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Current Opinion in Chemical Engineering

Ultra scale-down approaches to enhance the creation of bioprocesses at scale: impacts of process shear stress and early recovery stages

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The sensitivity of biological materials to shear stress conditions encountered during large-scale bioprocessing makes successful scale up from the bench challenging. Ultra scale-down technologies seek to use just millilitre quantities to enhance our understanding of the impact of the process environment as a basis for process optimisation. They can help speed translation of new biological discoveries to market and reduce risks encountered in scale up. They are important both as process discovery tools and as preparative tools to yield material for study of subsequent stages. In this review the focus is on the early recovery stages post fermentation or cell culture and in particular the use of continuous-flow and dead-end centrifugation integrated with preparative stages (e.g. flocculation) and subsequent depth filtration. Examples range from therapeutic antibodies, to rationally engineered (synthetic biology) host strains, to stem cells for therapy.

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Current Opinion in Chemical Engineering 2016, **14**:150–157

This review comes from a themed issue on **Biotechnology and bioprocess engineering**

Edited by **Eleftherios Terry Papoutsakis** and **Nigel J Titchener-Hooker**

For a complete overview see the Issue and the Editorial

Available online 26th October 2016

http://dx.doi.org/10.1016/j.coche.2016.09.012

Bench-scale technologies for application of process-scale shear stress

All stages of an industrial sequence for the preparation of a biological therapeutic involve exposure of the material to various forms of stress, that is, mechanical or different types of hydrodynamic stress. In this review we are concerned with hydrodynamic stress as commonly occurs during flow within and through process equipment. In some cases the stress applied is similar at bench and industrial scale but often the stress applied at full scale far exceeds that at bench scale. Notable examples include pump transfers (e.g. [1]), flow through valves, feed within continuous-flow high speed centrifuges [2] and the discharge of supernatant and sediment phases [3]. There are also examples of high stress occurring at bench scale not reproduced at full scale, for example, flow through pipettes [4•], mixing using surface mounted (grinding) magnetic stirrers.

A key objective of ultra scale-down technologies (Figure 1) is to determine if the biological material is susceptible to applied process shear stress, and if so to quantify the effect of shear stress on the material and on the performance of subsequent unit operations, for example, filtration or chromatography. Two geometries are commonly used at bench scale to expose process material to hydrodynamic shear stress:

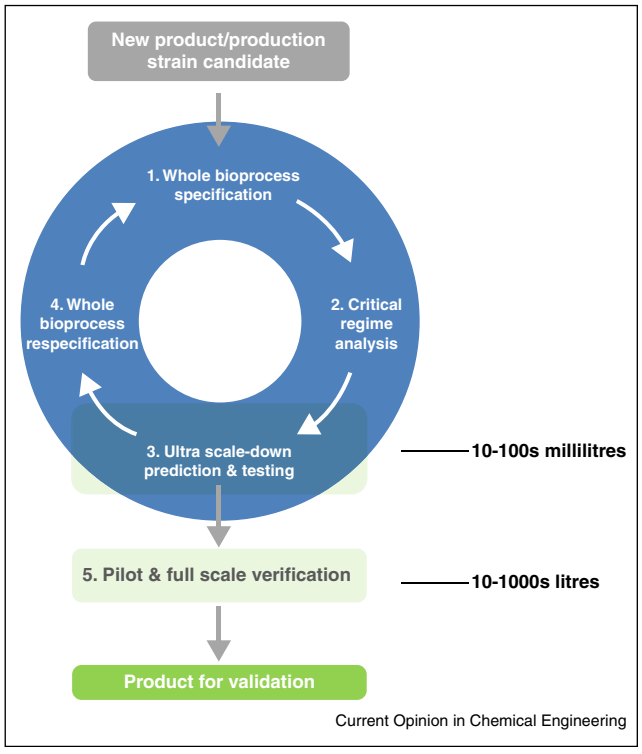
(a) Capillary flow — here, exposure to stress occurs on entry to and exit from the capillary, flow through the capillary and impact of the discharged jet on the collection surface. The flow in the capillary progresses from undeveloped to laminar rather than turbulent flow as opposed to turbulent flow in many large-scale applications.
(b) Rotating disc flow — here, flow occurs over the rotating disc surface housed within a chamber (Figure 2). The greatest stress occurs at the disc tip with both rotational flow and radial flow impacting on the chamber surface. The flow is generally highly turbulent but heterogeneous in nature.

Examples of materials studied include for capillary flow: mammalian cells (e.g. [5•,6,7]) and cells for therapy [8,9]; and for rotational flow: precipitates (e.g. [10]), mammalian cells (e.g. [11•]), cells for therapy (e.g. [12]), adjuvants (e.g. [13]), flocs (e.g. [14]), phages (e.g. [15]) and antibodies (e.g. [16,17]).

Bench-scale technologies for centrifuge ultra-scale down

All bioprocess stages pose particular challenges in achieving successful scale-down in a conventional sense, that is, maintaining geometric similarity. For example, for packed bed chromatography the need to maintain the same bed height and superficial velocity means that scale down is ultimately limited by the column diameter beyond which it is necessary to reduce column height and hence residence time for binding or superficial flow velocity. Similarly for membrane separation when it is

Figure 1



The role of ultra scale-down in speeding bioprocess development. Process characterisation is needed to evaluate manufacturability of a new product or to evaluate the process impact of introducing a new host, that is, through screening or rational engineering (synthetic biology) approaches. (1) Specification of whole bioprocess from fermentation/cell culture to formulation. (2) Identification and characterisation of critical regimes, for example, of high shear stress, long hold times, hostile interfaces, etc. (3) Use of ultra scale-down methods to prepare material and for testing of sensitivity to critical regimes and impact on rest of process. (4) Use of new insight to respecify process sequence, for example, remove regimes resulting in product damage. (5) With increased confidence in ability of ultra scale-down methods to predict pilot and full-scale operations focus on process refinement rather than process reinvention.

no longer possible to maintain the same membrane length and hence profile of transmembrane pressure drops. However, the translation from small (ca 10s mL) bench scale (where scale down rules cannot be followed) to pilot scale is eased by the availability of large (ca 100s mL) capacity bench-scale devices which can keep to scale down rules [18–20]. Such devices are not available for pilot-scale continuous-flow centrifugation and, here, scale down requires a major change in geometry for operation at scales of less than 1000s mL.

The classical approach for scale down of full-scale (*FS*) centrifugation is based on the definition of an equivalent area of a gravity settling pond, Σ_{FS}. Here, the geometry and rotational speed of a centrifuge is used to compute the settling of particles during suspension flow through the centrifuge. A characteristic clarification performance is used to define Σ_{FS} for the centrifuge. This theoretical analysis needs to be accompanied by an experimental study to help characterise non-idealities in flow, for example, due to turbulence or due to particle resuspension. Within this review this calibration factor is incorporated within the value of Σ_{FS}. On this basis, the centrifuge performance is simply characterised by the ratio of flow rate, Q_{FS}, to settling area. While this does not allow scaling based on dewatering or on sediment capacity, it does provide a useful basis for clarification, the main function of many centrifugation stages during recovery.

The translation from a full-scale continuous-flow centrifuge to a bench-scale batch test tube centrifuge is a considerable challenge. The equivalent settling area, Σ_{USD}, of a test tube housed in a centrifuge head is defined in terms of the inner and outer radii of the suspension (R_1 and R_2) and the rotational speed, N, and using the same separating criteria as for the full-scale centrifuge. The suspension volume, V_{USD}, and the spin time, t_{USD}, give the equivalent flow rate such that we have the following expressions:

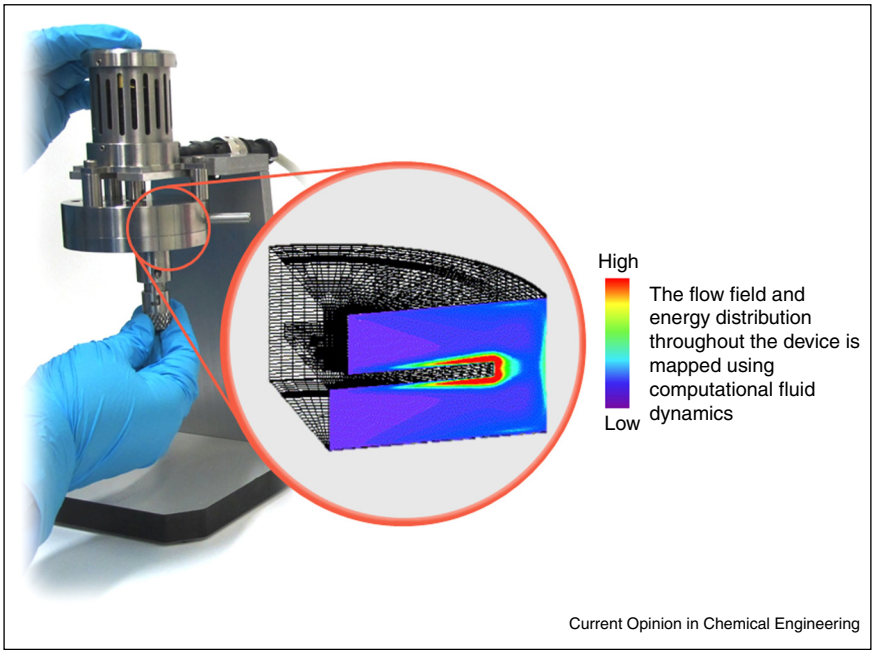
$$\sum_{USD} = \frac{V_{USD}(2\pi N)^2}{6g\ln(2R_2/(R_2+R_1))}$$

$$S = f\left(\frac{V_{USD}}{t_{USD}\Sigma_{USD}}\right) = f\left(\frac{Q_{FS}}{\Sigma_{FS}}\right)$$

where S is solids remaining in supernatant. Typical values of $(V_{USD}/t_{USD}\Sigma_{USD})$ range from 1×10^{-9} m^3 m^{-2} s^{-1} to mimic a high speed centrifuge operating at low flow rate to 100×10^{-9} m^3 m^{-2} s^{-1} to mimic a high speed centrifuge operating at high flow rate, the upper limit to recover low density difference 0.1–100 μm sized biological particles. Typical values of Σ_{USD} are 0.01–0.5 m^2, typical values of Σ_{FS} for pilot-scale centrifuges are 500–5000 m^2.

Careful attention is needed to make sure the acceleration and deceleration phases of bench-scale centrifuge operation do not unduly affect the extent of clarification (see [21] for correction factors) and that supernatant withdrawal does not lead to resuspension of the often loosely compacted sediment [22]. However, the greatest challenge is the nature of the feed to a continuous centrifuge and the impact of process shear stress. Incoming material is subject to considerable acceleration forces and estimated maximum energy dissipation rates range from 45 W/mL to 530 W/mL [2,14]. These are considerably higher stresses than those which occur in preceding stages such as reactors for cell growth, or for cell lysate, precipitate, floc or crystal preparation and are probably of similar orders of magnitude as occur in devices used for mechanical cell disruption (high pressure or bead mill homogenisers). The design of

Figure 2



An ultra representation of energy dissipation rate a ratescale-down approach to investigating process material shear sensitivity at the laboratory scale. This is achieved using a rotating disc device (kompAs^{TM}) which produces defined levels of hydrodynamic shear stress (energy dissipation rate: 1–1000 W mL^{-1}), comparable to those found in industrial equipment such as centrifuges or pumps. The total volume of process material required (20 mL) is sufficient for further studies such as USD centrifugation and/or USD filtration as well as analytical measurements of the product and impurities. Note that the colour scale is a logarithmic representation of energy dissipation rate.

the feed zone to the centrifuge determines the level of shear stress, for example, hermetic or hydrohermetic designs to eliminate or minimise the air core in the entry feed zone allow more gentle acceleration of the feed suspension. It is important to determine if this is a critical design feature for the material being processed as results of primary recovery operation could vary [10,11•].

Other sources of stress can include the discharge of the supernatant that can be via a centripetal pump into a pipe or by high velocity flow over a weir into a capture vessel. The discharge of the solids can either be via intermittent high velocity nozzle discharge [3] or continuous nozzle discharge at a velocity determined by the centrifuge design, for example, discharge at the outer bowl [23] or via the bowl axis with a relationship determined by the extent of dewatering achieved [24,25]. The resuspension of high viscosity sediment can also be a high stress process especially via manual pipetting at bench scale [4•].

The relative importance of these various stress effects is determined by the material being processed and the product form/location, for example, supernatant or sediment and the place of the centrifuge operation in the bioprocess sequence. At present, in the absence of a generic design procedure, this is best studied via particular examples of process sequences.

Ultra scale-down studies of bioprocess sequences

A general overview of scale down of biochemical engineering options has been provided elsewhere [26]; the focus here is on use of ultra scale-down and the enhanced bioprocess understanding which is gained in particular downstream processing sequences in the early recovery stages.

Interaction of homogenisation and centrifugation

One route for the recovery of intracellular products is via high pressure homogenisation followed by centrifugal removal of the cell debris. Adaptive focused acoustics has been used to mimic the high pressure homogenisation of recombinant *Escherichia coli* for the release of an antibody fragment and the formation of cell debris of specific size distribution as well as the release and disruption of nucleic acids [27]. A homogeniser operating on a recycle loop followed by a disc stack centrifuge for debris removal was successfully mimicked by a combination of bench-scale adaptive focused acoustics and ultra scale-down centrifugation (as described above) to demonstrate how challenging is the removal of micronized cell debris formed from the need for multiple recycles to achieve near complete protein product release [28]. The impact has also been demonstrated of cell wall weakening during extended fermentation leading to reduced centrifuge

clarification performance [29]. Recently, synthetic biology approaches have been studied using ultra scale-down methods to rationally engineer cells with improved characteristics for bioprocessing. In one example, the use of a strain engineered with an inducible nuclease has been shown using to enhance the processability of recombinant *E. coli* homogenates [30]. Similarly, ultra scale-down methods predicted the effect of the micellar state of lipids and their removal to avoid fouling for hydrophobic resins [31].

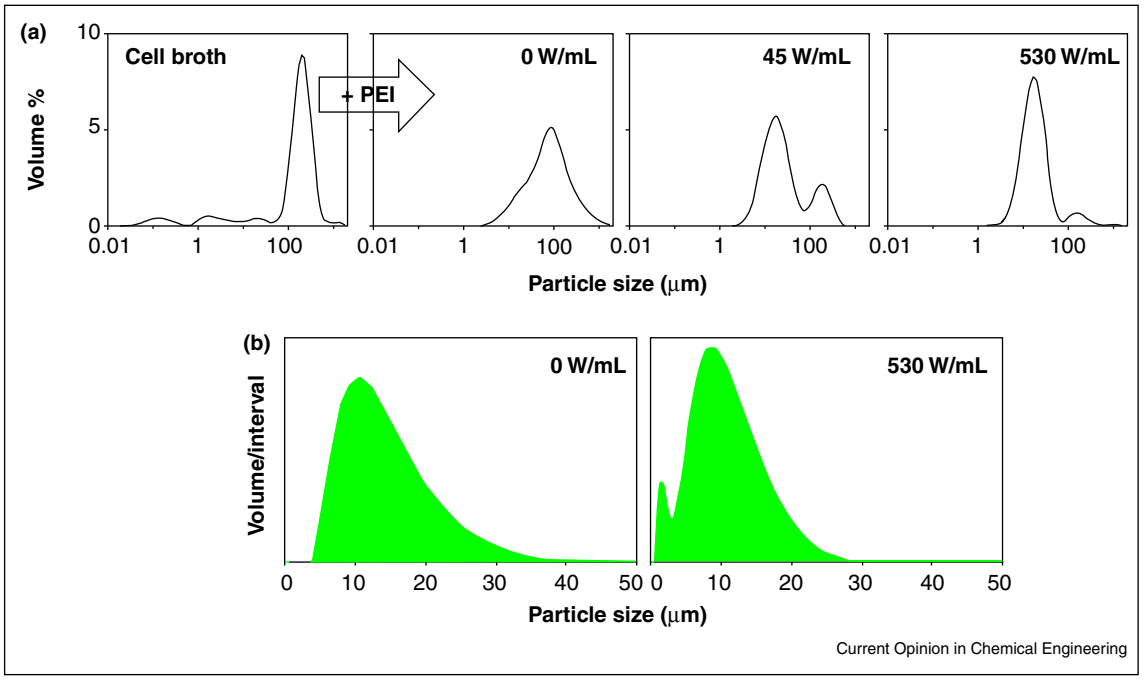
Interaction of flocculation, centrifugation and depth filtration

Flocculation in this review relates to use of polymeric reagents (e.g. polyethyleneimine (PEI) or polydiallyldimethylammonium chloride (PDADMAC)) for the selective precipitation (e.g. of nucleic acids) and aggregation (e.g. of colloidal proteins, lipopolysaccharides, colloidal proteins) of biological contaminants (e.g. [14,32,33–35]). It is used in a process sequence to offer a rapid clean-up of a complex bioprocess stream before progression to a sensitive chromatographic or selective adsorption separation stage. The ultra scale-down of floc preparation follows the same rules as for a precipitate (see below) with regards to mixing conditions and times. The ability to work at small scale allows study of the effect of floc agent properties (e.g. molecular weight, concentration, degree of molecular unravelling etc.), as well as sensitivity to mixing conditions [36]. Key findings include the use of flocculating agents to remove the presence of small (micron sized) particles to form large flocs that are sensitive to the effects of shear stress. However, the micron sized particles do not reappear and, because the particles remain well above the critical size for centrifugal removal, high levels of clarification are achieved (Figure 3a). For example, in one study combining ultra scale-down flocculation, shear stress and centrifugation the use of flocculation was predicted to reduce contaminant carry-over by >50-fold [14] with greater ease of depth filtration of the subsequent supernatant [14,37•]. This was then verified at pilot scale [14,32,37•]. Verification at pilot scale of ultra scale-down predictions is critical to provide confidence for design predictions and has been achieved for other flocculated systems (e.g. [38]).

Interaction of precipitation and centrifugation

In the context of this review, precipitation is generally achieved via a change in the process environment, for example, pH, ionic strength, dielectric constant etc. or combinations thereof. Precipitation continues to be explored to provide novel solutions to bioprocessing (e.g. [39–41]). The product or the contaminants may be subject to precipitation and then generally removal/recovery by centrifugation [21] or possibly filtration [20] especially

Figure 3



(a) Flocculation is used to process an autolysised *E. coli* cell broth removing all sub-micron cell debris and also precipitating soluble contaminants, for example, nucleic acids, while the exposure (for 20 s) to high shear stress decreases the average particle size, there is no reappearance of sub-micron particles leading to ultra scale-down predictions of high levels of clarification in continuous flow centrifugation [14] that are verified at pilot scale [37•]. **(b)** The fine particles formed as a result of the application of shear stress (20 s) on mammalian cell broth will be difficult to remove by centrifugation and subsequent filtration [11•].

if the product remains in the soluble phase. The control of the properties of the precipitate is achieved by the choice of reactor conditions of the mean velocity gradient ($G = (P/V\mu)^{0.5}$) where P is power dissipated in a volume, V, with suspension viscosity, μ, and residence time, t. The precipitate particle size is generally inversely proportional to G. The dimensionless Camp number ($Ca = Gt$) determines the strength of the precipitate with maximum resistance to disruption to shear stress being observed for $Ca > 10^5$ [42]. However, even after such ageing the precipitate is still strongly susceptible to shear stress induced break up, for example, in pumps or in centrifuge feed zones. Examples of successful USD prediction of precipitation and precipitate recovery by centrifugation have been achieved, for example, salt precipitation of microbial proteins or ethanol precipitation of blood plasma proteins [10].

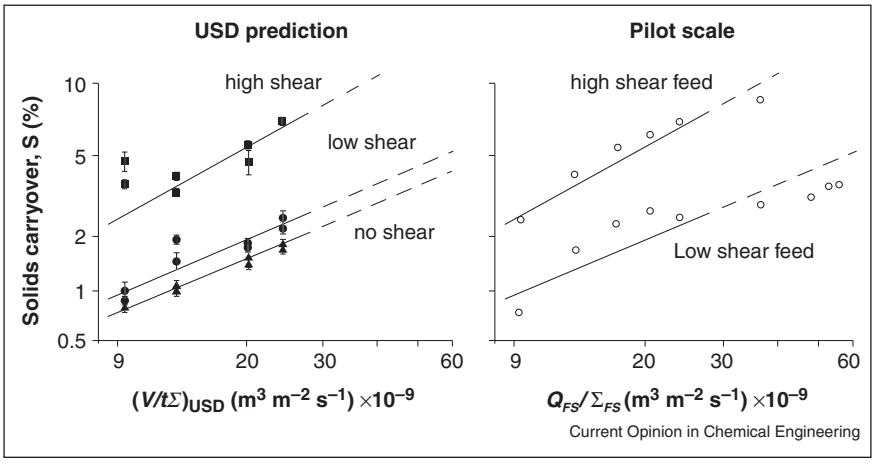
Interaction of suspension cell culture, harvest, centrifugation and filtration

The production of monoclonal antibodies and other protein products via mammalian cell culture involves firstly a sequence of solid-liquid separation stages to prepare a clarified broth suitable for chromatographic or adsorption based separation. The cells are cultured in a relatively low shear stress environment and high levels of shear stress may lead to considerable attrition (Figure 3b) and release of intracellular components. The impact of such disruption on the centrifugal clarification of cell broth has been studied with the use of a high stress feed zone predicted to lead to over 10-fold increase in solids carryover compared with no shear stress (Figure 4). The use of a low stress feed zone reduces such carryover very significantly [11•,22,43,44] and using ultra scale-down filtration tools [45–47] is shown to enhance the performance of subsequent filtrations stages [48]. The correct back pressuring of a centrifuge to maintain a low shear stress (hydrohermetic) feed zone may be defined as a Critical Process Parameter when the validation of such a bioprocess is addressed. The impact of such shear stress on the protein product might need to be considered, for example, for a monoclonal antibody no impact on glycosylation pattern was observed but the presence of half antibodies increased [49] while for a fusion protein a large impact on extent of glycosylation was observed [50•]. Improvements in mammalian cell biology have been predicted to be best realised by use of low shear stress processing options [51]. The effect of surface interactions under shear stress has been reported for antibodies leading to loss of functionality in solutions without the appropriate protective agents [16,17,52].

Interaction of surface-attached cell culture, centrifugation and resuspension

The preparation of cells for therapy is one of the most challenging areas in terms of understanding the impact of the processing environment on the product [53]. For allogeneic therapies, such as multi-potent adult stem cells (MPACs), the scale of production is often near the limit of analytical techniques so the challenge is to understand the effect of the processing environment. For autologous therapies, such as induced pluripotent stem cells (iPS cells) or T-cells, there is also the challenge of scale-down. As might be expected different cell lines respond very differently to the application of process stress [12,54]

Figure 4



Ultra scale-down shear stress analysis along with ultra scale-down centrifugation was used to predict the carryover of solids during clarification of industrial scale mammalian cell culture broths. The predicted large increase in solids carryover was verified at pilot scale (Σ_{FS} = ~2500 m^2, Q_{FS} = 80–500 L/h), as was the effect of increasing the flowrate through the centrifuge. The use of no shear bench-scale centrifugation significantly underestimates the performance of even low-shear pilot-scale centrifugation [11•]. Solid lines are fits by regression of the ultra scale-down data, dashed lines are extrapolation of relationship; the lines for high and low shear ultra scale-down data fits are transposed to show match with pilot-scale results.

and for a particular cell line the cell passage number, generation number and method of growth all affect the response to shear stress [55]. Capillary based studies have been used to reduce the number of cells required for process characterisation [9]. As predicted [7,56,57] the main reason for damage is due to extensional flow at the capillary entrance but careful computational analysis of the particle and fluid dynamics is needed to identify regimes of exposure of cells to stress. The translation of such results has led to an understanding of the reasons for cell loss during operation of scale-down mimics of dead-end centrifugation where the combination of pipette flow velocities and sediment rheology [58] led to stresses exceeding the predicted process stress for damage and solutions to reduce such stress [4•]. Capillary flow has been used to gain further understanding of the reasons for other causes of cell damage [8,59]. Similar ultra scale-down tools are needed to explore new options for cell recovery, for example, cross flow filtration or expanded bed centrifugation [4•] and washing and administration.

Conclusions

An increasing number of stages in a bioprocess are becoming accessible to investigation using ultra scale-down methods, including continuous operations. This review has focused on one of the major challenges for scale down, that is, centrifugation, especially as a key stage between cell culture/fermentation and chromatographic purification. Examples are provided of the successful application of ultra scale-down methods for a range of bioprocess sequences.

The ability to complete ultra scale-down studies of whole bioprocess sequences will allow low cost development of a full-scale process while assurance is awaited of the clinical safety and efficacy of the candidate therapy. Then it will be possible to move at speed to large-scale manufacture.

As more complex therapies become the deliverable of value, the use of ultra scale-down to help enhance process development at low cost is likely to become increasingly important. Ultra scale-down methods also provide a quantitative and predictive methodology by which to assess the benefits of host cell engineering (synthetic biology) on whole bioprocess performance.

Acknowledgements

The funding of the UK Engineering and Physical Sciences Research Council (EPSRC) for the Innovative Manufacturing Research Centre in Bioprocessing hosted at UCL is gratefully acknowledged as is the support of the collaborating companies including: BioProducts Laboratories, BioVex, Eli Lilly, FujiFilm Diosynth, GE Healthcare, GSK, ImmunoBiology, Lonza Biologics, MedImmune, Merck, Novo Nordisk, OnyVax, Pall Life Sciences, Pfizer, Puridify, The Automation Partnership (TAP, now Sartorius Stedim), UCB, Westfalia (now GEA).

The support of the Higher Education Funding Council for England (HEFCE) Catalyst Fund to support access to ultra scale-down technologies and the knowledge transfer activities is also gratefully acknowledged.

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