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Technical note

An optical alignment system improves precision of soluble aggregate quantitation by sedimentation velocity analytical ultracentrifugation

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ABSTRACT

Appropriate characterization of soluble aggregates is an important aspect of biologics development and manufacturing, and sedimentation velocity analytical ultracentrifugation (SV-AUC) is often used an orthogonal technique to size-exclusion chromatography (SEC) for this purpose. Precise quantification of low levels of soluble aggregates by SV-AUC can be adversely impacted by improper cell alignment. This report describes the development of an optical system capable of quantifying cell alignment that affords a substantial improvement compared to historical approaches.

The characterization and control of aggregation is an important aspect of biologics development. Size-exclusion chromatography (SEC) is the common method of choice in the biopharmaceutical industry for the quantification of soluble aggregates [1,2]. However, SEC is subject to several potential limitations such as interactions of the protein with the stationary phase, differences between formulation and mobile phase matrices preventing direct analysis under formulated conditions, the inability to quantify very large aggregates, or characterization of reversible self-association. Due to these potential limitations, an orthogonal technique is typically required to characterize aggregation [1–3]. Sedimentation velocity analytical ultracentrifugation (SV-AUC) is not subject to the same limitations and is commonly employed in this role.

The precision of aggregate quantification by SV-AUC may be decreased by convective flow, particularly for low levels of aggregates [4–7]. The primary sources of convection in SV-AUC include centerpiece geometry, quality, alignment, and thermal control [8]. Temperature-driven convection can be minimized by adequate incubation of the sample and rotor at the run temperature inside the centrifuge [4]. The interaction of solutes with the centerpiece walls is minimized in sector-shaped centerpieces [9], given they are properly aligned. If sector walls are not aligned parallel to the centrifugal force, the accumulation of solute molecules on one wall and corresponding depletion on the opposite wall causes convection [8,10,11]. Misalignment of cells has been shown to lead to a systematic increase in apparent aggregate levels [4–6]. Imperfections in the sector walls such as scratches or pitting can also lead to convective flow [8], but recent advancements in centerpiece manufacturing have improved the precision of SV-AUC data [5].

Typically, cells are aligned either visually (using scribe marks on the rotor and cell housings) or mechanically. Different approaches for mechanical alignment have been proposed, including commercially available tools such as the cell alignment tool from Spin Analytical (Spin CAT) and custom-built models such as that described in Ref. [4]. Although mechanical alignment has been demonstrated to be superior to visual alignment [4–6], both of these techniques indirectly align the sector walls using the scribe lines or rectangular slots on the bottom of the cell housing. None of these can necessarily ensure that the sector walls of the centerpiece are parallel to the centrifugal force; therefore, a means to directly align sector walls in filled cells loaded into rotors is highly desirable.

Building on principles outlined in previous work from Hersh and Schachman [12], we have developed and built a custom optical alignment (OA) system based on a digital vision system. A loaded rotor is mounted into the OA system using the rotor drive hole, and rotated until a spring-loaded indexing pin engages the bottom of a rotor hole. The cell to be aligned is located directly opposite from

Abbreviations used: SD, standard deviation; SEC, size-exclusion chromatography; CW, clockwise; CCW, counter-clockwise; IP, intermediate precision; mAb, monoclonal antibody; OA, optical alignment; SV-AUC, sedimentation velocity analytical ultracentrifugation; Spin CAT, AUC Cell Alignment Tool.

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the indexing pin, thus the OA system can accommodate both the Beckman An-50 Ti and An-60 Ti rotor types. Cells are illuminated from the bottom and a digital image is captured from above using a digital vision system equipped with a telecentric lens (Fig. 1A). The resulting images are processed to yield the alignment angle and sector angles (Fig. 1B). If the resulting cell alignment is outside of the target range, the cell is manually rotated, another image is captured, and the process is repeated until a satisfactory alignment is achieved.

The uncertainty of cell alignment when using the OA system, expressed as the standard deviation (SD), was assessed in a series of related experiments using a Beckman An-60 Ti rotor. The uncertainty attributable to image analysis was determined by collecting ten images of an empty cell (SD = 0.008°, n = 10). When repeated on cells filled with water, the uncertainty increased approximately two-fold (SD = 0.015°, n = 10), consistent with blurring of sector edges caused by refraction. Removing and remounting the rotor in the OA system further increased the uncertainty by approximately three-fold (SD = 0.046°, n = 30). Finally, the uncertainty of the difference between pre- and post-run (approximately 3 h at 60,000 rpm) alignment was determined (SD = 0.086°, n = 107). These results indicate that the typical experimental uncertainty of alignment, putatively caused by inadvertent cell movement during rotor handling and/or centrifuge operation, is more than five-fold greater than the measurement capability of the OA system. The accuracy of the OA system was assessed by comparing the mean of the measured sector angles (2.47°, n = 208 total determinations from N = 21 unique centerpieces) to the value of 2.5° specified by the manufacturer.

The relative performance of a mechanical (Spin CAT) and a visual (scribe lines) alignment techniques was evaluated using the OA system. While the Spin CAT demonstrated overall higher precision compared to visual alignment (Fig. 1C), the spread in alignment angles across different cells suggests that the alignment accuracy may be impacted by 'internal misalignment' [5,6], a situation in which proper external alignment of the cell housing does not necessarily result in the radial alignment of the sector walls. With minimal manual adjustments cells can be routinely aligned to

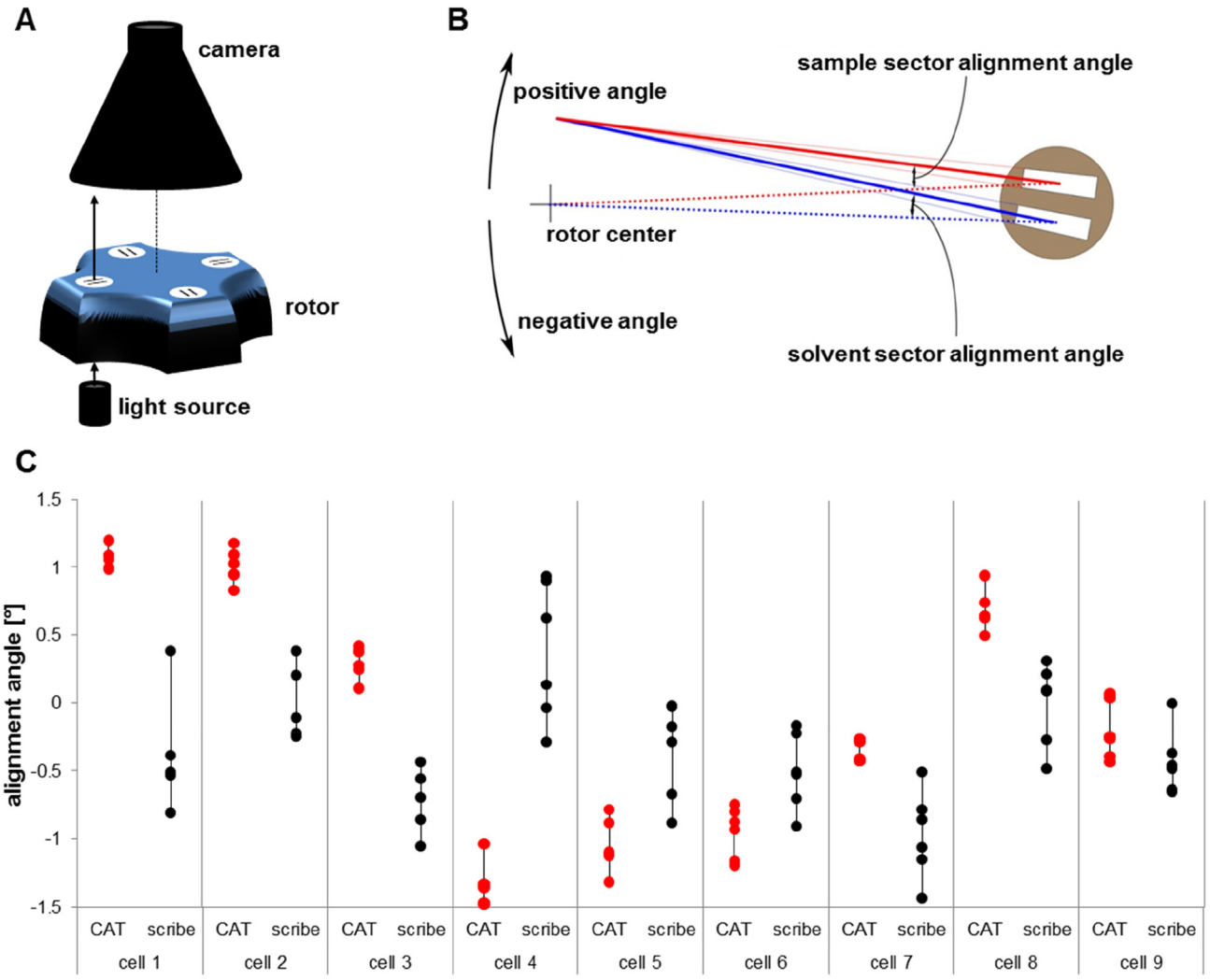

Fig. 1. **(A)** OA system consisting of a light source and camera with telecentric lens. The system can accommodate both the Beckman An-50 Ti and An-60 Ti rotor types.
(B) Schematic of alignment angle determination as implemented in the OA system, as viewed from top of rotor (not drawn to scale). Following image acquisition the center of the rotor and edges of the sample and reference sectors are located using image processing software embedded within a commercially available machine vision system. Alignment angles and sector angles are then calculated from these points/segments.
(C) The relative performance of the mechanical Spin Analytical CAT tool (red circles) and visual alignment using the scribe marks (black circles). Nine cells (Beckman cell housings and two-sector centerpieces) were aligned a total of six times each using both alignment techniques, and the OA system was used to determine alignment angles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)