run and a curve is generated for each performance characteristic over time. In doing so, the shear stress parameter(s) is(are) varied in each of the one, two, three or more control cultivation runs such that the obtained curves converge towards the curve of the selected cultivation process about which further
20 information is desired.

In step (6') replacing step (6) as already described the curve from the set of curves in step (5') for a performance characteristic is selected that is closest to the cultivation process curve for said performance characteristic.
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In step (7') replacing step (7) as already described it is then determined how the shear stress intensity needs to be changed to move from the control cultivation process to the cultivation process by comparing the obtained curves. By comparing the curves, it is obtained the approximate conditions to mimic the selected cultivation process. In other words, the control cultivation process is therefore calibrated to the
30 selected cultivation process as a quasi reference run. That is the absolute shear stress values are not required, but only the change of the equipment settings, e.g. the rotational speed of the pump, to which these can be attributed.

The cultivation process in step (2') can be chosen arbitrarily and can be any type of cultivation process
35 known by the skilled person.

Furthermore, it is ensured that the shear stress intensity occurring in the cultivation process is higher than the shear stress intensity occurring in the control cultivation process. Usually, the cultivation process of step (2') has a higher shear stress intensity than the control cultivation process of step (4'), which is carried out
40 at a lower shear stress intensity. Only in very rare exceptional cases this is not the case or not possible. Then, however, this embodiment cannot be carried out. Therefore, it is ensured in this embodiment that the shear stress intensity occurring in the cultivation process is higher than the shear stress intensity occurring in the control cultivation process.

It goes without saying that the selected conditions and parameters of the cultivation process of step (2') and the control cultivation process of step (4') should be as similar as possible in order to obtain meaningful results. For example, the cultivation type such as batch, fed-batch, continuous (perfusion) should be the same, the type of organism to be cultured selected from a variety of organisms (e.g., eukaryotic cells) should be the same, and the variety of performance characteristics that change over time should be the same, etc.

This embodiment may be also used to investigate one or more modifications of the cultivation process compared to the control cultivation process. For example, this embodiment can be used as a scale-down model: The cultivation process is then performed on a large scale, for example, and the control cultivation process is performed on a smaller scale. The small-scale control cultivation process is then modified until it runs like the large-scale cultivation process and information on the shear stress levels would be obtained.

For example, this embodiment could also be used as a scale-up model: The cultivation process is then carried out on a small scale and the control cultivation process is carried out on a larger scale. The large-scale control cultivation process is then modified until it runs like the small-scale cultivation process and information on the shear stress levels would be obtained.

To better understand this embodiment of the present invention, an exemplary embodiment will be explained by way of example. In this example embodiment, the cultivation process is a cultivation process in production-scale and the control cultivation process is the same cultivation process but at a small-scale.

Often, a scale-up of a process with similar input parameters shows lower performance at production-scale than at small-scale or laboratory-scale^{(22),(23)}. For example, despite scaling up the process according to the general principles of bioprocess engineering, viable cell density and titer in the bioreactor are lower at production-scale than at small-scale. One parameter that is usually not held constant during scale-up is the hydrodynamic shear stress, which could explain the performance disparity between scales.

Therefore, simulating the shear stress of a production bioreactor at small-scale could enable comparable process performance to be achieved during scale-up.

In Figs. 11A and 11B exemplary curves of a cultivation process using a CHO cell line performed in a production-scale bioreactor (curve P) and in a small-scale bioreactor (curve S) are shown with two performance characteristics, namely viable cell density (VCD) in [10^6 cells/mL] and the titer in [g/L] plotted against the time in [h], respectively. It can be seen that the small-scale bioreactor provides better performance than the production-scale bioreactor. To investigate the shear stress as a possible cause of the poor performance of the production-scale bioreactor, the method according to the invention is used. The process of the production-scale bioreactor is used as the cultivation process. The process of the small-scale bioreactor is used as the control cultivation process which is the same as the selected cultivation process but only modified in the scale used.

To investigate the maximum hydrodynamic shear stress that occurs, the shear stress sensitivity device is, for example, operated as explained in Case 1, Fig. 10A. For this purpose, one, two, three or more cultivation process runs are performed as in the control cultivation process by varying one shear stress

parameter, namely the maximum shear stress, from cultivation run to cultivation run. However, it should be noted that this method is not limited to this exemplary embodiment and it is understood that other variations of shear stress parameter(s) are also possible.

5 In this exemplary embodiment, parallel control cultivation runs may be performed with identical conditions but increasing the rotational speed of the pump (Note: the same procedure is performed in experiment 1 described in "Experimental part I", but with calibration and characterization of the shear stress sensitivity device according to step (4); see Figures 13A-13F). The obtained curves with varying rotational speed of the pump are shown in Figures 12A and 12B.

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In the present example the rotational speed of the pump is adjusted so that $\text{rpm A} < \text{rpm B} < \text{rpm C}$. Finally, the resulting curves A, B, and C of the performance characteristics (in the present example: VCD and titer) are compared to the production-scale curve P. If the curves of the performance characteristics exhibit a similar profile over time, it can be concluded that the maximum shear stress conditions achieved with the shear stress sensitivity device match those of the production-scale bioreactor. In the present example the curve B matches with curve P.

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The shear stress sensitivity device therefore allows the performance of, for example, production-scale cultivation processes to be achieved by mimicking the maximum shear stress conditions. The advantage of this approach is that there is no need to determine the maximum shear stress per se, nor is there a need for complex calibration or characterization of the shear stress sensitivity device. By systematically increasing the shear stress intensity using shear stress parameter(s), for example, via the rotational speed of a pump (as shown in Figure 10A, Case 1), the shear stress conditions can be matched to e.g. production-scale bioreactors by simply comparing the profiles or curves of the performance characteristics over time.

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This way of mimicking stress conditions is thereby very broadly applicable and not limited to the conditions given in this exemplary embodiment.

30 The invention is also directed to a process for culturing cells in a liquid culture medium, wherein the shear stress sensitivity determined according to the method of the present invention is used in the same cultivation process but performed on a larger or smaller scale as the method for determining the shear stress sensitivity.

35 Subject of the present invention is also a process for culturing eukaryotic or prokaryotic cells in a liquid culture medium in a bioreactor and performing a method for determining the shear stress sensitivity of cells in a shear stress sensitivity device during a cultivation process according to the present invention.

The invention is also directed to a process for producing a recombinant protein, the process comprising the steps of:

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step I) culturing eukaryotic or prokaryotic cells expressing a recombinant protein in a liquid culture medium in cell culture in a bioreactor;

step II) harvesting the recombinant protein;

step III) purifying the recombinant protein;

wherein performing in step (I) a method for determining the shear stress sensitivity of the cells in a shear stress sensitivity device during the cultivation process according to the present invention.

The invention also relates to the use of a device comprising

- 5 - a bioreactor,
 - a closed loop system connected with and located outside the bioreactor;
 - a pump placed in the loop system, the pump being a kinetic pump and
 - means to control the hydraulic resistance in the loop system;
as a shear stress sensitivity determination device or a shear stress sensitivity device,

- 10 wherein the device has no nozzle,
 for culturing cells in a liquid culture medium, wherein a defined shear stress is set during the culturing.

- The defined shear stress which is set during the culturing may be a maximum shear stress value which is obtained by varying shear stress parameter(s) in the device. The maximum shear stress value is preferably
15 determined on the basis of a shear stress-dependent physical quantity, in particular the shear stress-dependent physical quantity being the aggregate size of shear-sensitive aggregates. Preferably, the maximum shear stress value being determined on the basis of a shear stress-dependent physical quantity, as follows: The shear stress dependent physical quantity is exposed to defined, predetermined conditions, whereby the physical quantity varies according to the magnitude of the maximum shear stress. The defined,
20 predetermined conditions are conditions where the maximum shear stress values are each known from a state-of-the-art calibration procedure. The shear stress dependent physical quantity is exposed to unknown conditions in the device, whereby the physical quantity varies according to the magnitude of the maximum shear stress. The unknown conditions in the device are conditions in which the maximum shear stress values in the device are not known, but can be determined by the state-of-the-art calibration procedure,
25 whereby the maximum shear stress values occurring in the device are established (characterization procedure). Then, the magnitude of the maximum shear stress value in the device can be inferred from the physical quantity obtained under the defined, predetermined conditions. The same magnitude for the physical quantity means that the same maximum shear stress value is present.

- 30 According to another embodiment, as defined shear stress relative shear stress levels may be used which result from two runs of a cultivation process in which different shear stress levels are present, one of the runs is used as a control run and the control run is repeated and modified by varying the shear stress parameter(s) until it runs like the other run. In this case, the calibration and characterization of the device is omitted; this procedure has also already been explained in detail.

- 35 It is understood that the explanations for the method of the invention should apply equally to the use of the device and vice versa.

- 40 Further experiments have shown that shear stress sensitivity is also applicable to different cells, for example, different cell lines expressing a completely different recombinant protein. It was found that each cell line has an individual sensitivity towards similar levels of shear stress. Any type of living organism growing in a suspension bioreactor can be investigated in terms of shear stress sensitivity. Besides the maximum hydrodynamic shear stress this invention also allows to independently investigate parameters, such as duration of shear stress exposure and shear stress frequency (Figures 10A-10E). These

parameters are shown to influence the cultivation performance and are essentially relevant during bioprocess scale-up or scale-down.

There are many different advantages of the present invention:

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With the present invention, it is possible for the first time to examine and understand the effects of shear stress in detail. The shear stress is determined directly in a cultivation process during the cultivation process, whereby the total shear stress can be determined as a value of the shear stress intensity by combining all shear stress parameters in the form of the maximum shear stress, the duration of shear stress exposure and the shear stress frequency. This means that the shear stress is defined for the first time in its entirety and describes what it is composed of (maximum shear stress, duration of shear stress exposure and shear stress frequency), whereby each of these parameters can be set individually and contribute to the total shear stress. It is therefore possible to vary one, two or three shear stress parameters simultaneously in the shear stress sensitivity device, while keeping all other cultivation parameters constant. The shear stress sensitivity device and the different shear stress parameters that can be modified provide an extraordinary amount of variance and flexibility for various process control strategies.

It has been found that the shear stresses induced by gassing or stirring in a conventional bioreactor contribute only insignificantly, if at all, to the overall shear stress and therefore have a rather negligible influence on the performance of a cell culture. In particular, the shear stresses never reach a level of >10 Pa, regardless of the conditions applied. A shear stress value of 10 Pa is generally much too low in the context of biotechnological equipment, for example, even for a larger scale, especially when, for example, recirculation pumps are used in perfusion processes. Therefore, stirring and gassing are only present as a kind of background shear stress and play practically no role in determining the shear stress sensitivity of living organisms. Therefore, the actual shear stress parameters are the decisive factors in a cultivation system and not the gassing or the stirring.

The method according to the invention is not only highly variable in terms of the shear stress parameters, but also with regard to the cultivation method to be investigated. Any cell cultivation method, with any cells, any liquid culture medium, any cultivation mode and any cultivation conditions can be used for the method according to the invention. The only limitation for the cultivation process is that it must be carried out in a liquid medium so that the cell culture can be circulated in the shear stress sensitivity device. In fact, any kind of living organism that grows in a suspension bioreactor can be examined for its sensitivity to shear stress. Experiments have demonstrated that the sensitivity to shear stress can also be applied to different cells, e.g. different cell lines expressing a completely different recombinant protein. It was also found that each cell line has an individual sensitivity to similar shear stress values.

The process of the invention can also be carried out during various process operations (batch, fed-batch, perfusion), whereby the other parameters in the bioreactor are not affected.

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Since the shear stress exerted on the cells is quantified by a calibration, the result is independent of the specifically shear stress sensitivity device as used, such as the individually inserted pump, and reflects the conditions of the respective complete cultivation system.

- 5 The method according to the invention also allows the equipment used for the shear sensitivity device to be reflected in the shear stress intensity. Each inner surface, tool, device, etc. that is part of the shear sensitivity device interacts with the set process conditions to obtain an overall shear stress value that acts on the cells. In other words, there is an interaction of all the individual features of the shear sensitivity device (e.g. the set-up of the shear sensitivity device) and the set conditions, which lead to a synergistic overall
10 effect that goes far beyond the isolated individual effects (e.g. determining the shear stress on a wall of a device or on a cell).

- The conversion of process conditions/equipment used for the shear stress sensitivity device into shear stress values in [Pa] allows the shear stress sensitivity or shear stress limit of the cells to be determined or
15 to be estimated. This allows a very accurate assessment of the cells' behaviour under the cultivation conditions. This enables both a scale-up and a scale-down of a cultivation process. The shear stress can be adjusted in a targeted manner, so that it is possible to calculate back from a larger to a smaller system (scale-down) or from a smaller to a larger system (scale-up).

- 20 The shear stress behavior of the cells directly affects the performance characteristics, so that the curves obtained in the method of the present invention directly reflect how the cells cope with a given shear stress intensity and also respond in each case to the level of shear stress intensity exerted.

- The shear stress sensitivity device as designed and used in the context of this invention holds the
25 advantage to being close to the cultivation methods of a cell culture of interest. For example the bioreactor used for culture can be equipped in such a way that measurements of the shear stress sensitivity of the respective cells directly under these cultivation conditions are made possible.

- Therefore, the shear stress sensitivity device provides insights into cell behavior and hydrodynamic shear
30 stress that allow for accurate conclusions about the level of shear stress applied, contributing to predictions for other cultivation procedures, of equal, larger or smaller scale using similar or different hardware components. In particular, this allows reliable predictions to be made as to how a cultivation process can best be carried out. The investigation of the cells sensitivity towards shear stress intensity at small scale may allow to accurately predict potential variations in process performance and product quality during
35 scale-up or scale-down and/or thereby enables to pick clones during cell line development based on small or large scale shear stresses. This makes it possible to select at a very early stage which clones are suitable for production purposes and which shear stress resistance they exhibit. This is a selection procedure, for example, how to distinguish good-producing clones from poor-producing clones.

- 40 According to another embodiment of the invention the complex characterization procedure including calibration of the shear stress sensitivity device may be omitted. That is, only the relative shear stress levels are compared, the absolute values do not have to be determined. In a simplified manner, a cultivation

process is selected and a control process (reference) of the cultivation process with a low or the lowest possible shear stress level is selected, and then the control process is reworked in the shear stress sensitivity device by varying the shear stress parameter(s) in the direction of the cultivation process, so that from the variation of the shear stress parameter(s) the relevant information can be derived.

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Also this embodiment according to the invention can, for example, be used to investigate the shear stress as a possible cause for the poor performance of a bioreactor in a production scale. For example, this embodiment can be used as a scale-down model: The small-scale control cultivation process is then modified until it runs like the large-scale cultivation process and information on the shear stress levels would be obtained. This embodiment can also be used as a scale-up model: The large-scale control cultivation process is then modified until it runs like the small-scale cultivation process and information on the shear stress levels would be obtained.

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The present invention therefore provides surprising insights and knowledge about cell behavior and hydrodynamic shear stress of cells, which allow accurate conclusions about the level of applied shear stress and contribute to predictions for other cultivation processes on the same, larger or smaller scale.

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Experimental part I

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Experiment 1

Effect of hydrodynamic shear stress during fed-batch cultivation

Defined levels of shear stress were investigated in fed-batch mode according to the set-up of the shear stress sensitivity device shown in Fig. 1A. In experiment 1, the effect of hydrodynamic shear stress was investigated in CHO fed-batch cultures. The used cell line was a CHO cell producing a recombinant protein (Cell line A). The shear stress parameter selected and studied was the maximum shear stress (represented by the rotational speed of the pump).

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For all cultivation runs and tested stress conditions, the same fed-batch process settings (same shear stress sensitivity device with the same cultivation conditions) were applied. All parameters and conditions were kept constant for all cultivation runs. The glucose addition threshold was <3 g/L. The cells consume the glucose and this is recorded accordingly. Details of the cultivation process can be found in experimental part II.

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Only the selected shear stress parameter was varied from cultivation run to cultivation run, i.e. the rotational speed of the pump was kept constant during each cultivation run, but increased with each subsequent cultivation run. The pump is a magnetically levitating centrifugal pump (in experiment 1: PuraLev i30 SU, Levitronix, Switzerland). The cell culture was continuously circulated in the shear stress sensitivity device as explained earlier.

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The results for the measured performance characteristics of the cell cultivations over time are shown in Figs. 13A to 13F. That is Figs. 13A to 13F show the offline data of CHO cell fed-batch cultures exposed to various levels of hydrodynamic shear stress. The offline data were obtained by sampling in the shear stress

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sensitivity device and measuring outside the shear stress sensitivity device. Details are given in experimental part II.

The control in Figs. 13A to 13F represents a standard fed-batch set-up without bypass loop and without pump, so that the shear stress value is assumed to be negligible.

Figures 14A and 14B show the online data of the shear stress set-up, i.e., the values measured in the shear stress device itself during the cultivation process. Specifically, Figs. 14A and 14B show the online data of the varied rotational speed in [rpm] of the magnetically levitating centrifugal pump as already mentioned (Fig. 14A) and the online measured constant bypass flow in [L/min] during fed-batch cultivation (Fig. 14B). The corresponding set-up of the shear stress sensitivity device is shown in Fig. 1A and operated according to case 1 shown in Fig. 10A, but without hollow fiber filter module.

The shear stress levels were increased by increasing the rotational speed in [rpm] of the magnetically levitating centrifugal pump (Figure 14A). The rotational speeds in [rpm] were calculated into shear stress values (Pa) by the equation given in Fig. 9C as explained earlier (step (4) of the method according to the invention, see Figs. 8A and 8B, and Figs. 9A, 9B and 9C). The online signals are measured very frequently (every few seconds) and are thus displayed as lines. In order to be able to indicate a symbol in addition to the lines, every 800th data point was represented as a symbol. The shear stress values calculated with the equation given in Fig. 9C were as follows (see Fig. 14A):

Table 3:

cultivation run No.	rotational speed [rpm]	shear stress value [Pa]
control	-	-
1	2950	30
2	4143	64
3	5450	118
4	5820	137
5	6492	174

In detail, Figure 14A depicts the online data of the fed-batch cultivation with elevated shear stress levels. To keep the shear stress level constant during the cultivation the magnetically levitating centrifugal pump operates at a defined rotational speed setpoint, which is kept constant throughout each cultivation run (Figure 14A). To keep the bypass flow ≈ 0.5 L/min, the length of the tubing was adjusted according to the rotational speed, as can be seen from Fig. 10A and the 3 exemplary set-ups for the shear stress sensitivity device with the increasing rotational speed of the pump from the left to the right side. However, due to increasing relative viable cell density (VCD) and the thereby increasing viscosity the bypass flow tends to drop off slightly over the course of the cultivation (Figure 14B). A fed-batch process is characterized by an increasing volume over time. As a result, the shear stress frequency changes over time (Equation 1). Since the fed-batch processes (all cultivation runs thereof) were kept identical, and thus also the filling volume over time, the observed effects can be solely attributed to the different levels of hydrodynamic shear stress.

The results for the performance characteristics of the cell cultivation runs over time are shown in Figs. 13A to 13F. Therefore, 6 runs of the cultivation process were performed, i.e. the cultivation process with the selected cells (here: CHO cells) was performed 6 times under the same conditions (fed-batch) with the same parameters in the shear stress sensitivity device of Fig. 1A, i.e. 1 control run and 5 examination runs.

5 The selected time period for each run was 14 days. For each run, the time course for performance characteristics for the cultivation was determined as offline data.

In experiment 1, the characteristic parameters were relative viable cell density (VCD) (Fig. 13A), viability (Fig. 13B), lactate dehydrogenase (LDH) activity in the supernatant (Fig. 13C), glucose concentration (Fig. 13D), lactate concentration (Fig. 13E) and the relative titer (product concentration) (Fig. 13F).

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For each of the performance characteristics, the values were determined as offline data over time. For each run of the cultivation process, a curve was drawn for each performance characteristic over time, with the values for the performance characteristic plotted against time and for the respective shear stress value.

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Furthermore, in a control run a control cultivation process was performed using the same cultivation process as before, with the same cells, the same liquid medium, the same cultivation mode, the same conditions of the cultivation process and the same shear stress sensitivity device but without loop system and pump over the same period of time. Due to the missing pump and loop system, the shear stress sensitivity device of the control process in this example case was now only a bioreactor. For the control run a control curve was also generated for the same performance characteristics as before, but the cell culture was not subjected to the shear stress due to the missing pump and loop system. Of course, in the control cultivation process, the same performance characteristic(s) for the cell culture are measured over the same selected period of time as in cultivation runs nos.1 to 5.

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25 The analysis of Figs. 13A to 13F gives the following picture:

Within the exponential growth phase relative viable cell densities (VCD) are decreased for conditions with increasing shear stress (cf. Figure 13A). The peak viable cell density (VCD) is lower for the condition with the highest stress of 174 Pa whereas final viable cell densities (VCD) at day 14 are comparable. Viability profiles highlight that until day 8 viabilities are lower for conditions with higher stress with a shift to higher viabilities from day 9 onwards when compared to conditions with lower stress (Figure 13B). This might be explained by the mechanism of the determination of the viability via trypan blue staining. This method allows to detect dead cells, however lysed cells can not be detected. With higher shear stress rates dead cells might be sheared towards the end of the culture, which means they cannot be measured by trypan blue staining (CEDEX HiRes, Roche Diagnostics), thus viability is increased. Until day 10 lactate dehydrogenase (LDH) activities are elevated for conditions with higher stress followed by a shift (Figure 13C). This shift might be because towards the end of the culture the LDH activity is more strongly driven by the total cell density (TCD), which is higher for conditions with lower shear stress ($TCD = VCD/Viability$). Figure 13D indicates that glucose concentrations increase with conditions of higher stress. This is due to the negative effect of shear stress on growth. Due to the decreased growth, less lactate accumulates and after the shift in the lactate metabolism from production to consumption lactate decreases faster for conditions with lower shear stress (Figure 13E). Finally, also the relative titer shown in Figure 13F is diminished for conditions with higher stress (118, 137 and 174 Pa). Product quality measurements revealed no major difference (data not shown).

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Based on these results it can be concluded that this CHO cell line demonstrates to be resistant to shear stress levels up to ≈ 64 Pa during fed-batch cultivation. The curves at 64 Pa are closest to the control curve overall. Conditions with ≥ 118 Pa show reduced growth and titer. This suggests that the shear stress limit for this cell culture is about 64 Pa.

Experiment 2

Effect of hydrodynamic shear stress during perfusion cultivation

Defined levels of shear stress were investigated in continuous mode according to experiment 2 in perfusion mode according to the set-up of the shear stress sensitivity device shown in Fig. 1B. In experiment 2, the effect of hydrodynamic shear stress was investigated in CHO perfusion cultures. The used cell line was a CHO cell producing a recombinant protein (Cell line A), the same cell line as used for the fed-batch cultivation as already described. Glucose addition threshold is < 3 g/L. The shear stress parameter selected and studied was the maximum shear stress (represented by the rotational speed of the pump).

For all cultivation runs and tested shear stress conditions, the same perfusion process settings (same shear stress sensitivity device with the same conditions) were applied. All parameters and conditions were kept constant for all cultivation runs. Details of the cultivation process can be found in experimental part II.

Only the selected shear stress parameter was varied from cultivation run to cultivation run, i.e. the rotational speed of the pump was kept constant during each cultivation run, but increased with each subsequent cultivation run. The pump is the magnetically levitating centrifugal pump as indicated in experiment 1. The cell culture was continuously circulated in the shear stress sensitivity device, as already explained. The shear stress sensitivity device also contains a hollow fiber filter module as shown in Fig. 1B.

The results for the performance characteristics of the cell cultivations over time are shown in Figs. 15A to 15D. Figs. 15A to 15D show the offline data of CHO cell perfusion cultures subjected to different hydrodynamic shear stresses. The same perfusion process settings were used for all shear stress conditions tested.

The control in Figures 15A to 15D represents a standard perfusion set-up, wherein the pump, loop system and hollow fiber module are still present, but a lower shear stress than in the cultivation process runs is given by means of the rotational speed of the pump. Optimally, the control represents the minimum shear stress level with which perfusion runs by default. The feedback-controlled bypass flow is 0.5 L/min.

Figures 16A and 16B show the online data of the shear stress set-up, i.e. the values measured in the shear stress sensitivity device itself during the cultivation process. These are the varied rotational speed in [rpm] of the magnetically levitated centrifugal pump (here: i30SU type pump) (Fig. 16A) and the online measured constant bypass flow in [L/min] during perfusion cultivation (Fig. 16B). The corresponding set-up of the shear stress sensitivity device is shown in Figure 1B and was operated according to Case 1 shown in Figure 10A, i.e., with the hollow fiber filter module shown.

The shear stress levels were increased by increasing the rotational speed in [rpm] of the magnetically levitating centrifugal pump (Figure 16A). The adjusted rotational speeds in [rpm] were calculated into shear

stress values (Pa) according to the equation indicated in Fig. 9C as already explained (step (4) of the process according to the invention, see Figs. 8A and 8B, and Figs. 9A, 9B and 9C). The online signals are measured very frequently (every few seconds) and are thus displayed as lines. In order to be able to indicate a symbol in addition to the lines, every 350th data point was represented as a symbol. The shear stress values calculated with the equation indicated in Fig. 9C were as follows (see Fig. 16A):

Table 4:

cultivation run No.	rotational speed [rpm]	shear stress value [Pa]
control	≈2500-3000	≈21-32
1	2950	30
2	4143	64
3	5045	100
4	5820	137
5	6492	174

In detail, Figures 16A and 16B show online data of the shear stress set-up with varied rotational speed of the pump in [rpm] and constant bypass flow during perfusion cultivation. The rotational speed of the magnetically levitating centrifugal pump (here: pump of type i30SU) are indicated in Figure 16A and the online measured bypass flow is shown in Figure 16B.

Figures 16A and 16B depict the online data of the perfusion cultivation with elevated shear stress levels. Despite the control condition, elevated shear stress levels were achieved by increasing the rotational speed in [rpm] while adjusting the tubing to keep the bypass flow ≈ 0.5 L/min (Figure 16A and 16B). Similar as for the fed-batch cultivation (Figure 14B), the bypass flow tends to drop off slightly over the course of the cultivation due to increasing relative viable cell density (VCD) and the thereby increasing viscosity (Figure 16B).

The results for the performance characteristics of the cell cultivation runs over time are shown in Figs. 15A to 15D. Therefore, 6 runs of the cultivation process were performed, 1 control run and 5 cultivation runs, i.e. the cultivation process with the selected cells (here: CHO cells) was performed 5 times under the same conditions (perfusion) with the same parameters in the shear stress sensitivity device of Fig. 1B. The selected period of time for each cultivation run was 6 days. For each cultivation run, the time course for the selected performance characteristics for cultivation was obtained as offline data. In experiment 2, the performance characteristics were the relative viable cell density (VCD) and viability (Figure 15A), the lactate dehydrogenase (LDH) activity in the supernatant (Figure 15B), glucose concentration (Figure 15C) and lactate concentration (Figure 15D), each of which was determined over time as offline data. For each cultivation run, a curve was drawn for each performance characteristic over time, with the measured values for the performance parameter plotted over time and for the respective shear stress value. Conditions with increased shear stress levels were achieved by increasing the rotational speed in [rpm] of the magnetically levitating centrifugal pump (see Figure 16A).

Furthermore, in a control run a control cultivation process was performed using the same cultivation process as before, with the same cells, the same liquid medium, the same cultivation mode, the same conditions of

the cultivation process over the same period of time but a minimum shear stress level possible for the perfusion mode was given by the rotational speed of the pump. For this control cultivation process a control curve was also generated for the same performance characteristics as already measured (Figs. 15A to 15D). As already explained, a standard perfusion set-up was used where the flow was controlled at 0.5 L/min. Of course, in the control cultivation process, the same performance characteristic(s) for the cell culture are measured over the same selected period of time as in cultivation runs nos.1 to 5 of experiment 2.

The analysis of Figs. 15A to 15D gives the following picture:

- 10 Relative viable cell density (VCD) and viability are reduced for the condition with the highest stress of 174 Pa (Figure 15A). Due to reduced growth, less glucose is consumed, and less lactate produced, while LDH activity is increased (Figures 15B, 15C and 15D). The shown offline samples in Figures 15A-15D also indicate reduced growth as well as reduced glucose consumption and lactate production for the condition with 137 Pa. Perfusion cultivation with shear stress levels up to 100 Pa perform comparable to the control.
- 15 The slightly elevated LDH activity of the 100 Pa condition between day 1 and 5 reveals that the LDH activity in the supernatant is a sensitive indicator for increased stress levels (Figure 15B). Abruptly rising LDH activities between day 5 and 6 for the control, 100 Pa and 137 Pa condition may be explained by incorrect sample handling or measurement error.
- 20 Based on the above data it can be concluded that during pre-stage perfusion process cultivations with stress levels of ≤ 100 Pa show a similar performance in terms of growth. That is, the shear stress limit for this cell culture is about 100 Pa.

Experiment 3

25 Effect of hydrodynamic shear stress during perfusion cultivation

In experiment 3 the effect of constant and high (174 Pa) hydrodynamic shear stress with changing bypass flow was investigated in CHO perfusion cultures. For all tested bypass flow conditions, the same perfusion process settings were applied. The shear stress sensitivity device according to Fig. 1B was used. The cell line used for this experiment was again a CHO cell producing a recombinant protein (Cell line A). Glucose addition threshold was < 3 g/L. This is an embodiment wherein 2 shear stress parameters are varied: The shear stress sensitivity device was operated according to Case 4, Fig. 10E. In Case 4 two shear stress parameters were varied from cultivation run to cultivation run, namely the duration of shear stress exposure was increased, the shear stress frequency was decreased, while the maximum shear stress was kept constant.

35 For all cultivation runs and tested stress conditions, the same perfusion process settings (same shear stress sensitivity device with the same conditions) were applied. All parameters and conditions were kept constant during all cultivation runs. Details of the cultivation process can be found in experimental part II.

40 The cell culture was continuously cycled in the shear stress sensitivity device, as explained earlier. The shear stress sensitivity device also contained a hollow fiber filter module as shown in Fig. 1B.

The results for the characteristic parameters of the cell cultivations over time are shown in Figs. 17A to 17D. Figs. 17A to 17D show the offline data of CHO cell perfusion cultures subjected to different

hydrodynamic shear stresses. The same perfusion process settings were used for all shear stress conditions tested.

No control cultivation process was performed, since only the individual correlations between different shear stress parameters were to be investigated. The cultivation with the setpoint medium bypass flow was used for orientation, since the experiment has already been carried out once in this way (cf. Figures 15A to 15D and 16A and 16B).

Figures 18A and 18B show the online data of the shear stress set-up, i.e. the values measured in the shear stress device itself during the cultivation process. These are the constant rotational speed in [rpm] of the magnetically levitated centrifugal pump (here: pump of type i30SU) (Fig. 18A) and the online measured varying bypass flow in [L/min] during perfusion cultivation (Fig. 18B). The corresponding set-up of the shear stress sensitivity device is shown in Figure 1B and is operated according to Case 4 shown in Figure 10E, i.e., with the hollow fiber filter module shown. The online signals are measured very frequently (every few seconds) and are thus displayed as lines. In order to be able to indicate a symbol in addition to the lines, every 350th data point was represented as a symbol. Figures 18A and 18B depict the actual rotational speed in Figure 18A and bypass flow in Figure 18B over time. The difference for the bypass flow between the low and high condition is ≈ 10 -fold (Figure 18B).

The adjusted rotational speed in [rpm] was calculated into a shear stress value (Pa) according to the equation indicated in Fig. 9C as already explained (step (4) of the process according to the invention, see Figs. 8A and 8B, and Figs. 9A, 9B and 9C). The shear stress value calculated with the equation indicated in Fig. 9C were as follows (see Fig. 18A):

Table 5:

Cultivation run No.	rotational speed [rpm]	shear stress value [Pa]	bypass flow [L/min]
1	6492	174	≈ 2.0
2	6492	174	≈ 0.5
3	6492	174	≈ 0.25

The results for the performance characteristics of the cell cultivation runs over time are shown in Figs. 17A to 17D. Therefore, in experiment 3, 3 runs of the cultivation process were performed, i.e. the cultivation process with the selected cells (here: CHO cells) was performed 3 times under the same conditions (perfusion) with the same parameters in the shear stress sensitivity device of Fig. 1B. The selected period of time for each cultivation run was 6 days. For each cultivation run, the time course for the selected characteristic parameters for cultivation was obtained as offline data. In experiment 3, the performance characteristics were relative viable cell density (VCD) (Fig. 17A) and viability (Fig. 17A), lactate dehydrogenase (LDH) activity in the supernatant (Fig. 17B), glucose concentration (Fig. 17C), and lactate concentration (Fig. 17D), each of which was determined over time as offline data. For each cultivation run, a curve was drawn for each performance characteristic over time, with the values for the respective performance characteristic plotted over time and for the respective shear stress value.

In detail, Figures 17A to 17D show the offline data of CHO cell perfusion cultures exposed to a high level of hydrodynamic stress (174 Pa) with variable bypass flow. Shear stress levels were kept constant by keeping the rotational speed in [rpm] constant at 6492 rpm while the bypass flow was varied by introducing tubes of variable length.

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The analysis of the figures gives the following picture:

Despite the constant maximum hydrodynamic shear stress of 174 Pa for all three cultivation runs it is visible that the culture with the highest bypass flow has the highest relative viable cell densities (VCDs), while the culture with the lowest bypass flow has the lowest relative viable cell densities (VCDs) (Figure 17A). This agrees with the metabolite concentrations, showing the lowest glucose and highest lactate concentrations for the culture with high bypass flow and vice versa for the culture with low bypass flow (Figures 17C and 17D). LDH activities are rather comparable (Figure 17B).

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The results in Figures 17A to 17D highlight, that the growth performance is not attributed to maximum hydrodynamic stress alone. According to Figure 10E (Case 4) and equation 1 to 2, the bypass flow influences the shear stress frequency ($f_{req.}$) as well as the duration of shear stress exposure (residence time/length of exposure) (t_{res}). The bypass flow correlates positively with shear stress frequency and negatively with duration of shear stress exposure (residence time).

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Since the cultivation with a low bypass flow shows the lowest and the cultivation with a high bypass flow shows the highest performance in terms of growth, it can be concluded that the duration of shear stress exposure (residence time/length of exposure) (t_{res}) has a stronger influence on performance when compared to the shear stress frequency ($f_{req.}$).

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It can therefore be derived from experiment 3 that 2 shear stress parameters can be varied at the same time and thus the parameter with the greater effect can be identified.

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Experiment 4

Effect of hydrodynamic shear stress during perfusion cultivation

The effect of hydrodynamic shear stress was investigated during perfusion cultures using a CHO cell line expressing a different recombinant protein (Cell line B) than in experiments 1 to 3. The rotational speed of the pump in [rpm] was increased to increase shear stress without controlling the bypass flow, which causes an increasing bypass flow with increasing rotational speed in [rpm]. However, an increasing bypass flow means that the shear stress frequency and the duration of shear stress exposure also changes, since the bypass flow correlates positively with the shear stress frequency and negatively with the duration of shear stress exposure. Therefore, in experiment 4 all 3 shear stress parameters are varied.

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For all cultivation runs and tested shear stress conditions, the same perfusion process settings (same shear stress sensitivity device with the same conditions) were applied. All parameters and conditions were kept constant during all cultivation runs. Only the selected shear stress parameters and the bypass flow change from cultivation run to cultivation run. The pump is a magnetically levitating centrifugal pump (here: PuraLev i30 SU, Levitronix, Switzerland). The cell culture was continuously circulated in the shear stress sensitivity device, as explained earlier. The shear stress sensitivity device also contains a hollow fiber filter module as shown in Fig. 1B. Details of the cultivation process can be found in experimental part II.

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5 The results for the performance characteristics of the cell cultivations over time are shown in Figs. 19A to 19D. Figs. 19A to 19D show the offline data of CHO cell perfusion cultures subjected to different hydrodynamic stresses. The same perfusion process settings were used for all shear stress conditions tested.

The control in Figures 19A to 19D represents a standard perfusion set-up. The feedback-controlled bypass flow is 0.2 L/min.

10 Figures 20A and 20B show the online data of the shear stress set-up with varied rotational speed in [rpm] and bypass flow during perfusion cultivation. The rotational speed of the magnetically levitating centrifugal pump i30SU is shown in Figure 20A and the online measured bypass flow in Figure 20B. The corresponding set-up of the shear stress device is depicted in Figure 1B. The rotational speed in [rpm] was increased to increase shear stress without controlling the bypass flow, which causes an increasing bypass flow with
15 increasing rotational speed in [rpm]. The online signals are measured very frequently (every few seconds) and are thus displayed as lines. In order to be able to indicate a symbol in addition to the lines, every 350th data point was represented as a symbol.

20 The adjusted rotational speeds in [rpm] were calculated into shear stress values (Pa) according to the equation indicated in Fig. 9C as already explained (step (4) of the process according to the invention, see Figs. 8A and 8B, and Figs. 9A, 9B and 9C). The shear stress values calculated with the equation indicated in Fig. 9C were as follows (see Fig. 20A):

Table 6:

cultivation run No.	rotational speed [rpm]	shear stress value [Pa]	bypass flow [L/min]
Control	≈3000-3300	≈32-39	0.21
1	5050	100	≈0.52-0.46
2	6490	174	≈0.76-0.69
3	7660	251	≈0.94-0.85

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The analysis of the figures gives the following picture:
Figures 19A to 19D show the offline data of CHO cell perfusion cultures exposed to various levels of hydrodynamic shear stress with varying rotational speed and bypass flow. In experiment 4, the performance characteristics were the relative viable cell density (VCD) and viability (Figure 19A), lactate dehydrogenase
30 (LDH) activity in the supernatant (Figure 19B), glucose concentration (Figure 19C) and lactate concentration (Figure 19D).

For all tested stress conditions, the same perfusion process settings were applied. Relative viable cell density (VCD) reveals decreased growth and viability with a maximum hydrodynamic shear stress of 174
35 and 251 Pa compared to the control are shown in Figure 19A. The cultivation with 100 Pa shows a comparable relative VCD and slightly reduced viabilities and lactate concentrations as well as slightly higher values for glucose and LDH activity (Figures 19A to 19D). These effects are more pronounced with

increasing hydrodynamic shear stress levels of 174 and 251 Pa. The shear stress limit in the present experiment would therefore be in the range between 100 and 174 Pa.

Figures 20A and 20B depict the online data of rotational speed in Figure 20A and bypass flow in Figure 20B showing that with increasing rotational speed (rpm) also the bypass flow is increased. This is because the experimental set-up with elevated hydrodynamic shear stress (100, 174 and 251 Pa) is similar to the control set-up. If no experimental adjustments are made to increase the pressure drop, bypass flow positively correlates with rotational speed in [rpm] (cf. Figures 4A and 4B). Applying this set-up requires no adjustments to a standard perfusion set-up, and thus, represents the simplest experimental approach to investigate the effect of hydrodynamic shear stress. However, it should be pointed out again that the bypass flow is related to shear stress frequency (*freq.*) (equation 1) and duration of shear stress exposure (residence time/length of exposure) (*t_{res}*) (equation 2). Thus, the effects shown in Figures 19A-19D cannot be considered just an effect of the maximum hydrodynamic shear stress, as every shear stress parameter was changed.

These data highlight that the set-up to measure the shear stress sensitivity is also applicable to different CHO cell lines expressing a completely different recombinant protein. It further shows that each CHO cell line has an individual sensitivity towards similar levels of stress. The here made invention, however, is not only limited to CHO cells. Any type of living organism growing in a suspension bioreactor can be investigated in terms of shear stress sensitivity. Besides the maximum hydrodynamic stress this invention allows to independently investigate shear stress parameters, such as duration and frequency of shear stress exposure (Figures 10A to 10E). These parameters were shown to influence the cultivation performance and are essentially relevant during bioprocess scale-up.

Experimental part II

1. Set-up shear stress sensitivity device

Experimental set-up for fed-batch, batch or perfusion cultivation:

A tank (e.g. 3L tank) is connected to a tube (e.g. silicone tube 10 mm x 6 mm x 2 mm with a length of 100 mm, connection e.g. CPC coupling). The tube is then connected to the suction side of the pump (e.g. via AseptiQuik S 3/8"). The pump is a magnetically levitating centrifugal pump (PuraLev i30 SU, Levitronix, Switzerland). On the discharge side, the pump is again connected (e.g. via AseptiQuik S 3/8") to a tube (e.g. silicone tube 10 mm x 6 mm x 2 mm with a length of 100 mm). This tube is then connected via CPC coupling with an additional tube of variable length. Hereby the length and diameter can be adjusted depending on the operating conditions. The tube is either connected directly to the bioreactor (fed-batch or batch mode) or to a hollow fiber filter module. A hollow fiber filter module which may be used is e.g. Repligen S02-E65U-07. A flowmeter (e.g. LFSC-i10X) was installed between tank and hollow fiber filter module or variable tube respectively. Pressure sensors may be used to determine the pressure drop over the hollow fiber filter module and the tubes of variable length.

2. Bioreactor cultivation conditions

Cell expansion

The seed train was mainly conducted in shake flasks following a standard protocol to assure optimal cell growth (temperature = 36.5° C). Before the N-1 stage at least one pre-stage cultivation (N-2) was performed under controlled conditions of pH (6.7 – 7.2) and dissolved oxygen concentration (50%). Cultures were split every ≈ 3 days.

Fed-batch cultivation

The fed-batch bioreactor cultivations were performed in a controlled 3 L bench-top bioreactor with a CHO-K1 GS cell line (Cell line A) and a proprietary chemically defined basal and feed medium. The concentrated feed medium was continuously added by a feed pump. Feed addition was started at day 2 with a feeding rate of 30 mL/L/d. The seeding cell density was $< 1.0 \times 10^6$ cells/mL, while the dissolved oxygen concentration was controlled at 50% with pure oxygen gassing through a submerged sparger. The standard process format has a pH range from 6.7 – 7.2 and a constant specific power input (P/V). Temperature was maintained at 35.0° C. Glucose was fed on demand once the measured concentration was < 3 g/L. Fed-batch processes were conducted for 14 days.

Perfusion cultivation

The perfusion bioreactor cultivations were performed in a controlled 3 L bench-top bioreactor with CHO-K1 GS cell lines (Cell line A and B) and a proprietary chemically defined basal medium. The perfusion system consisted of a centrifugal pump (PuraLev i30 SU, Levitronix, Switzerland), a clamp-on flow meter, a tangential flow filtration (TFF) membrane (hollow fiber filter module) and scales to control the reactor weight and perfusion rates. Perfusion rates were adjusted every 24 h based on pre-defined rates. As perfusion medium the chemically defined basal medium was used. The recirculation flow or the rotational speed of the centrifugal pump was adjusted according to the experimental requirements. The seeding cell density was $< 2.0 \times 10^6$ cells/mL. Dissolved oxygen concentration, pH range and specific power input (P/V) was similar between fed-batch and perfusion processes. Temperature was maintained at 36.5° C. Glucose was added on demand once the measured concentration was < 3 g/L. Perfusion processes were conducted for 5 to 6 days.

Offline sample analysis

Bioreactor samples (fed-batch and perfusion) were analyzed to determine the viable cell density (VCD), cell viability, glucose concentration, lactate concentration and lactate dehydrogenase (LDH) activity. Viable cell density (VCD) and cell viability were analyzed using the Vi-CELL BLU (Beckman Coulter GmbH, USA) in combination with trypan blue and Accumax™ (Innovative Cell Technologies, USA). The concentration of glucose and lactate as well as LDH activity was determined with the Konelab Prime 60i (Thermo Fisher Scientific Inc., USA).

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List of reference signs

	100	shear sensitivity device
	110	bioreactor
5	114	outlet of the bioreactor
	115	liquid culture medium
	116	inlet of the bioreactor
	118	stirrer
	125a, 125b, 125d1,	
10	125d2, 125d3	tubes
	125c	variable tube
	130	pump
	131	pump housing
	132	pump head
15	133	impeller
	134	a rotating magnet ring
	135	motor/bearing winding
	136	motor/bearing stator
	125c1, 125c2	reducer
20	140	flowmeter
	150	hollow fiber filter module
	A, B, C, D, E	arrows

Legends to the figures:**Figs. 1A and 1B:**

- Arrow A, Arrow B flow direction of the cell culture in the loop of the shear sensitivity device
 5 Arrow C permeate flow

Fig. 2:

- T_{max} maximum shear stress
 v_{vm} volume of air per volume of culture medium per minute
 10

Figs. 3A and 4A:

- ID inner diameter of a tube
 dP pressure drop

Figs. 3B and 4B:

- HF short short hollow fiber filter module
 HF long long hollow fiber filter module
 dP pressure drop
 15

Fig. 5:

- R_g radius of gyration
 R² coefficient of determination
 ID inner diameter of a tube
 HF short short hollow fiber filter module
 20 HF long long hollow fiber filter module
 25

Fig. 6:

- Arrow D entry into the pump
 Arrow E exit from the pump
 30

Fig. 7:

- u(r) flow rate [m/s]
 μ dynamic viscosity [Pa s]
 Δp pressure difference [Pa/m]
 35 R radius pipe [m]
 r radial distance [m]
 whereby $0 \leq r \leq R$.

Fig. 8A:

- 40 NPs nanoparticles
 PMMA poly(methyl methacrylate)
 R_g radius of gyration
 T_{max} maximum shear stress
 R² coefficient of determination

Fig. 8B:

	NPs	nanoparticles
	PMMA	poly(methyl methacrylate)
5	R _g	radius of gyration
	T _{max}	maximum shear stress
	SSD	shear stress sensitivity device
	R ²	coefficient of determination

10 Figs. 9A and 9B:

	R _g	radius of gyration
	R ²	coefficient of determination

Figs. 10A to 10E:

15	rpm	rotational speed of the pump
	freq.	shear stress frequency
	t _{res}	duration of shear stress exposure (residence time of the cell culture within the pump)

20 Figs. 11A, 11B, 12A and 12B:

	Curve P:	production-scale bioreactor
	Curve S:	small-scale bioreactor
	Curve A	small-scale bioreactor/shear stress sensitivity device: rpm A
	Curve B	small-scale bioreactor/shear stress sensitivity device: rpm B
25	Curve C	small-scale bioreactor/shear stress sensitivity device: rpm C

Figs. 13A, 15A, 17A, 19A

relative VCD	relative viable cell density
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30 Figs. 13C, 15B, 17B, 19B:

LDH	lactate dehydrogenase
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Claims

1. A method for determining the shear stress sensitivity of cells in a shear stress sensitivity device (100) during a cultivation process comprising a liquid culture medium (115), which comprises the following steps:
- 5
- (1) providing a shear stress sensitivity device (100) comprising
- a bioreactor (110),
 - a closed loop system connected with and located outside the bioreactor (110);
 - 10 - a pump (130) placed in the loop system, the pump (130) being a kinetic pump and
 - means to control the hydraulic resistance in the loop system;
- (2) selecting cells, liquid culture medium (115), cultivation mode and cultivation conditions for the cultivation process to be performed;
- 15
- (3) selecting one, two or three shear stress parameters to be investigated from the group consisting of:
- maximum shear stress represented by a rotational speed of the pump;
 - duration of shear stress exposure represented by a residence time of the cell culture within the
 - 20 pump (130); and/or
 - shear stress frequency represented by a number of passages of the cell culture comprising the liquid culture medium (115) and cells through the loop system per time;
- (4) characterizing the shear stress sensitivity device (100) by determining the shear stress values that occur at varying maximum shear stress in the shear stress sensitivity device (100) by using a calibration method or by using computer calculations and simulations;
- 25
- (5) performing one, two, three or more cultivation runs of the same cultivation process of step (2) in the shear stress sensitivity device over the same selected period of time, by successively increasing or decreasing one, two or three shear stress parameters in each subsequent cultivation run, and measuring one or more performance characteristics of the cells during each run of the cultivation process and generating a curve for each performance characteristic in each run over time;
- 30
- (6) performing a control cultivation process in the shear stress sensitivity device (100) for each performance characteristic using the same cultivation process as in step (5), wherein a lower maximum shear stress than in step (5) is used and generating a control curve for each performance characteristic over time;
- 35
- (7) selecting the curve from the set of curves in step (5) for a performance characteristic that is closest to the control curve for said performance characteristic of step (6) which represents the shear stress sensitivity of the cells and constitute the shear stress limit for the cells;
- 40

whereby it is ensured that the maximum shear stress occurring in the closed loop system is higher than the maximum shear stress occurring in the bioreactor (110).

2. The method according to claim 1,
characterized in that
the cultivation mode in step (2) is selected from batch or fed-batch
5 or
the cultivation mode in step (2) is selected from continuous cultivation mode, particularly perfusion, whereby
a cell retaining means is then additionally provided in the shear stress sensitivity device (100), the cell
retaining means is particularly selected from a hollow fibre filter module (150).
- 10 3. The method according to claim 1 or 2,
characterized in that
the means to control the hydraulic resistance in the loop system are selected from a variable tube (125c)
having a variable length and/or a variable diameter, reducers (125c1, 125c2) for the variable tube, valves,
such as pinch valves, and variable winding of the variable tube (125c).
- 15 4. The method according to any of the preceding claims,
characterized in that
the calibration method in step (4) is selected from a method wherein a shear stress-dependent quantity is
determined and correlated with determined occurring maximum shear stress values, in particular the shear
20 stress-dependent quantity is the aggregate size of shear sensitive aggregates.
5. The method according to claim 4,
characterized in that
the same shear stress-dependent quantity is used in the calibration method and in the characterization of
25 the shear stress sensitivity device, particularly the shear stress-dependent quantity is the aggregate size,
more particularly the radius of gyration of particle aggregates, especially the aggregates are selected from
poly(methyl methacrylate) nanoparticle aggregates or Blauton polymer floc system.
6. The method according to any of the preceding claims,
30 characterized in that
the calibration method in step (4) is performed as follows:
- the aggregates size of shear sensitive aggregates is selected as shear stress-dependent quantity;
- a calibration method using a calibration device is selected which uses the aggregate sizes and
correlates the aggregate sizes with determined maximum shear stress values;
35 - reworking the selected calibration method and measuring the aggregates sizes of the aggregates;
- generating a calibration curve in which the measured aggregates size values are plotted against
the determined maximum shear stress values,

particularly the aggregate size is selected to be the radius of gyration of particle aggregates and the
40 aggregates are selected from poly(methyl methacrylate) nanoparticle aggregates or Blauton polymer floc
system.
7. The method according to claim 6,
characterized in that

the characterization in step (4) is performed as follows:

- selecting the same calibration method as used in claim 6;
 - reworking the selected calibration method using the shear stress sensitivity device (100) instead of the calibration device, in particular with the same aggregates as used in the calibration method;
 - 5 - performing continuous recirculating runs of the aggregates in the shear stress sensitivity device (100) by the pump (130) until the aggregates no longer change their size by increasing or decreasing the rotational speed of the pump in each subsequent run, and measuring the aggregate size after each run;
 - generating a curve, wherein the measured aggregate size is plotted against the rotational speed of the pump (130) and
 - 10 - converting the measured aggregates size to maximum shear stress values using the calibration curve of the calibration method or using the calibration curve of claim 6;
- in particular the aggregate size is selected to be the radius of gyration of particle aggregates and the aggregates are selected from poly(methyl methacrylate) nanoparticle aggregates or Blauton polymer floc system.

15

8. The method according to any of the preceding claims, characterized in that

the cultivation process in step (5) is performed as follows:

- performing a 1st cultivation process in the form of a 1st cultivation run over a 1st period of time,
- 20 performing a 2nd cultivation process in the form of a 2nd cultivation run over a 2nd period of time,
- performing a 3rd cultivation process in the form of a 3rd cultivation run over a 3rd period of time,
- optionally performing further cultivation processes in the form of further runs over further periods of time,
- wherein all periods of time are selected to be of equal length,
- whereby in each cultivation run the cell culture comprising the liquid culture medium (115) and the cells are
- 25 continuously circulated in the loop system of the shear stress sensitivity device (100),
- whereby the selected shear stress parameter(s) is(are) set to a predetermined value in the 1st cultivation run and kept constant during the 1st cultivation run, and for each subsequent cultivation run each selected shear stress parameter is set to a higher or lower value from cultivation run to cultivation run.

- 30 9. The method according to any of the preceding claims, characterized in that

the control cultivation process in step (6) is performed

- in case the cultivation mode is batch or fed-batch in the same shear stress sensitivity device (100) as used in step (5) but without loop system and without pump (130);
- 35 - in case the cultivation mode is continuous mode, especially perfusion, in the same shear stress sensitivity device (100) as used in step (5), wherein the maximum shear stress is adjusted lower as used in step (5), particularly as low as possible.

- 40 10. The method according to any of the preceding claims, characterized in that one, two, three or more of the following features are selected:

- the kinetic pump (130) is selected from a centrifugal pump, in particular a magnetically levitating centrifugal pump;
- the loop system connects an outlet (114), particularly located in the lower part, of the bioreactor (110) with an inlet (116), particularly located in the head part, of the bioreactor (110);

- the means to control the hydraulic resistance is placed in the loop system downstream of the kinetic pump (130);
- the pump (130) is placed in the loop system downstream of the outlet (114) from the bioreactor (110);
- 5 - the cell retaining means is arranged in the loop system between the means to control the hydraulic resistance and the bioreactor (110);
- a flowmeter (140) is placed in the loop system, preferably between the bioreactor (110) and the variable tube (125c) or between the bioreactor (110) and the hollow fibre filter module (150);
- one or more pressure gauges are provided in the loop system;
- 10 - the bioreactor (110) comprises a stirrer (118);
- in step (5), in each cultivation run the performance characteristic(s) for the cell culture are measured on a regularly basis;
- in step (6), the performance characteristic(s) for the control cell culture are measured on a regularly basis;
- 15 - in step (7), an analysis of the shear stress behavior of the cells is performed with the obtained curve(s) for one or more performance characteristics, in particular in comparison to the control curve or without a control curve;
- a kinetic pump (130) is used that has a replaceable pump head (132) that may be replaced after all cultivation runs of a cell cultivation process have been performed;
- 20 - in step (5) and/or step (6) the period of time is at least 1 day, in particular up to 30 days;
- the characterization of the shear stress sensitivity device in step (4) is performed by reworking a calibration method, in particular by reconstructing the device of the calibration method and thus reworking the calibration method with the reconstructed device;
- if the calibration method in step (4) is selected from a method wherein a shear stress-dependent
- 25 quantity is determined and the aggregate size is selected as shear stress-dependent quantity, the same batch of aggregates is used for both the calibration and the characterization methods;
- only one, two or three shear stress parameters are varied in step (5) while all other parameters of the shear stress sensitivity device (100) are kept constant; and
- only one, two or three shear stress parameters and in addition the bypass flow are varied in step
- 30 (5) while all other parameters of the shear stress sensitivity device (100) are kept constant.

11. The method according to any of the preceding claims, characterized in that

the one or more performance characteristics for the cultivation process or control cultivation process are

35 selected from parameters which change over time during cell cultivation, in particular from the group consisting of the viable cell density, the viability, the glucose concentration, the lactate concentration, the LDH concentration, the titer, growth, productivity of the cell culture, and product quality.

12. The method according to any of the preceding claims,

40 characterized in that

one of the following modifications according to case 1 to case 4 is selected for the variation of one, two or three shear stress parameters in the shear stress sensitivity device (100) over all runs of the cultivation process:

- case 1 increasing the maximum shear stress from cultivation run to cultivation run while keeping constant the duration of shear stress exposure, and shear stress frequency in all cultivation runs;
- or
- 5 case 2 increasing the duration of shear stress exposure from cultivation run to cultivation run while keeping constant the maximum shear stress and shear stress frequency in all cultivation runs;
- or
- 10 case 3 increasing the shear stress frequency from cultivation run to cultivation run while keeping constant the maximum shear stress and the duration of shear stress exposure in all cultivation runs;
- or
- 15 case 4 increasing the duration of shear stress exposure from cultivation run to cultivation run while keeping the maximum shear stress constant and decreasing the shear stress frequency from cultivation run to cultivation;

whereby particularly one or more of the following conditions are selected to control one, two or three shear stress parameters:

- Keeping the maximum shear stress constant in the form of the rotational speed by keeping the rotational speed of the pump (130) at the same value or
- Increasing or decreasing the maximum shear stress in the form of the rotational speed by setting the rotational speed of the pump (130) higher or lower;
- Keeping the duration of shear stress exposure constant if the rotational speed of the pump (130) is increased or decreased by using the means to control hydrodynamic resistance, particularly by varying the length and/or diameter of the variable tube (125c), in the loop system of the shear stress sensitivity device (100);
- Increasing the duration of shear stress exposure if the rotational speed of the pump (130) is kept constant by adding additional pumps with the same rotational speed in series in the loop system;
- Increasing or decreasing the duration of stress exposure if the rotational speed of the pump (130) is kept constant by using the means to control hydrodynamic resistance, particularly by varying the length and/or diameter of the variable tube (125c), in the loop system of the shear stress sensitivity device (100);
- Keeping the shear stress frequency constant by using the means to control hydrodynamic resistance, particularly by varying the tube length and/or the diameter of the variable tube (125c), in the loop system of the shear stress sensitivity device (100);
- Increasing the shear stress frequency if the rotational speed of the pump (130) is kept constant by adding one or more external loops to the bioreactor (110) or by adjusting the working volume of the bioreactor (110); and/or
- Increasing the shear stress frequency if the rotational speed of the pump (130) is kept constant by providing one or more additional pumps, which are connected in parallel and all pumps operate with the same value of the rotational speed.

13. The method according to any of the preceding claims, characterized in that

step (2) is replaced by step (2'): performing a cultivation process or using a cultivation process already performed and providing one or more cultivation process curves of the cultivation process, wherein one or more performance characteristics of the cells are plotted over a selected period of time;

- 5 step (4) is replaced by step (4'): performing a control cultivation process or using a control cultivation process already performed and providing one or more cultivation process curves of the control cultivation process, wherein one or more performance characteristics of the cells are plotted over a selected period of time, wherein the control cultivation process is the same cultivation process as in step (2'), but wherein a lower shear stress intensity is used than in the cultivation process and the same performance characteristics of the cells and the same period of time is used as in step (2');

- 15 step (5) is replaced by step (5'): performing one, two, three or more control cultivation runs of the control cultivation process of step (4') in the shear stress sensitivity device over the same selected period of time, by successively increasing or decreasing one, two or three shear stress parameters in each subsequent control cultivation run, wherein each subsequent control cultivation run is modified by varying the shear stress parameter(s) toward the selected cultivation process of step (2') and measuring one or more performance characteristics of the cells during each run of the control cultivation process and generating a curve for each performance characteristic in each run over time;

- 20 step (6) is replaced by step (6'): selecting the curve from the set of curves in step (5') for a performance characteristic that is closest to the cultivation process curve for said performance characteristic;

- 25 step (7) is replaced by step (7'): determining how the shear stress intensity needs to be changed to move from the control cultivation process to the cultivation process by comparing the obtained curves;

- whereby it is ensured that the shear stress intensity occurring in the cultivation process is higher than the shear stress intensity occurring in the control cultivation process.

14. The method according to claim 13,
30 characterized in that
the cultivation process in step (2') is selected to be carried out on a larger scale than the control cultivation process in step (4').

15. A process for culturing cells in a liquid culture medium, wherein the shear stress sensitivity of the cells determined in the method of any one of claims 1 to 12 is used in the same cultivation process but performed on a larger or smaller scale.

16. A process for culturing eukaryotic or prokaryotic cells in a liquid culture medium in a bioreactor (110) and performing a method for determining the shear stress sensitivity of cells in a shear stress sensitivity device (100) during the cultivation process according to any one of claims 1 to 14.

17. A process for producing a recombinant protein, the process comprising the steps of:
step I) culturing eukaryotic or prokaryotic cells expressing a recombinant protein in a liquid culture medium in cell culture in a bioreactor (110);

step II) harvesting the recombinant protein;
step III) purifying the recombinant protein;
wherein performing in step I) a method for determining the shear stress sensitivity of the cells in a shear stress sensitivity device (100) during the cultivation process according to any one of claims 1 to 14.

5

18. Use of a device (100) comprising

- a bioreactor (110),
- a closed loop system connected with and located outside the bioreactor (110);
- a pump (130) placed in the loop system, the pump (130) being a kinetic pump and

10 - means to control the hydraulic resistance in the loop system;

as a shear stress sensitivity device (100),

wherein the device (100) has no nozzle,

for culturing cells in a liquid culture medium (115), wherein a defined shear stress is set during the culturing.

15 19. Use of the device (100) according to claim 18,
characterized in that

- as the defined shear stress, a maximum shear stress value is used which is obtained by varying shear stress parameter(s) in the device (100), the maximum shear stress value being determined on the basis of
- 20 a shear stress-dependent physical quantity, in particular the shear stress-dependent physical quantity being the aggregate size of shear-sensitive aggregates;

or

25 - as the defined shear stress, relative shear stress levels are used which result from two runs of a cultivation process in which different shear stress levels are present, one of the runs is used as a control run and the control run is repeated and modified by varying the shear stress parameter(s) until it runs like the other run.

20. Use of the device (100) according to claim 19,

30 characterized in that

the maximum shear stress value being determined on the basis of a shear stress-dependent physical quantity, in that

- the shear stress dependent physical quantity is exposed to defined, predetermined conditions, whereby the physical quantity varies according to the magnitude of the maximum shear stress;

35 - the shear stress dependent physical quantity is exposed to unknown conditions in the device (100), whereby the physical quantity varies according to the magnitude of the maximum shear stress;

- the magnitude of the maximum shear stress value in the device (100) can be inferred from the physical quantity obtained under the defined, predetermined conditions, whereby the same magnitude for the physical quantity means that the same maximum shear stress value is present.

40