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Development of a flow cytometry–based pulse-width assay for detection of aggregates in cellular therapeutics to be infused by catheter

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Abstract

Background aims. Delivery of cell-based therapies through the carotid artery with the use of an intra-arterial catheter could introduce aggregates and cause focal ischemia in the brain. We developed a pulse-width flow cytometry method for aggregate detection and quantification. The assay was designed to be used as a cell product release assay in a clinical trial seeking to treat ischemic stroke with sorted cells brightly expressing aldehyde dehydrogenase ($ALDH^{br}$ cells) delivered through intra-arterial catheters. *Methods.* The forward light scatter pulse-width axis of a flow cytometer was calibrated for particle diameter measurements through the use of traceable standard microspheres and linear regression. As a positive control, Concanavalin A–aggregated cells were counted manually and sorted onto slides to compare with pulse width–determined values. Known numbers of aggregates were spiked into purified singlet cells for quantification. A clinical standard for aggregate count and diameter was determined. The assay was used to qualify catheters with the use of $ALDH^{br}$ cells. *Results.* The pulse-width axis was highly linear for microsphere diameter ($r^2 > 0.99$), which allowed for size calibration. Microscopically determined counts and diameters corresponded to pulse width-determined values. Known aggregate counts were linear with pulse width–determined aggregate counts ($r^2 = 0.98$). The limit of detection was determined to be 0.004%. Flow of $ALDH^{br}$ cells through catheters did not generate aggregates. The final method to be used as a release assay for the stroke clinical trial was tested successfully on samples from volunteer donors. *Conclusions.* The pulse-width aggregate detection assay provides a reliable, reproducible, accurate and rapid means of detection, classification and quantification of aggregates in cell therapy products.

Key Words: *bone marrow, cell aggregation, flow cytometry, pulse width, stem cells, stroke*

Introduction

Cell-based therapies are currently being investigated to treat cardiovascular diseases such as stroke, heart disease and peripheral artery disease. These therapies often require the use of micro-catheters to deliver cells directly to the site of injury (such as damaged myocardium or ischemic cortex), which eliminates cell loss in the systemic circulation but is frequently more invasive than an intravenous infusion. The safety of intra-arterial delivery must be assessed to determine the suitability of its use in the delivery of cellular therapeutics. Because biological products can respond to physical and chemical stimuli by adhesion or aggregation, the US Food and Drug Administration recommends testing protocols for cardiac delivery devices that assess cellular viability, adhesion to the device and aggregation [1]. The use of catheters to infuse cells into the ischemic cortex has the additional risk of delivering cellular aggregates that may cause additional focal ischemic infarcts in the brain. Therefore, the occurrence of cellular aggregates in cell therapies designed to be delivered into the brain should be determined, as well as the effects of catheter delivery on aggregation of the cellular product. Pulse-width flow cytometry offered a viable option as a rapid method for reliable aggregate sizing and quantification.

A current Phase 1/2 clinical trial is investigating the safety and efficacy of aldehyde dehydrogenase (ALDH) bright cells in subjects aged 30–83 years who have had unilateral, predominately cortical, ischemic strokes in the middle cerebral artery

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distribution in the prior 13–19 days [2]. Treated subjects receive injections of bone marrow derived stem cells that express high levels of the enzyme ALDH by intra-carotid injections through a standard radiologic micro-catheter into the area of ischemia. These cells are characterized by low side scatter (SSC^{lo}) on flow cytometry and bright (br) expression of a fluorescent substrate for ALDH. These cells, known as $ALDH^{br}$ SSC^{lo} cells (or simply $ALDH^{br}$ cells), are a heterogeneous stem and progenitor cell population containing hematopoietic, endothelial and mesenchymal progenitors that have broad regenerative potential [3–12]. The safety of delivering $ALDH^{br}$ cells has been studied in clinical trials of critical limb ischemia and ischemic heart failure [3,5].

Limiting the number of aggregates above a specified size is thought to be important in reducing the potential for injury during infusion of cells into the arterial system. Prior investigators used intra-carotid infusion of 100 times the number of cells and 7–33 times the number of particles >30 μm than would have been infused in a maximal dose of $ALDH^{br}$ cells from a single manufacturing run with 5% aggregates >30 μm. In those studies, no adverse neurological events were reported in more than 80 patients treated [13,14]. Therefore, we set our release criteria for aggregates in an $ALDH^{br}$ patient dose as ≤5% of all non-debris events and ≤150,000 total particles >30 μm. This aggregate diameter and count should have no clinical significance, given the number and diameters of cerebral capillaries and as demonstrated through safe infusion by others.

We therefore sought to develop a method to rapidly and accurately determine the number of aggregates in a cell therapy product. Whereas automated methods of cell detection and counting (including flow cytometry) are routinely found to be more reliable, less subjective and less time-consuming than microscopy-based methods of cell quantification, the automated method must be validated to demonstrate that this is the case before implementation [15–18]. We developed and tested a method to use pulse-width flow cytometry to quantitatively determine the diameter, percentage and number of cellular aggregates in samples of $ALDH^{br}$ cells.

Pulse width provides an accurate measure of particle diameter. In flow cytometry, a particle passing through a laser beam generates a detectable light pulse with a particular intensity (pulse height) for a particular period of time (pulse width) for any given parameter of detection (fluorescence or light scatter). The integral of the two is the pulse area. Forward (or low angle) light scatter height or area (FSC-H or FSC-A) in flow cytometry are often referred to as relative measures of the size of the cell; however, it is well attested that cytometer optics and particle physics cause wide variations in the relationship between actual particle diameter and FSC-H or FSC-A signal intensity [19]. Meanwhile, the pulse width is usually independent of the types of optical variances that affect pulse height. Pulse width is a factor of laser beam height, particle velocity and particle diameter. Because constant sheath pressure provides uniform velocity of particles as they pass through the beam, and the beam height is constant within an instrument setup, the pulse width can be directly correlated to the diameter of the particle (assuming uniform shape and consistent angle of incidence during flight between different detected particles, which is optimized by minimizing sample core stream diameter). Therefore, pulse-width flow cytometry can distinguish between single cells and aggregates [20]. The most common application of pulse width is in the exclusion of doublets during cell cycle analysis [21–23]. In DNA ploidy analysis, the fluorescence pulse width generated by the nuclear dye is most often analyzed by pulse processing [24]. However, with the use of known size standards, the diameter of the particle can be correlated directly to light scatter pulse width independent of the use of a fluorescent label (for particles with a diameter larger than the beam height or when using mathematical normalization on smaller particles) [19,25–28].

Because it is important to detect large particles of any composition, not only labeled cells, we sought to develop an assay that did not rely on fluorescence. Additionally, the assay should use existing electronics and optics on a commercial instrument without need for beam height subtraction. Whereas side scatter typically has a higher quality detector (a photomultiplier tube rather than a photodiode), others have used FSC pulse width (FSC-W) successfully to measure small particles [27]. Furthermore, the forward scatter parameter on the FACSAria cell sorter with the use of FACSDiva software (BD Biosciences, San Jose, CA, USA) is provided with an FSC area scaling control; thus, it is the only parameter that allowed precise range scaling of its width axis without excluding events through "zooming" in the software (FACS indicates fluorescence-activated cell sorting). In practice, FSC-W provided high linearity from 6–75 μm, sufficiently verifying the quality of this parameter.

Experimentation showed that diameters of positive control biological aggregates determined by linear regression of known microsphere standards correlated well with diameters determined by quantitative microscopy of sorted aggregates. Testing also showed the assay to have a high level of accuracy and sensitivity to detect known counts of aggregates spiked into suspensions of purified singlet cells.

The method was then applied to test the aggregation of $ALDH^{br}$ cells formulated for delivery into