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Inverse problem analysis of pluripotent stem cell aggregation dynamics in stirred-suspension cultures

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Abstract

The cultivation of stem cells as aggregates in scalable bioreactor cultures is an appealing modality for the large-scale manufacturing of stem cell products. Aggregation phenomena are central to such bioprocesses affecting the viability, proliferation and differentiation trajectory of stem cells but a quantitative framework is currently lacking. A population balance equation (PBE) model was used to describe the temporal evolution of the embryonic stem cell (ESC) cluster size distribution by considering collision-induced aggregation and cell proliferation in a stirred-suspension vessel. For ESC cultures at different agitation rates, the aggregation kernel representing the aggregation dynamics was successfully recovered as a solution of the inverse problem. The rate of change of the average aggregate size was greater at the intermediate rate tested suggesting a trade-off between increased collisions and agitation-induced shear. Results from forward simulation with obtained aggregation kernels were in agreement with transient aggregate size data from experiments. We conclude that the framework presented here can complement mechanistic studies offering insights into relevant stem cell clustering processes. More importantly from a process development standpoint, this strategy can be employed in the design and control of bioreactors for the generation of stem cell derivatives for drug screening, tissue engineering and regenerative medicine.

Keywords

Stem cells; bioreactors; population balance equation model; inverse problem; aggregation kernel; tissue engineering

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6. Disclosure statement

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1. Introduction

Stem cells self-renew extensively in vitro and can adopt fates of all somatic cells including those which are central to the regeneration or repopulation of injured or diseased tissues. Enabling this potential necessitates the development of scalable processes for the robust production of therapeutics in quantities which meet clinical demand. Human stem cells including embryonic (ESCs), induced pluripotent (iPSCs) and mesenchymal stem cells (MSCs) can be propagated in stirred-suspension vessels and/or coaxed to particular phenotypes. Stem cells can be cultured in stirred suspension attached on microcarriers, encapsulated or as aggregates.

Stem cell aggregate cultivation involves less cumbersome preparatory (pre-culture) and downstream (post-culture; mainly separation) procedures. Aggregation promoted through agitation-induced collisions among cells and/or cell clusters, is a central determinant of the process outcome. Cluster size influences the transport of nutrients, O₂, metabolites and growth factors while cells residing within large clusters potentially experience hypoxic conditions with poor nutrient supplementation (Van Winkle et al., 2012; Wu et al., 2014). In addition to the growth and viability, the aggregate dimensions strongly influence the differentiation trajectory and heterogeneity of cultured cell populations (Niebruegge et al., 2009). Despite its significance, stem cell aggregation in stirred-suspension vessels is treated empirically.

Predicting and controlling the aggregate size distribution in this context are highly desirable for stem cell engineering. This demands a quantitative framework linking the size distribution of stem cell clusters to measurable and adjustable culture parameters. Population balance equation (PBE) models, which are based on mass or number balances, have been employed in the study of the aggregation dynamics among cells such as platelets and neutrophils under shear (Belval and Hellums, 1986; Neelamegham et al., 1997) and plant cells in suspension culture (Kolewe et al., 2012). We also have shown previously the application of a PBE model in the aggregation of stem cells cultured in suspension (Kehoe et al., 2010). The rate of aggregation in a PBE is largely dictated by the ‘coalescence’ (more appropriately termed ‘aggregation’) kernel which provides a functional dependence of the aggregation rate on the size (and other properties) of cell clusters, and the process conditions. In the aforementioned studies however, a particular aggregation function has been assumed. The widely utilized von Smoluchowski aggregation kernel (von Smoluchowski, 1917) describing the collision of two spheres moving by diffusion or in a laminar flow field, is not appropriate given the hydrodynamics of stirred suspension cultures. Empirical variations of this kernel have also been employed in coagulation or aggregation processes (Aldous, 1999; Smit et al., 1994). While convenient to use, such kernels involve adjustable parameters requiring fitting and the numerical values may not apply to the same experimental setup after is been even slightly modified. More importantly, empirical kernels are selected by trial and error offering limited insight into the aggregation process.

The extraction of PBE functions from dynamic experimental data constitutes an inverse problem. Solution of the inverse problem is ideally suited for stem cell bioreactors where obtaining functions describing the behavior of single aggregates is not possible by direct observation. Solving the inverse problem allows the prediction of the steady state or equilibrium condition for cluster size distribution, validation of physical models of aggregate formation (and breakup) rates and affords insights on reciprocal effects among agglomerates and their milieu (Mahoney et al., 2002). Despite their significance, inverse problems are often ill-posed requiring sophisticated numerical methods for their solution. Additionally, discretization and noise in experimental data affect approximate analytical solutions (Ramkrishna and Mahoney, 2002). Wright and coworkers (Wright et al., 1990) demonstrated elegantly the derivation of the coagulation frequency in a PBE model from transient (C_6H_6 - CCl_4 mixture in water) droplet size data in a turbulent flow field. The resulting frequencies showed significant deviation from previous simple models of particle interactions in turbulent flow.

In this study, the inverse problem approach was taken to obtain kernel expressions in a PBE model for the evolution of the distribution of ESC aggregates cultured in a stirred-suspension bioreactor flask at different agitation speeds (60–100 rpm). The aggregation kernel was recovered from experimental data as an inverse problem solution via self-preserving distributions (see next section). Subsequent PBE model predictions using the extracted kernels were in agreement with our experiments supporting the implementation of inverse problem strategies for developing a quantitative framework for stem cells cultured as aggregates in scalable stirred-suspension vessels. Such frameworks are essential for the design and control of bioprocesses for the production of stem cell therapeutics.

2. Material and methods

2.1 Mouse ESC culture

Mouse E14Tg2a ESCs (passages 35–50; Mutant Mouse Regional Resource Centers (MMRRC), University of California-Davis, CA) were cultured in plates coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) in phosphate buffer saline (PBS) at 5% CO_2 /95% air and 37 °C. Cells were adapted to defined serum-free medium (DSFM): Dulbecco's modified Eagle's medium (DMEM; Sigma), 20% KnockOut serum replacer, 0.1 mM nonessential amino acids, 0.055 mM β -mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μ g/mL) (all from Invitrogen, Carlsbad, CA), and 1,000 U/mL leukemia inhibitor factor (LIF; EMD Millipore, Billerica, MA) (Kehoe et al., 2008). Medium was replaced daily and cells were subcultured every 2–3 days. Cells were passaged after incubation with TrypLE™ (Invitrogen) in PBS and cell clump dissociation into single cells by gentle pipetting. After spun down, cells were suspended in DSFM and plated in gelatin-coated dishes. For stirred-suspension culture, cells harvested from dishes were counted in a hemacytometer or a cell counter (TC20, Bio-Rad, Hercules, CA), suspended as single cells in DSFM and transferred to 125-ml ProCulture spinner flasks at 1×10^5 cells/ml. The agitation rate was kept constant throughout each run (60–100 rpm).

2.2 Stem cell aggregate micrograph acquisition and analysis

Samples withdrawn from the spinner flasks were replaced by an equal volume of fresh medium (constant total volume) and transferred to 96-well plates where images were taken at 4× magnification with a camera (Moticam 2300, Motic, Richmond, BC) connected to the microscope. To generate aggregate size distributions, raw images were processed with NIH ImageJ (<http://imagej.nih.gov/ij/index.html>). After background subtraction (rolling ball method), the diameter of each aggregate was obtained as the mean of two perpendicular diameters. The number of cells per aggregate was estimated assuming that the aggregates are spherical and taking into account aggregate porosity (Wu et al., 2014).

2.3 Population balance equation (PBE) model

The evolution of stem cell cluster size distribution is described by a one-dimensional PBE model:

$$\frac{\partial n(x,t)}{\partial t} + \frac{\partial [n(x,t) \frac{\partial x}{\partial t}]}{\partial x} = \frac{1}{2} \int_0^x n(x_c, t) n(x', t) K(x_c, x') dx' - \int_0^\infty n(x, t) n(x', t) K(x, x') dx' \quad (\text{Eq. 1})$$

The population density function $n(x,t)$ is defined such that $n(x,t)dx$ is the number of aggregates of size (mass or volume) x to $x+dx$ in a unit culture volume. The rate of change of $n(x,t)$ (first term) and the “loss” of ESC aggregates with size x (second term) due to

proliferation $\left(\frac{\partial x}{\partial t}\right)$ -induced increase of their size are balanced by the birth of ESC aggregates of size x due to agglomeration of clusters with sizes $x' < x$ and $x_c = x - x'$ (third term) and the disappearance of clusters with size x due to aggregate formation with clusters of any mass (fourth term). We assumed a batch process with randomly mixed aggregates which form by the combination of two smaller clusters/cells. Negligible attrition is also accepted given the high viability of cultured cells (typically >90% (Kehoe et al., 2008; Wu et al., 2014)).

The aggregation rate or frequency is typically the product of the collision frequency and aggregation efficiency presuming that collision is the rate determining step of the aggregation process. While the aggregation rate is proportional to the product of the number concentrations of the colliding particles (for dilute systems), the aggregation kernel $K(x, x')$ is proportional to the aggregation efficiency and can be seen as a rate constant representing the ‘reaction rate’ between clusters with sizes x and x' . The kernel form depends on the physical process (e.g. aggregation, flocculation, coalescence, coagulation etc.) under consideration.

2.4 Self-similarity solution to a PBE

The solution of the inverse problem capitalizing on the self-similarity of a PBE distribution has been described systematically by Ramkrishna (Ramkrishna, 2000; Wright and Ramkrishna, 1992) and the same methodology was essentially employed here. A self-similar (or self-preserving) distribution exhibits over time a shape that is preserved and a maximum whose position increases. The self-similar solution can be decomposed into time-invariant and -variant components by appropriate scaling of the variables. As a result, all curves of the

self-preserving distribution collapse into a single curve. Here, $n(x,t)$ can be written as (Ramkrishna, 2000):

$$n(x,t)=g(t)f(z) \quad (\text{Eq. 2})$$

where the variable z corresponding to a dimensionless normalized particle size is defined as:

$$z=\frac{x}{S(t)} \quad (\text{Eq.3})$$

The functions $s(t)$, $g(t)$, and $f(z)$ (time-invariant) to be determined are nonnegative and smooth. The function $s(t)$ expressing the mean aggregate size is taken as the ratio of successive moments $M_i(t)$, $M_{i+1}(t)$ of the distribution:

$$S(t)=\frac{M_{(i+1)}(t)}{M_i(t)} \quad (\text{Eq.4})$$

where

$$M_i(t)=\int_{x_{min}}^{x_{max}} x^i n(x,t) dx \quad (\text{Eq. 5})$$

The PBE can be expressed in terms of the cumulative volume fraction, which is a probability distribution function:

$$\frac{\partial F(x,t)}{\partial t} + \frac{\partial [X(t) \frac{M_1 \partial F(x,t)}{x \partial x}]}{\partial x} = - \int_0^x dF'(x',t) \int_{x-x'}^{\infty} \frac{dF(x'',t)}{x''} K(x',x'') \quad (\text{Eq.6})$$

The self-similar form of the number density function yields the time-invariant cumulative function:

$$F(x,t)=\frac{\int_0^z z' f(z') dz'}{\int_0^{\infty} z' f(z') dz'} = \Phi(z) \quad (\text{Eq. 7})$$

$$n(x,t)=\frac{M_1}{S(t)^2} \frac{\Phi'(x)}{x} \quad (\text{Eq.8})$$

Substitution of the transformed $F(x,t)$ yields:

$$z\Phi'(z) - \frac{M_1}{S(t)^2 S'(t)} \frac{\partial [\Phi'(z) \alpha \ln(\frac{M}{zS(t)})]}{\partial z} = \frac{1}{S'(t)} \int_0^z \Phi'(x) dx \int_{z-x}^{\infty} \frac{\Phi'(y)}{y} K(xS(t), yS(t)) dy \quad (\text{Eq. 9})$$

Numerical calculations for the inverse problem were performed in MATLAB. The PBE was solved using a forward finite difference method implemented in Fortran 90. More details on pertinent numerical methods are in the Appendices.

2.5 Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM) or mean $\pm$ standard deviation (SD) as stated. ANOVA and the *post hoc* Tukey test were performed using Minitab (Minitab Inc, State College, PA) with $p < 0.05$ considered as significant.

3. Results

Two stages were identified in the cultivation of mESCs over 4 days in stirred suspension: The first stage encompasses approximately the first 12 hours of culture in which the growth term was neglected making this a pure mESC aggregation process. This is in line with the doubling time of 11.7 hours for mESCs in spinner flask cultures (Wu et al., 2014). Thus, equation 9 becomes:

$$z\Phi'(z) = \frac{1}{S'(t)} \int_0^z \Phi'(x) dx \int_{z-x}^{\infty} \frac{\Phi'(y)}{y} K(xS(t), yS(t)) dy \quad (\text{Eq. 10})$$

The time-variant component $S(t)$ of the self-similar solution is calculated followed by the computation of the similarity distribution $\Phi(z)$ and eventually of the kernel $K(x,y)$.

In the second stage of the culture, cell growth was dominant (vs. aggregation) driving the evolution of the size distribution and the problem was formulated accordingly.

3.1 Similarity distribution of ESC aggregate size

Aggregate size distributions were generated after sampling stirred suspension cultures under different agitation rates. From approximately 24 μm for all agitation rates (60, 80 and 100 rpm) at 2 hours post-seeding, the average diameter of mESC aggregates reached $30.89 \pm 8.17 \mu\text{m}$ (60 rpm), $31.71 \pm 8.64 \mu\text{m}$ (80 rpm) and $32.08 \pm 8.65 \mu\text{m}$ (100 rpm) after 12 hours. At day 4, mESC aggregates at 60 rpm ($308.14 \pm 28.29 \mu\text{m}$; approx. 6750 cells calculated per aggregate with a single cell radius of $\sim 7.5 \mu\text{m}$ and porosity ~ 0.22 (Wu et al., 2014)) were larger compared to those at 80 rpm ($211.23 \pm 32.66 \mu\text{m}$ or ~ 2170 cells per cluster; $p = 0.018$) and 100 rpm ($188.90 \pm 19.24 \mu\text{m}$ or ~ 1560 cells per cluster; $p = 0.006$) (Wu et al., 2014). Distributions at intermediate time points were also obtained and the corresponding $n(v,t)$ (describing the aggregate size by volume) was calculated (Fig. 1A).

3.1.1 Calculation of the $S(t)$ function—The function $S(t)$ (Eq. 4), which represents the scaled average aggregate volume, is the ratio of successive moments of the experimental size distributions. The second ($M_2(t)$) and first ($M_1(t)$) moments provided good approximations of $S(t)$. After 12 hours, $S(t)$ was equal to $3.33 \pm 0.07 \times 10^4$ at 60 rpm, $4.17 \pm 0.11 \times 10^4$ at 80 rpm and $2.83 \pm 0.15 \times 10^4$ at 100 rpm (Fig. 1B). However, the slope $dS(t)/dt$ (or $S'(t)$) is used for estimation of B and λ (Eq. A9; Table 1). The highest slope was observed for 80 rpm. In all agitation rates, λ values were negative whereas B was lowest at

100 rpm ($2.483 \pm 0.407 \times 10^3$). B corresponds to the average ‘coagulation’ rate (Wright and Ramkrishna, 1992) as:

$$B = \int_0^\infty \int_0^\infty x \frac{\Phi'(x)}{x} y \frac{\Phi'(y)}{y} b(x, y) dx dy \quad (\text{Eq. 11})$$

The highest and lowest average rates were noted at 80 rpm and 100 rpm, respectively.

3.1.2 Calculation of the time-invariant function $\Phi(z)$ —Inspection of the above expression for B (Eq. 11) reveals that the similarity distribution $\Phi(z)$ should be estimated for the calculation of $b(x, y)$. We thus proceeded with the calculation of $\Phi(z)$ so that the experimental distributions at different time points can be transformed into a single, time-invariant distribution. This allows rewriting the PBE simplifying considerably the inverse problem for the calculation of the kernel. The trend of the similarity distribution ($\Phi(z)$) (Fig. 2), cumulative similarity distribution ($\Phi(z)$) and cumulative volume fraction ($\Phi(v)$) functions for stirred-suspension cultures at different agitation rates are depicted in Figure S1. The z values were between 0.04–5.3 for 60 rpm and 0.08–3.3 for 100 rpm. The disparate z ranges reflect the different values of $S(t)$ calculated at 60–100 rpm. The distribution $\Phi(z)$ at each time point was calculated (Eq. 8) and collapsed with the typical scale $S(t)$ (Eq. 4). As suggested previously (Wright and Ramkrishna, 1992), the Γ (gamma) distribution was chosen (Eq. A12) to approximate $\Phi(z)$ analytically. This approximation simplifies the inverse problem ensuring that the self-similarity distribution is continuous and reducing effects due to experimental errors. The parameters of the approximated $\Phi(z)$ for different agitation rates are shown in Table 2. The parameter values decreased with decreasing stirring speeds ($p < 0.05$). The results show that the similarity distribution at different agitation rates is time-invariant as expected further supporting the calculation of the $S(t)$ described above. Thus, the time-invariant component, which is not straightforward to extract from the respective number density function plots (Fig. 1A), is recovered.

3.2 Aggregation kernel – first stage of culture

During the first stage of cultivation, the changes in cluster size distribution are attributed mainly to collisions among cells/aggregates while the effect of cell proliferation ($T_d \sim 12$ hours) is less pronounced. Moreover, there is an initial lag in ESC growth likely due to cell adaptation to the environment with agitation. In our analysis therefore, we neglected the growth term due to proliferation in the PBE (Eq. 1). The kernel function was calculated from Eq. 10 solving the inverse problem utilizing $(z, \Phi(z))$ pairs (Eq. 10 and Eq. A11). The solution was constrained by kernel symmetry ($K(x, y) = K(y, x)$; particles x and y conform to the same physical laws) and positivity ($K(x, y) > 0, \forall x, y > 0$; kernel properties include a probability and no breakage is assumed) (see Appendices).

In addition, Tikhonov regularization (Tikhonov and Arsenin, 1977) was implemented because of the ill-posed character of this problem with small uncertainties in $\Phi(z)$ leading to large errors in the $K(x, y)$. To that end, we sought to minimize the norm of the residual, $\|X\delta - Y\|$, between the right- (Y) and left-hand side values ($X\delta$; X is the integral operator acting on the quantity $\delta = b(x, y)/B$) of Eq. A11. The method introduces a non-negative

regularization parameter (λ_{reg}), which should be chosen appropriately to obtain an estimate of the aggregation kernel via the inverse problem (see Appendix B). The L-curve criterion is a reliable way of selecting the best value for λ_{reg} (Johnston, 2001). This consists of a log-log plot of $\|\delta\|$ versus $\|X\delta - Y\|$ with λ_{reg} as the parameter. The two curve segments represent the dominant effects of the regularization error or the perturbation error, respectively (Fig. 3A) with the optimal λ_{reg} value corresponding near the vertex. The largest acceptable λ_{reg} value for all available data at different agitation rates was 1×10^{-5} .

Upon calculating the unknown quantity $b(x,y)/B$ representing the ratio of the scaled aggregation frequency to its mean, the kernel $K(v,v')$ was determined for different agitation rates from B , λ (Eq. A9) and $b(x,y)$ (Eq. A10) as shown in Figures 3B and S2. The kernel function increased with z reaching 0.55 1/hr at 60 rpm, 0.76 1/hr at 80 rpm and 0.98 1/hr for 100 rpm. It should be noted that $K(x,y)$ was not monotonically increasing throughout the z domain and local minima were observed, e.g., at aggregate size volumes of 3.1×10^4 , 4.2×10^4 and $2.6 \times 10^4 \mu\text{m}^3$ for 60, 80 and 100 rpm, respectively.

3.3 Prediction of stem cell aggregate size distribution

The obtained kernel was then applied to the PBE to predict the temporal evolution of the distribution of mESC aggregates in stirred-suspension at different stirring speeds (Figs. 4A, S3). The agreement between the predicted number density functions and the experimental data illustrates that the solution of the inverse problem $K(x,y)$ allowed the accurate prediction of the transient size distribution. By taking the zeroth moment of the predicted distribution, it became obvious that the total aggregate number decreased faster at 100 rpm (Fig. 4B).

Moreover, sensitivity analysis was performed to assess the robustness of the kernel estimation and its effect on the PBE prediction. For this purpose, a 10% error was added to the self-similarity size distribution $\Phi(z)$ and the $S(t)$. The parameters of the Γ distribution (Eq. A12) approximating $\Phi(z)$ after perturbation are shown in Table 3. Similarly, the λ and B values obtained after perturbation of the $S(t)$ (Eq. A9) are shown in Table 4. The general shape of the kernel remained unchanged despite the perturbation (Figs. 4C, S4). The resulting size distributions showed no appreciable difference among various stirring rates regardless of perturbations in the kernel calculation.

3.4 Aggregation kernel – Second stage of culture

After the first 12 hours and up to day 4 of culture, the rate of aggregate size change due to cell proliferation was modeled as a Gompertz process:

$$\frac{\partial x}{\partial t} = r(x, S) = \mu x \ln \left(\frac{M}{x} \right) \quad (\text{Eq.12})$$

where M is the cluster volume limit (at $t \rightarrow \infty$) and μ is a constant characteristic of cell proliferation. Both parameters ($M = 9.71 \times 10^6$, $\mu = 5.72 \times 10^{-3} \text{ hr}^{-1}$) were estimated from experiments (Wu et al., 2014). Because the number of aggregates remained fairly constant over culture days 1–4, we investigated whether aggregation could be ignored. For this reason, we set $K(x,y) = 0$. For comparison, we also chose the analytical expression below (Eq.

13) out of several ones proposed for aggregation processes (Aldous, 1999; Smit et al., 1994) (Tobin et al., 1990) (Wu et al., 2014).

$$K(x, y) = \exp\left(-k_1\left(\frac{x+y}{2}\right)^c\right) \left(x^{\frac{1}{3}} + y^{\frac{1}{3}}\right)^{\frac{7}{3}} k_1, c > 0 \quad (\text{Eq. 13})$$

The aggregate radius, r , was normalized with respect to the radius of a single cell (7.5 μm). After mapping the density function on a logarithmic scale of the radius, aggregate size distributions were generated from experimental data and PBE simulations with or without aggregation at different agitation rates (Fig. 5). Even without aggregation ($K(x, y) = 0$) the transient experimental aggregate size distributions were closely predicted. With the above analytical expression for $K(x, y)$ (Eq. 13), there was also no considerable difference from the PBE prediction for $K(x, y) = 0$.

These results indicate that for cultivation beyond the initial stage (days 1–4 here), the aggregate size is affected by proliferation rather than collision-induced clustering. This is evidenced by the agreement between experiments and simulation results for $K(x, y) = 0$. Our posit is thus confirmed that the evaluation of the kernel by solution of the inverse problem is not critical for the later stage of cultivation unlike the first day post-seeding.

4. Discussion

Although the cultivation of hPSCs as aggregates in scalable vessels represents an attractive modality for stem cell-based manufacturing in clinically relevant quantities, a detailed quantitative study of the clustering phenomena is lacking. The aggregate size is intimately linked to the rate of exchange of nutrients, O_2 and differentiation factors, the cell-cell interactions and cluster ultrastructure. In turn, these features and processes impact the viability, proliferation and fate decisions of cultured stem cells.

Here, a PBE was cast to model the aggregation of stem cells cultured in spinner flasks. The aggregation kernel capturing the kinetics of cluster formation was obtained by solving the inverse problem based on experimental data for different agitation speeds. Subsequent PBE simulations incorporating the obtained kernel expressions led to predictions of the transient aggregate size distributions in excellent agreement with the culture data. Thus, this study provides a proof of concept about the applicability of the inverse problem methodology to the scalable stirred-suspension culture of stem cells.

The kernel was extracted from culture data for the first 12 hours post-seeding. Subsequently, a less pronounced contribution of aggregation than of proliferation to the ESC cluster size increase was considered. This was in line with our observations of the total number of aggregates leveling off after about 12 hours. Moreover, the exclusion of cell proliferation simplified the inverse problem. Ramkrishna and co-workers have discussed the self-similar behavior of microbial systems with growth (Ramkrishna, 1994; Ramkrishna and Schell, 1999). Given that stem cell populations are not synchronized, the prospect of combined effects due to proliferation and clustering is interesting and warrants further investigation.

The same analysis can be applied to the cultivation of human stem cells in stirred suspension vessels. In fact, the longer doubling time of hPSCs (vs. mESC) supports neglecting growth initially for extracting the aggregation kernel. We have noticed (data not shown) nonetheless that hPSC aggregation appears to be slower than for mESC although a more detailed study is necessary. Unlike mESC, hPSCs are cultured typically at lower stirring rates and require the use of a Rho-associated protein kinase (ROCK) inhibitor for single-cell survival but those differences do not pose complications to the inverse problem.

Despite the ill-posed nature of the recovery of the kernel from transient data, this methodology can be advantageous compared to empirical approaches or kernels derived via theoretical studies. The hydrodynamic environment in a stirred suspension bioreactor can be significantly complex depending on the operating conditions (e.g. agitation rate, seeding cell densities etc.), impeller geometry, and the presence of various ports (e.g. for sampling), probes (e.g. for dissolved O_2) and baffles. These make almost impossible the derivation of theoretical models of aggregation particularly those pertaining to cells rather than liquid droplets. Moreover, empirical expression we tested yielded results which did not match our experiments even after fitting kernel parameters (Fig. S5). In contrast, solving the inverse problem requires temporal aggregate size data which are relatively straightforward to collect. Moreover, these data can be combined with additional information (e.g. pluripotency marker expression via flow cytometry (Wu et al., 2014)) for a multidimensional state vector in the PBE thereby expanding the model's predictive capabilities.

The estimated kernel encompasses the effects of operational variables such as the agitation rate, substrate availability and cell seeding density although establishing explicit functions for such dependencies may be difficult. To that end, an expression between a kernel sampled at different conditions and a particular variable may be inferred from physical models or auxiliary relations. For example, the laminar shear coalescence kernel by von Smoluchowski (von Smoluchowski, 1917) is proportional to the shear rate and was utilized for platelet aggregation in a shear field (Belval and Hellums, 1986). We also adopted previously a similar expression to illustrate the application of a PBE model to mESC aggregates in spinner flasks (Kehoe et al., 2010). Relations are available linking the shear rate in a bioreactor to the geometric characteristics of the vessel, agitation rate, and fluid properties (e.g. (Cherry and Papoutsakis, 1986; Croughan et al., 1987)). Combined with the kernel calculation from transient size information, more detailed models can be constructed for the design and predictive control of stirred-suspension processes for stem cells.

The inverse problem kernel increased with the similarity variable z at different agitation rates but local minima were observed. In fact, the kernel functions differed among agitation rates evidencing the strong dependence of $K(x,y)$ on stirring. In addition, the total number of aggregates, predicted by the PBE utilizing the inverse problem kernel, decreased faster at 80 and 100 rpm. Interestingly, the average aggregate size and its rate of change (slope in Fig. 1B) were greater at 80 rpm than at 60 rpm and 100 rpm. A possible explanation is that faster agitation leads to greater velocities boosting the collision rate among particles therefore promoting more rapid aggregate formation (differences in aggregation efficiency notwithstanding). As a tradeoff however, the increased shear imposes a limit on clustering and the emergence of larger aggregates. On top of supporting the dependence of the kernel

on the shear rate as discussed, these findings illustrate the intricacies of the aggregation phenomena in stem cell cultures under stirring.

It should also be noted that we did not consider the breakage of aggregates largely because we have observed a monotonic increase of aggregate size. The calculated kernel thus represents the net effect of aggregation and possible breakage suggesting that the inclusion of a term for the latter would not alter significantly the conclusions of our study. Having only an aggregation term also simplifies the inverse problem although systems featuring both aggregation and breakage processes have been discussed mainly when a steady-state balance is achieved (Ramkrishna, 2000; Vigil and Ziff, 1989). Combined aggregation and disruption kinetics have been reported for baby hamster kidney (BHK) cells cultured as clusters in spinner flasks (Moreira et al., 1995). The aggregate size distribution was represented by a bimodal distribution ('small' and 'large' aggregates) as the time-dependent, two modified β distributions. The rate of change in aggregate size was modeled by taking into account collisional formation and break-up, and disruption due to hydrodynamic shear but not cell proliferation. Despite its restrictive assumptions (e.g. linear dependence of hydrodynamic shear on aggregate diameter), this approach sheds light on the contributions of each process considered. In principle, PBE models also can be extended to include terms describing separately the contribution of processes, which were not considered in detail here.

The numerical analysis of PBE inverse problems has been discussed in depth but the accuracy of the solution can be improved by collecting distributions at more time points thereby getting better estimates, for example, of the parameters λ and $\langle b \rangle$ (related to $S(t)$). Additionally, the use of methods for automated image acquisition, processing and data smoothing with noise dampening facilitate the reduction of errors in the extracted distributions.

Stem cell clustering is mediated by E-cadherin adhesion and E-cadherin^{-/-} mESCs fail to aggregate in static cultures (Larue et al., 1996). Moreover, loss of E-cadherin is linked to epithelial-mesenchymal transition which is an essential process in various stages of development and human stem cell differentiation (Eastham et al., 2007; Thiery, 2003). Aggregation also modulates signaling directly affecting the stem cell state (Azarin et al., 2012). These observations, combined with the envisioned use of bioreactors for the integrated expansion and directed differentiation of stem cells, raise interesting mechanistic questions immediately relevant to the bioprocess outcome. Yet, direct surveillance of aggregation phenomena is impractical in stirred vessels. To that end, the solution of the inverse problem represents underlying rate laws based on the behavior of single particles without necessitating the observation of single clusters. Hence, this method can complement mechanistic studies including computational models of aggregation (Graner and Glazier, 1992; White et al., 2013).

During the later stage of cultivation aggregation is no longer a driving force for the temporal evolution of stem cell cluster size. Our PBE simulation yielded similar results using either the kernel in Eq. 13 or $K(x,y)=0$. The change of the distribution was attributed to cell proliferation and was captured by the Gompertz equation with parameters estimated from

single aggregates under static conditions as discussed (Wu et al., 2014). Despite not taking into account possible effects of nutrient consumption (compensated through daily medium exchange) on cell proliferation, this can be incorporated in the PBE model (Mantzaris and Daoutidis, 2004) constraining the maximum aggregate size.

More importantly however, we show here that selecting appropriate culture conditions initially rather than later is critical for controlling the cluster size distribution of stem cells and their progeny in stirred suspension. In our experimental system for example, setting the agitation rate at 80 rpm for the first ~12 hours results in faster rate of increase of the average aggregate size while reducing stirring later may minimize the risk for shear-induced cell damage. Caution is suggested for generalizing conclusions from this study regarding specific agitation rates and their effects on stem cell clustering to other large-scale bioreactor systems. While aiding our understanding of stem cell aggregation phenomena in bioreactors, the experimental methodology and PBE framework presented in this work can be employed in such bioprocesses yielding kernels thereby facilitating the design, control and optimization of the production of stem cell therapeutics for regenerative medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Inverse problem calculations

For the initial stage of the culture, cell/cluster aggregation was considered to be the dominant factor influencing the aggregate size distribution. Hence, the growth term in (Eq. 9) was ignored:

$$z\Phi'(z) = \frac{1}{s'(t)} \int_0^z \Phi'(x) dx \int_{z-x}^{\infty} \frac{\Phi'(y)}{y} K(xS(t), yS(t)) dy \quad (\text{Eq. A1})$$

Taking the partial derivatives of both sides yields the feasibility condition for self-similarity:

$$\frac{\partial}{\partial t} \left[\frac{1}{s'(t)} \int_0^z \Phi'(x) dx \int_{z-x}^{\infty} \frac{\Phi'(y)}{y} K(xS(t), yS(t)) dy \right] = 0 \quad (\text{Eq. A2})$$

Further differentiation leads to the condition:

$$\frac{\partial}{\partial t} \left[\frac{K(xS(t), yS(t))}{s'(t)} \right] = 0 \quad (\text{Eq. A3})$$

which is necessary for self-similarity and by differentiation:

$$K(xS(t), yS(t)) = S(S(t))b(x, y) \quad (\text{Eq. A4})$$

$$S'(t) = BS(S(t)) \quad (\text{Eq. A5})$$

B is a constant corresponding to the average aggregation rate. Assuming the kernel is a homogeneous function of the aggregate size, then:

$$K(\alpha x, \alpha y) = \alpha^\lambda K(x, y) \quad (\text{Eq. A6})$$

When the PBE exhibits a self-preserving distribution, the degree of homogeneity, λ , should be -1 . $S(S(t))$ is an arbitrary positive function of $S(t)$ defined here as $S(t)^\lambda$ since most frequency models possess this property.

$$S(S(t)) = S^\lambda \quad (\text{Eq. A7})$$

$$K(x, y) = b(x, y) \quad (\text{Eq. A8})$$

The differential equation (Eq. A5) becomes:

$$\frac{dS(t)}{dt} = BS(t)^\lambda \quad (\text{Eq. A9})$$

$$K(xS(t), yS(t)) = S(t)^\lambda b(x, y) \quad (\text{Eq. A10})$$

Parameters λ and B are determined by fitting the above equation to $S(t)$ values. Replacing (Eq. A4) and (Eq. A7) in the self-similar cumulative distribution function yields (Wright and Ramkrishna, 1992):

$$z\Phi'(z) = \int_0^z \Phi'(x) dx \int_{z-x}^\infty \frac{\Phi'(y)}{y} \frac{b(x, y)}{B} dy \quad (\text{Eq. A11})$$

This is the main equation for obtaining the kernel $b(x, y)/B$ as a solution to the inverse problem given $\Phi'(z)$. Small uncertainties in the input data $\Phi'(z)$ may lead to large errors in the solution $b(x, y)/B$ necessitating the use of Tikhonov regularization (see part **B**.

Regularization). Because the unknown function is bivariate, a large number of expansion coefficients is required but this may be tackled by choosing appropriate basis functions.

An analytical expression for the cumulative similarity distribution $\Phi'(z)$ was defined to simplify the matrix equations for determining expansion coefficients (Wright and Ramkrishna, 1992). The expression fitted to the self-similar distribution must display the behavior of the self-similar distributions. Such a form with respect to $\Phi'(z)$ is found in Γ distributions, which can be singular at the origin (Wright and Ramkrishna, 1992). Consequently, the expansion of the similarity distribution is:

$$\Phi'(z) = \sum_{k=1}^{n_{term}} A_k z^{a_k-1} \exp(-B_k z) \quad (\text{Eq. A12})$$

where n_{term} is the number of Γ distributions used in the expansion and A_k , a_k and B_k are parameters of the k^{th} Γ distribution. This function satisfies the two constraints $\int_0^\infty \Phi'(z) dz = 1$ and $\int_0^\infty z\Phi'(z) dz = 1$. We used two terms ($n_{term}=2$) for the fitting process. The fitted curves were qualitatively similar whether one, two or three terms were included in the Γ distribution (Fig. S6).

Appendix B. Regularization

Discretizing equation A11 on n points in the z domain leads to a linear algebraic equation system:

$$Y = X\delta \quad (\text{Eq. B1})$$

where Y is a vector representing left hand side of the (Eq. A11) and X is an integral operator acting on the unknown vector δ representing $b(x, y)/B$. The ill-posed problem can then be replaced by a well-posed approximation via Tikhonov regularization:

$$\min \|X\delta - Y\| + \lambda_{reg} \|\delta\| \quad (\text{Eq. B2})$$

λ_{reg} is the regularization parameter with a small non-negative value. The first term in this equation represents the residual, whereas the second term measures the norm of the solution with the regularization parameter λ_{reg} acting as a weighting factor. Selection of appropriate values for λ_{reg} can be accomplished via a reliable automated technique, namely the L-curve criterion. The L-curve is a log-log-plot of the solution norm, $\|\delta\|$, versus the residual norm, $\|X\delta - Y\|$, with λ_{reg} as the parameter. The L-curve consists of two parts: One where the regularization error dominates and another where the perturbation error is more pronounced. The optimal regularization parameter must lie somewhere near the L-curve's corner. If the corresponding L curve does not have a sharp elbow corner, a clear best value for λ_{reg} cannot be found. With the proper choice of λ_{reg} , it is possible to recover a reasonable estimate of the kernel.

For its determination the unknown function $b(x,y)/B$ is cast as a finite sum of m unknown expansion coefficient a_j and known real-valued basis function L_j :

$$\frac{b(x,y)}{B} = \sum_{j=1}^m a_j L_j(x,y) \quad (\text{Eq. B3})$$

Laguerre polynomials were used to construct the sets of basis function for this inverse problem (Abramowitz and Stegun, 1965):

$$L_n(x) = \sum_{k=0}^n \binom{n}{k} \frac{(-1)^k}{k!} x^k \quad (\text{Eq. B4})$$

$$L_n(x,y) = L_i(x) L_j(y) \quad (\text{Eq. B5})$$

The similarity variable z is discretized into n points transforming equation A1 into a matrix form. By substituting equation B3 into A1, the integral operator X and Y are given as:

$$X_{ij} = \int_0^{z_i} \Phi'(x) dx \int_{z_i-x}^{\infty} \frac{\Phi'(y)}{y} L_j(x,y) dy \quad (\text{Eq. B6})$$

$$Y_i = z_i \Phi'(z_i) \quad (\text{Eq. B7})$$

The double integral in equation B6 can be re-written then:

$$\bar{Y}_{i,j}(z_n) = \int_0^{z_n} \Phi'(x) L_i(x) dx \bar{Y}_j(z_n - x) \quad (\text{Eq. B8})$$

$$\bar{Y}_j(z_n - x) = \int_{z_n-x}^{\infty} \frac{\Phi'(y)}{y} L_j(y) dy \quad (\text{Eq. B9})$$

We defined a_{ij} where $m = (i-1)m_{basis} + j$ and m_{basis} is the number of basis functions along $= m$ each axis. The unknown vector for expansion coefficients a_m in equation B3 can then be evaluated. Here, we utilized $m_{basis}=4$ with 10 independent expansion coefficients ($a_m=10$). The constraints used in conjunction with the calculation of the kernel are (i) symmetry (Eq. B10), and (ii) positivity everywhere (Eq. B11). The symmetry constraint was applied so that $a_{ij} = a_{ji}$. The numerical integral in equations B8 and B9 were calculated with the trapezoidal rule. We chose $n=50$ as the number of equidistant points along the z axis:

$$\sum_{j=1}^m a_j L_j(x, y) \geq 0 \quad (\text{Eq. B10})$$

$$\overline{X}_{i,j}(z_n) \overline{a}_{ij} \geq 0 \quad (\text{Eq. B11})$$

Highlights

- A PBE model was set up for stem cell aggregate size distribution over time.
- PBE kernels were obtained as inverse problem solutions for various stirring rates.
- The rate of change of average aggregate size was greater at intermediate stirring.
- Results from simulation were in agreement with transient aggregate size data.
- This framework can assist in better design of stem cell bioprocesses.

Figure 1.

Stem cell aggregate size distributions and time-variant component calculation. (A) Results for distributions of aggregate sizes at different time points post-seeding and different agitation rates are shown at 2 (*), 5 (□), 8 (△) and 11 hours (○). Representative runs are shown from each stirring speed. (B) Plots of $S(t)$ based on experimental data (○; Eq. A9) from three independent replicates ($n=3$) for each agitation rate and after fitting (—). Data points are shown as mean $\pm$ SD.

Figure 2.

Similarity distributions $\Phi(z)$ obtained from experimental size distributions of cultured ESC clusters at different agitation rates. Results from representative runs are shown at 2 (*), 5 ($\square$), 8 ($\triangle$) and 11 hours ($\circ$). The solid lines correspond to a fitted γ -distribution (Eq. A12).

Figure 3.

Calculation of the aggregation kernel $K(v, v')$ for ESCs cultured in stirred-suspension vessels. (A) L-curves obtained by applying minimization over λ_{reg} values (ranging from 10^{-8} to 1 logarithmically (base 10) equidistant) for different agitation rates (data pooled from at least three independent experiments per stirring speed). (B) For different agitation rates, the kernel function $K(v, v')$ is depicted as calculated from the inverse problem based on the experimental aggregate size data (3 independent runs) of cultured mESC's up to 11 hours after seeding.

Figure 4.

Predicted size distributions and sensitivity analysis. (A) The PBE simulation with the kernel function obtained as an inverse problem solution at different agitation rates is compared to the experimental data from representative runs at 11 hours. (B) Total number of aggregates (zeroth moment of $n(v,t)$) is shown over time for different agitation rates. (C) Kernel functions are shown when the inverse problem is solved after introducing a 10% error to the similarity distributions and $S(t)$.

Figure 5.

PBE simulation results represented as lines for days 1–4 of culture under various stirring conditions. Experimental data points from representative runs are also shown for comparison. (A) PBE with $K(x,y)=0$. (B) PBE with $K(x,y)$ as in Eq. 13. Simulation-derived distribution lines and points are shown for 24 (blue, *), 48 (red, □), 72 (green, ○) and 96 hours (magenta, △).

Table 1

Parameter values (mean $\pm$ SEM) calculated from $S(t)$ for different agitation rates (n=3 for each agitation rate).

Parameters	60 rpm	80 rpm	100 rpm
B	$1.210 \pm 0.099 \times 10^6$	$1.454 \pm 0.533 \times 10^6$	$2.483 \pm 0.407 \times 10^3$
λ	-0.627 ± 0.017	-0.598 ± 0.044	-0.02 ± 0.015

Table 2

Parameters of the approximating Γ distribution for the similarity distribution at different agitation rates (n=3 independent replicates per stirring speed).

Parameters	60 rpm	80 rpm	100rpm
A_1	32.36 ± 1.12	41.02 ± 2.32	78.57 ± 1.07
α_1	2.94 ± 0.09	3.05 ± 0.16	3.84 ± 0.10
B_1	4.40 ± 0.02	4.61 ± 0.12	5.04 ± 0.01
A_2	48.88 ± 2.63	54.36 ± 2.10	56.21 ± 1.24
α_2	11.27 ± 0.48	12.14 ± 0.36	13.11 ± 0.10
B_2	6.65 ± 0.13	6.97 ± 0.91	7.67 ± 0.08

Table 3

Parameter values (mean $\pm$ SEM, n=3) calculated from $S(t)$ for different agitation rates after introducing a 10% error.

Parameters	60 rpm	80 rpm	100 rpm
B	$1.407 \pm 0.123 \times 10^6$	$1.701 \pm 0.632 \times 10^6$	$2.734 \pm 0.319 \times 10^3$
λ	-0.626 ± 0.017	-0.598 ± 0.063	-0.002 ± 0.010

Table 4

Parameters of the approximating Γ distribution after a 10% error is introduced to the similarity distribution at different agitation rates with 3 independent replicates per condition.

Parameters	60 rpm	80 rpm	100rpm
A_1	54.19 ± 3.03	38.33 ± 7.24	99.26 ± 3.92
α_1	3.11 ± 0.44	2.96 ± 0.46	3.92 ± 1.26
B_1	5.03 ± 0.22	4.42 ± 0.59	5.20 ± 0.17
A_2	20.56 ± 4.91	130.74 ± 10.62	43.07 ± 7.74
α_2	7.73 ± 0.83	14.05 ± 0.81	12.56 ± 0.95
B_2	4.92 ± 0.33	8.02 ± 0.69	6.79 ± 0.22