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Application of Gravitational Field-Flow Fractionation (GrFFF) for Monitoring of Clustering Behavior of *Staphylococcus aureus*

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Staphylococcus aureus (*S. aureus*) is a resident micro-organism, which causes toxic shock syndrome by food poisoning and some nosocomial diseases through intra-vascular catheter. The shape of *S. aureus* is spherical with diameter of around 1 μm. They tend to clump together to form aggregates and eventually large grape-like clusters.[1-4]

Clumping of *S. aureus* is caused by interactions among the components of surface adhesines including several clumping factor proteins.[3,4] The sizes and the shapes of the *S. aureus* clusters vary with parameters such as the incubation time and temperature, the growth phase, and the composition of the culture medium, *etc.* A cluster composed of several cocci produces a single colony as does a single coccus in the same medium.

For the evaluation of the cells, the cell-counting is usually used, which counts the number of viable cells. The clustering phenomenon makes accurate evaluation of the bacteria difficult as the cell-counting measurement counts each cluster as a single cell regardless of its size. This generally requires additional analysis for more complete understanding of the *S. aureus* cells.

Field-flow fractionation (FFF) is a family of selective flow-assisted separation techniques suitable for separation of nano to micron-sized particles or macromolecules of various origins.[5] Gravitational FFF (GrFFF) is a member of FFF family, which employs the Earth's gravity as the external field. GrFFF has been shown to be suitable for the characterization of various types of micron-sized particles including latex particles,[6] cells,[7,8] starch granules,[9-11] and bacteria.[12] GrFFF provides size-based separation of micron-sized particles with larger ones eluting earlier than smaller ones.[13] For GrFFF calibration, retention times of a series of standards (with known diameters) are measured, and a plot of log t_r, *vs*. log d is established.[14,15]

In this study, *S. aureus* cells were cultured, and then analyzed by GrFFF, optical microscope (OM), and cell-counting to study the change in the morphology of the cells as a function of the incubation time.

Experimental

Bacterial incubation. The *S. aureus* used in this study was KCTC1621 (Korean Collection for Type Culture, Daejeon, Korea). Bacterium was stock in a saline solution containing 20% Glycerol (Sigma chemical, St. Louis, MO, USA) at −80 °C. Bacterium was cultivated at 30 °C in Nutrient Broth (NB, Becton Dickinson, MD, USA). The liquid culture was shaking-incubated in an Erlenmeyer flask at 180 rpm in a rotary incubator (Jeio Tech, Daejeon, Korea). In order to obtain the cell concentration, the optical density (OD) was measured at 600 nm with a spectrophotometer (Turner 340, Dubuque, USA) in the unit of colony-forming units (CFU) per milliliter. This measurement was repeated three times for each sample.

Gravitational Field-Flow Fractionation (GrFFF). The GrFFF channel was built in laboratory as described in a previous report.[6] The channel dimensions were 51 cm in tip-to-tip length, 2 cm in breadth, and 0.01 cm in the thickness. Samples (20 μL) were injected through a rubber septum using a 50 μL Hamilton hypodermic syringe (Reno, Nevada, USA). The carrier liquid was water containing 0.05% (w/v) sodium dodecyl sulfate (SDS) (Sigma chemical, St. Louis, MO, USA). The carrier flow was delivered by a HPLC pump (Young-Lin SP930D, Seoul, Korea) at a fixed flow rate of 1 mL/min throughout this study. The GrFFF eluent was monitored by a M720 UV/vis detector (Young-Lin Science, Anyang, Korea) set at 302 nm. All GrFFF analysis was repeated at least twice until the elution profiles are reproducible.

Optical Microscopy (OM). Optical microscopy (OM) was performed by using an Olympus BX51TF OM (Shinjuku Monolith, Japan). For all OM analysis, about 500-1,000 cells were measured by using the OM Image Inside software (Focus, Daejeon, Korea). For the OM size measurements of *S. aureus* clusters, the longest dimensions of the clusters were taken.

Results and Discussion

Figure 1 shows the GrFFF fractograms of *S. aureus* suspensions obtained at various incubation times (t) of 0, 4, 6, 12, 16, 24, 48, 120, and 168 hrs, respectively. In all fractograms, intense void peaks appear at around 1 min (void time), which are likely due to the elution of small

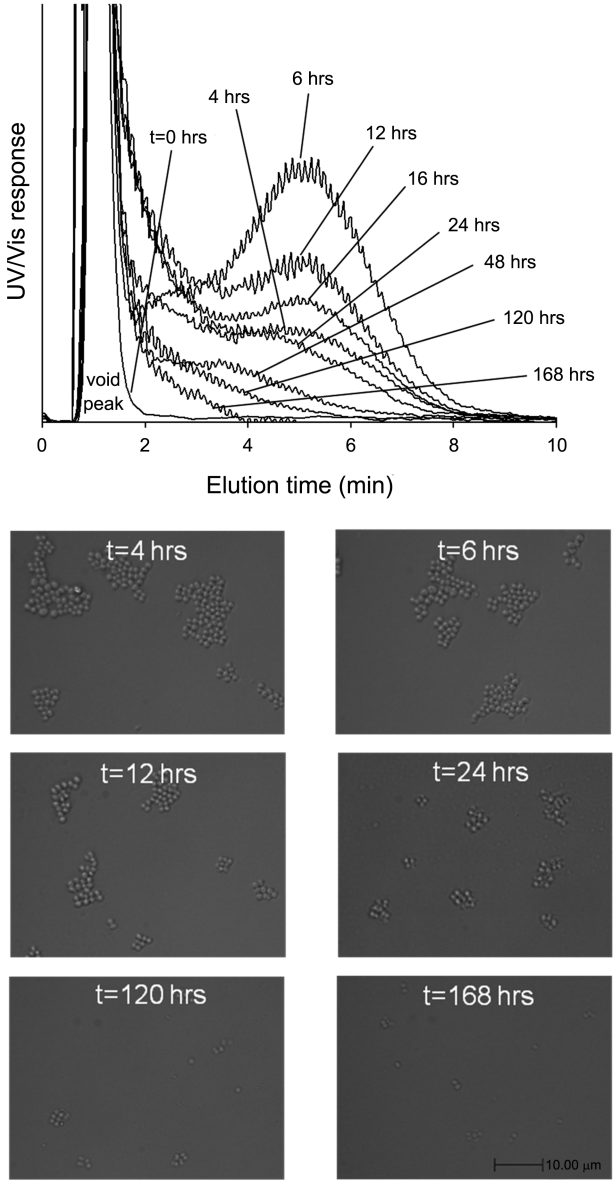

Figure 1. GrFFF fractograms and OM pictures of *S. aureus* clusters obtained at various incubation times.

unretained species including the residue in the culture medium, and single coccal cells. All GrFFF runs were completed within about 10 min.

As shown in Figure 1, at the beginning of the incubation ($t = 0$), no components are eluted after the void peak, suggesting there are no clusters large enough to be separated from the void peak. As the incubation proceeds, the *S. aureus* cells form larger clusters, which are eluted as a separate broad band. As the incubation time increases, this broad band of the clusters grows, suggesting more of the large clusters are formed. The band grew until the incubation time of 6 hrs, after which slowly shrinks probably due to disaggregation of the clusters. After about 7 days ($t = 168$ hrs), the GrFFF elution profile returns to where it started ($t = 0$), suggesting most of the large clusters were disaggregated to become smaller.

Figure 1 also shows OM pictures of the *S. aureus* clusters.

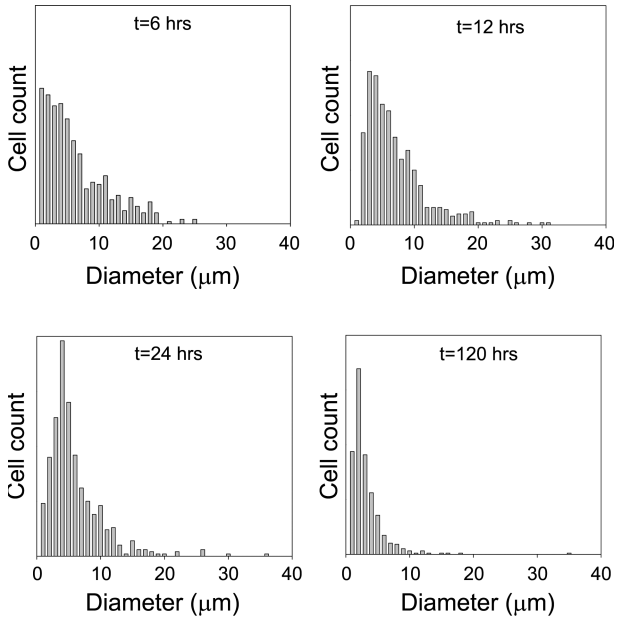

Figure 2. OM size distributions of *S. aureus* cells and clusters in the samples shown in Figure 1.

It can be seen that the cells aggregate to form clusters during incubation, as expected. At the incubation times of 4 and 6 hrs, the clusters of various shapes and sizes are seen, and some of larger ones are larger than about 10 μm in their longest dimensions. While the sizes and the shapes of the clusters are varied, the sizes of the coccal cells remain almost unchanged with the diameters around 1 μm. At the incubation times longer than 6 hrs, the sizes of the clusters decrease as the incubation time increases, probably due to disaggregation. This reduction in the cluster size is thought to be caused by the growth limitation or death of bacteria due to consumption of nutrients and accumulation of wastes in the medium. The overall population of the cluster was greatly reduced at the incubation times of around 120 hrs and longer.

Figure 2 shows the size distributions of the *S. aureus* cells and clusters determined by OM. As the incubation time increases longer than 6 hrs, the OM size distribution shifts toward the lower size, indicating the size of the clusters are decreasing by disaggregation. The size distribution measured at the incubation time of 120 hrs shows most of the cells are smaller than about 5 μm.

Figure 3 shows the number of viable cells measured as a function of the incubation time. The cell-counting was made at the unit of colony forming unit (CFU) per unit volume (mL). The population of viable cells gradually increases until the incubation time of 24 hrs. In cell-counting, each cluster is counted as a single cell regardless of its size. With the same number of viable cells, the cell-count will be lower if the sizes of the clusters increase. The increase in the viable cell-count after the incubation time of 6 hrs seems to be the result of disaggregation of the clusters, producing higher number of smaller clusters. It can be seen that, after the incubation time of about 24 hrs, the viable cell count quickly

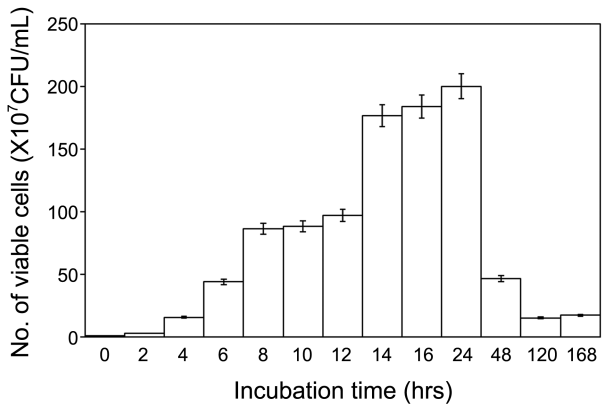

Figure 3. Number of viable cells measured at various incubation times. Each sample was measured three times, and error bars represent one standard deviation.

drops due to death of the cells.

Conclusion

The cell-counting measurement counts each cluster as a single cell regardless of its size. Thus the cell-counting cannot distinguish a single cell from a cluster, and requires additional analysis for more complete understanding of the clustering behavior. GrFFF provides useful and complementary information on the clustering behavior of the *S. aureus* cells. The GrFFF fractogram grows as the clusters become larger. With its capability of size-based separation, GrFFF could also provide information on the change in the size distribution of the clusters. GrFFF is gentle to living cells, and the analysis is relatively simple and fast with the total analysis time of less than about 10 min. With size-sensitive detectors such as the light scattering detector connected online, GrFFF may yield more information (e.g., absolute size and the density of the clusters) on the clustering behavior of the *S. aureus* cells. Also planned is further optimization of GrFFF for analysis of *S. aureus* cells, including quantitative measurement of the recovery during GrFFF analysis.

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