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Sedimentation field-flow fractionation device cleaning, decontamination and sterilization procedures for cellular analysis

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Abstract

In Sedimentation FFF (SdFFF) practice, it is known that a large number of cell elutions create aging phenomena of the separator, thereby reducing recovery and modifying elution characteristics. Systematic cleaning procedures are developed to enhance channel lifetime, together with microbial decontamination processes. Cells can be therefore reproducibly eluted for a large number of analyses and collected under sterile conditions, if needed. This is one of the most valuable aspect if further culture or transplantation is required. Decontamination was performed using, as contaminant probe, *Staphylococcus aureus*, highly adherent pathogenic bacteria that eluted from SdFFF as aggregates.

Keywords: Sedimentation field-flow fractionation; Cleaning; Sterilization; Cellular analysis

1. Introduction

Analysis and sorting of living cells in suspension is a major goal in life sciences. A wide panel of techniques and methodologies are available [1]. Field-Flow Fractionation (FFF) methodology was introduced in the late 1960s by Giddings [2]. This chromatographic-like separation family, in particular Sedimentation-FFF (SdFFF), appears to be particularly well suited for isolation and characterization of micron-sized species such as cells [1,3]. Like all other FFF methods, SdFFF is based on the differential elution of the species in a liquid (mobile phase) flowing through a ribbon-like capillary channel on a laminar mode [3]. Separation depends on the specific particle susceptibility to an external field applied perpendicularly to the greater surface of the ribbon. SdFFF elution mode for cells is known as ''Hyperlayer'' [1,3,4]. In such a mechanism, cell size, density, shape and rigidity are involved, as are channel geometry and flow-rate characteristics [1,3–6]. Extensive qualitative development of this elution mode has already been published [1,3,6–13]. SdFFF has shown a great potential for cell separation and purification with major biomedical applications. Fifteen years ago, Caldwell et al. [5] defined most of the basic rules and methodologies for cell separation. Since this pioneering report, many studies on cell

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separation and sorting have been performed on different cell types [14–23].

SdFFF cell separations require some specific methodological and technological feature development. SdFFF cell separation must respect cell integrity and viability as well as provide enhanced recovery and purified fraction collection under sterile conditions [24,25]. For this purpose, a carrier phase of isoosmotic buffer and containing biocompatible surfactant as well as channel wall material (polycarbonate) are selected to limit particle–particle or particle–channel wall interactions [26]. Nevertheless, cell suspension are themselves complex mixtures as well as many of their components: cell fractions, proteins, culture medium residues, or other undefined substances, may indeed absorb onto the channel wall, leading to surface characteristic modifications often described as ''channel poisoning'' [24,25]. This effect limits reproducibility, viability and cell recovery. We described, in this report, the systematic use of a specific cleaning procedure to improve cell viability and recovery at the separator outlet. This procedure can be associated with decontamination for defining instrumental and methodological sterile cell separation, which would allow for fraction collection and further cultivation.

Red blood cells (RBC) were used as a probe sample during the cleaning procedure. To study the efficiency of the decontamination procedure, the separation device was artificially contaminated by serial injections of *Staphylococcus aureus* (*S. aureus*). *S. aureus* is a model for channel contamination because of its ubiquitous distribution [27] and its pathological importance. Morphologically [28,29], *S. aureus* appeared as a Gram positive cocci which forms doublets, triplets or larger grape-like aggregates [30]. *S. aureus* aggregation is linked to cell surface expression of specific polysaccharides (adhesines), which constitute the microcapsule also called ''slime'' [31,32]. This latter component played an important role in surface adhesion [29]. Thus, *S. aureus* is a highly contaminating particle whose adhesion properties will considerably complicate decontamination procedures. Few data on FFF bacteria separation and elution mode have been published [33–38]. In our study, *S. aureus* in suspensions could not be eluted as single particle but rather in aggregates whom elution mode appeared to be ''Steric''.

2. Theory

The FFF elution mode theories have been extensively described elsewhere [3,8–13]. Briefly, three different elution modes are described: ''Brownian'', ''Steric'' and ''Hyperlayer''; depending on: (1) the intensity and the nature of the external field; and (2) particle characteristics such as size, density and shape. The elution order of separated species depends on their average velocity (average position in the channel thickness) and was measured in terms of the retention ratio R that is the ratio of the sample zonal velocity versus the mobile phase one.

Sub-micron-sized species elution mode is described as ''Brownian''. Retention ratio depends on the particles external field susceptibility, their diffusion coefficient and the external field strength [3,12]. At the same density, particles least affected by the field and/or having a high diffusion coefficient protrude into faster streamlines and are eluted first [1,3,12]. In the ''Brownian'' model, retention ratio is indirectly dependent on the external field strength and independent on the flow-rate [12].

Two elution models are described for micron-sized species: ''Steric'' and ''Hyperlayer'' [1,3,8–13]. In these cases, R depends on the particle size, and elution order is reversed in comparison to the ''Brownian'' model. In ''Hyperlayer'' mode, also called ''Focusing'' mode [12,13], the flow velocity/channel thickness balance generates a hydrodynamic lift force which drive the particles away from the accumulation wall. Species are then focused into a thin layer which corresponds to an equilibrium position in the channel thickness where the external field is exactly balanced by the hydrodynamic lift forces [1,3,8–13]. At equivalent density, large particles generate more lift forces and are focused in faster streamlines to be eluted first. Retention ratio is flow-rate and external field dependent. At constant field, the increase in flow-rate induce an increase in R, and at constant flow the increase in field decreased R.

If external fields can be increased sufficiently, or flow-rate decreased sufficiently to offset lift forces, micron-sized particles are confined into a very thin layer close to the accumulation wall. Then, elution mode was described as ''Steric'' [3], and appeared as a limit case of ''Hyperlayer''. Retention ratio is, as a consequence, only dependent on the particle radius

and channel thickness, but independent both of the external field and of the flow-rate [12]. That is, large particles protrude in faster flow streamlines and are eluted first [1,3,12].

3. Experimental

3.1. Biological samples

S. aureus strain (ATCC 6538P), was routinely cultured on Trypto–Casein–Soybean (TCS) agar (AES laboratories, Cambourg, France). Before SdFFF elutions, freshly cultured colonies (24 h) were suspended in sterile isotonic solution (NaCl 9.0 g/l, 0.2% m/v Bovine Serum Albumin [BSA]) to obtain 9.10^7 UFC/ml (UFC: Unit forming colonies) which correspond to an absorbance of 0.61 turbidimetrically measured at 550 nm (Stalabo spectrophotometer, Metrève, France). The UFC/absorbance value correspondence was verified as follows. The bacterial suspension was serially diluted from 10^0 to 10^{-6}; 0.1 ml of bacteria dilutions were cultured, in triplicate, on TCS agar. Petri dishes were incubated for 24 h at 37°C, and colonies (one colony corresponds to one UFC) were optically counted. Results were expressed as UFC/ml of bacteria suspension.

Morphology was systematically verified by microscopic examination after Gram coloration (Leica DM LB optical microscope, Leica microsystèmes, Reuil-Malmaison, France), before and after SdFFF elution, and on sonicated and unsonicated bacteria samples. Bacteria were sonicated (Bransonic 220, 10 min) to study the impact of *S. aureus* morphology on SdFFF elution mode.

Surface hydrophobicity of *S. aureus* was evaluated by using a previously described method [39] based on the selective distribution of bacteria suspension between an aqueous phase and a non-miscible organic phase (isooctane). A series of sterile culture tubes were prepared: to 1.2 ml of bacteria suspension (absorbance≈1.5 at 550 nm), an increasing volume of isooctane from 0 to 0.5 ml was added. After overnight incubation, the aqueous phase absorbance was measured at 550 nm and compared to the standard suspension (without isooctane). A decrease in aqueous phase absorbance could be linked to the bacteria affinity for the organic phase and thus demonstrated the hydrophobic characteristics of the bacteria surface.

Red blood cells (RBC): freshly drawn human blood was collected in EDTA containing tubes (Vacutainer, Meylan, France) and immediately stored at 4°C. RBC samples were prepared by 20-fold dilution in sterile isotonic phosphate buffered saline (PBS), pH 7.4, supplemented with 0.1% BSA (Sigma–Aldrich, Saint Quentin-Fallavier, France). Such a procedure led to a sample suspension less than 2% in volume. As described before [16], Coulter Counter® counting showed that the remaining platelets, ghosts and nucleated cells were not significant in number. The average cell size was analyzed using a Model TA II Coulter Counter® (Coultronic, Andilly, France). The average diameter of the RBC sphere equivalent volume was 5.1 μm±0.2 μm (n=10).

3.2. SdFFF system

The SdFFF separation device used in this study was derived from those previously described and schematized [16,20]. The separation channel was made up of two polycarbonate plates of 870×30×2 mm, separated by a Mylar® spacer in which the channel was carved. Channel dimensions were 785×10×0.250 mm with two V-shaped ends (50 mm) for the *S. aureus* elution mode study, and 785×10×0.125 mm with two V-shaped end (50 mm) for RBC retention analysis. Measured total void volumes (channel volume+connection tubing+injection and detection device) were respectively 2160±20 μl (n=15) and 1175±15 μl (n=15). Void volumes were calculated after injection and retention time determination of an unretained compound (0.5 g/l of Benzoic acid, UV detection at 254 nm). Inlet and outlet Peek® tubing (Upchurch Scientific, Oak Harbourg, USA) of 0.504 mm I.D. were directly glued to the external polycarbonate plate (accumulation wall). Then polycarbonate plates and Mylar spacer are sealed into a centrifuge basket. The channel-rotor axis distance was measured at r=13.8 cm. Sedimentation fields are expressed in units of gravity, 1 G=980 cm/s^2, and calculated using rotational speed (rpm: rotation per min) and r as below:

$$G = \left(\frac{\text{rpm} \times 2\pi}{60}\right)^2 \times r \quad (1)$$

Two rotating seals [16], drilled to allow 1.62 mm external diameter Peek® tubing to fit in, were used to permit the mobile phase to flow through the channel. An M71B4 Carpanelli engine associated with a pilot unit Mininvert 370 (Richards Systems, Les Ullis, France), controlled the rotating speed of the centrifuge basket. All devices involved in injection, cleaning and decontamination procedures are schematically represented in Fig. 1: (1) a Gilson Model 302 chromatographic pump (Gilson Medical Electronics, Middletown, WI, USA) was used to flow the sterile mobile phase, 3° sodium hypochloride solution and sterile distilled water (Fig. 1, device A); (2) prior to the sample injection device, two other injection devices were inserted: a Rheodyne® 7125i chromatographic valve (Fig. 1, device B), and a V-100L switching valve (Upchurh Scientific, Oak Harbour, NJ, USA) used to divert the flow off the channel during stop-flow injection (Fig. 1, device C); (3) samples were injected by means of a Rheodyne® 7125i chromatographic injection device (Rheodyne, Cotati, CA, USA) (Fig. 1, device D). The elution signal for RBC detection was recorded at 313 nm, by mean of a Knauer variable wavelength monitor (Knauer-France, Strasbourg, France) and a 14-byte M1101 (100 mV input) acquisition device (Keithley instrument, Palaiseau, France) operated at 1 Hz and connected to an Macintosh computer.

3.3. Cleaning and decontamination procedure

The different steps of cleaning and decontamination required a specific instrumentation set up, as schematically described in Fig. 1. Some devices are

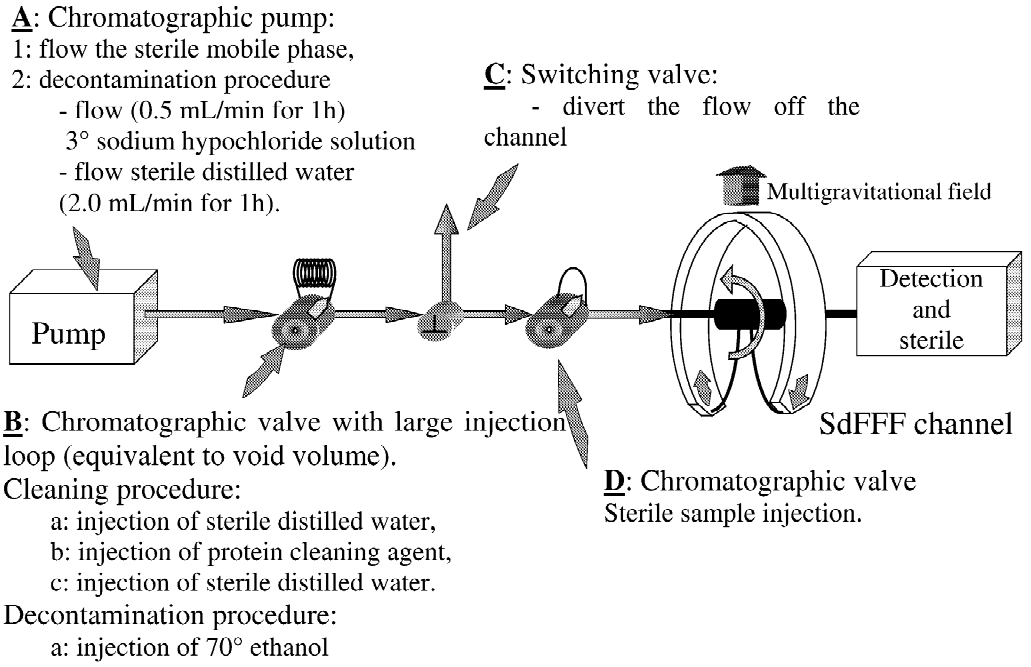

Fig. 1. Scheme of successive devices involved in sample injection and in SdFFF channel cleaning and decontamination procedure. Cleaning procedure takes place at the end of each sample elution and started with rotation stopped. In a first step, the carrier phase is flushed (**A**) at high flow-rate. In a second step, a void volume equivalent of sterile distilled water using the large volume loop (**B**) is injected. In a third step, a second void volume equivalent of a ''protein cleaning agent'' is injected (**B**). Finally, a multi-void volume sterile distilled water flush completed the procedure (**B**). Decontamination procedure: first after cleaning procedure, the whole SdFFF device was flushed with a 3° sodium hypochloride solution **(A)**. Second, the system was rinsed with sterile distilled water (**A**). Finally, the system was refilled with the sterile mobile phase. Prior cell injection, the channel could be flushed by means of a void volume equivalent injection of ethanol (70°) and rinsed by the sterile mobile phase (**B and A**).

specific for the cleaning procedure. Others were used for cleaning or decontamination as well as for elution (Fig. 1, device A). Fig. 1 caption describes the different specific purposes of the SdFFF system components.

Cleaning procedure takes place at the end of each sample elution and started with rotation stopped. First, the carrier phase was flushed at high flow-rate to allow the release of reversible adsorbed particles (Fig. 1, device A). Secondly, an osmotic shock was promoted by means of a void volume equivalent injection of sterile distilled water using the large volume Rheodyne® loop (Fig. 1, device B), and led to the release of cellular material. The remaining adsorbed material was released, in a third step, by means of a second void volume equivalent injection of a sterile (0.22 μm filtration) ''protein cleaning agent'', using the same large volume Rheodyne® valve (Fig. 1, device B). Finally, a multi-void volume sterile distilled water flush completed the procedure, using the large volume injection loop (Fig. 1, device B).

Decontamination procedure consisted of specific steps following the cleaning procedure. First, as described in Fig. 1, after cleaning, the whole SdFFF device was flushed at a 0.5 ml/min flow-rate for 1 h with a 3° sodium hypochloride solution (Fig. 1, device A). Secondly, the system was rinsed to eliminate the sodium hypochloride with sterile distilled water (2.0 ml/min for 1 h) (Fig. 1, device A). Finally, the system was refilled with the sterile mobile phase used for sample separation. Routinely, decontamination procedure was performed every day. Prior to cell injection, the channel was flushed by a void volume equivalent injection of ethanol (70°, Fig. 1, device B) and rinsed by the sterile mobile phase (Fig. 1, device A).

To study procedure efficiency, 2 ml of mobile phase were collected at the end of each decontamination procedure. A 0.1 ml aliquot of these 2 ml collected fractions was cultured on TCS agar (non-selective bacteria culture medium) for 48 h at 37°C. This analysis was performed before any sample injection and results are given in Table 1 in the ''Initial condition'' column. After experimental contamination of the SdFFF system by injection of 50 μl of a *S. aureus* 9.10^7 UFC/ml suspension, a new decontamination procedure and sterility control were performed, the corresponding results are reported in Table 1 under the ''Decontamination'' column. The procedure efficiency was determined in each case (initial and decontamination conditions), by the absence of any bacterial development after 48 h incubation at 37°C.

An end of the day decontamination was systematically performed after a *S. aureus* elution mode study. Results of these controls were shown in the ''End-Day decontamination'' column of Table 1.

Table 1
Decontamination procedure efficiency[a]

External field strength	Total bacteria (UFC) recovery	Bacterial development after 48 h incubation		
		Initial	Decontamination	End-Day decontamination
40 *G*	28.45%	Negative ($n=5$)	Negative ($n=5$)	
60 *G*	27.84%	Negative ($n=5$)	Negative ($n=5$)	Negative
80 *G*	28.20%	Negative ($n=5$)	Negative ($n=5$)	($n=20$)
100 *G*	23.34%	Negative ($n=5$)	Negative ($n=5$)	

[a] Decontamination procedure: the whole SdFFF device was flushed for 1 h with a 3° sodium hypochloride solution, rinsed and refill as described in ''Experimental'' section. Then 2 ml of mobile phase were collected at the end of each procedure, and 0.1 ml were subcultured on Trypto–Casein–Soybean agar for 48 h at 37°C. The procedure efficiency was determined by the absence of any bacteria development after 48 h incubation. Decontamination procedure, and its efficiency evaluation, were done before (Initial) and after (decontamination) the experimental SdFFF device contamination by stop-flow injection (5 min) of 50 μl of a *S. aureus* 9.10^7 UFC/ml suspension: channel dimension $785\times10\times0.250$ mm; mobile phase: sterile isotonic solution of 9.0 g/l NaCl, 0.2% m/v BSA; flow-rate: 1 ml/min; under variable external multigravitational field; off-line detection (counting) of eluted sample. Results displayed corresponded to the mean of 5 experiments ($n=5$). An End-Day decontamination was also realized and associated with a bacteriological control (''End-Day decontamination'').

3.4. SdFFF S. aureus elution mode

To study the *S. aureus* SdFFF elution mode, an off-line bacteria detection was chosen for signal selectivity enhancement. After stop-flow (5 min) injection of 50 μl of a *S. aureus* 9.10^7 UFC/ml suspension, eluted fractions of 1 ml were systematically collected during the entire elution process (mobile phase: sterile isotonic solution of 9.0 g/l NaCl, 0.2% m/v BSA; flow-rate: 1 ml/min). Then, 0.1 ml of each fraction was cultured, in triplicate, on Chapman agar. Petri dishes were incubated for 24 h at 37°C, and colonies (1 colony corresponds to 1 UFC) were counted optically. Thus, SdFFF fractograms which are a function of elution time and a mean of UFC number can be constructed. Off-line detection was chosen instead of on-line detection (DO measurement at 550 nm for example) to obtain selectivity for *S. aureus* and to control the correlation between the SdFFF elution mechanism and the nature of eluted particles, particularly by using *S. aureus* selective culture medium (Chapman agar) to determine the eluted UFC.

4. Results and discussion

4.1. Cleaning procedure

Many previously published reports [16,20,24–26], have demonstrated the interest in using BSA as a surfactant to reduce cell-channel wall interactions. Metreau et al. [16] showed that more than 90% of the injected species (RBC and synoviocytes) were collected at the outlet of the separating device. Fig. 2A is a representative RBC elution fractogram obtained by using a new or a clean channel, and after stopping channel rotation (End of Run: ER), no significant cell elution signal was recorded which demonstrate a very low level of cell release. A Coulter Counter® enumeration of collected fractions showed that 95.6±0.5% ($n=15$) of the injected cells were eluted. Thus, the low percentage (around 5%) of unrecovered cells observed after sample injection into a new or clean channel corresponded to a cell sticking (reversible or not) into the SdFFF system. With repeated separations, proteins either from the carrier phase or from cell samples, led to channel

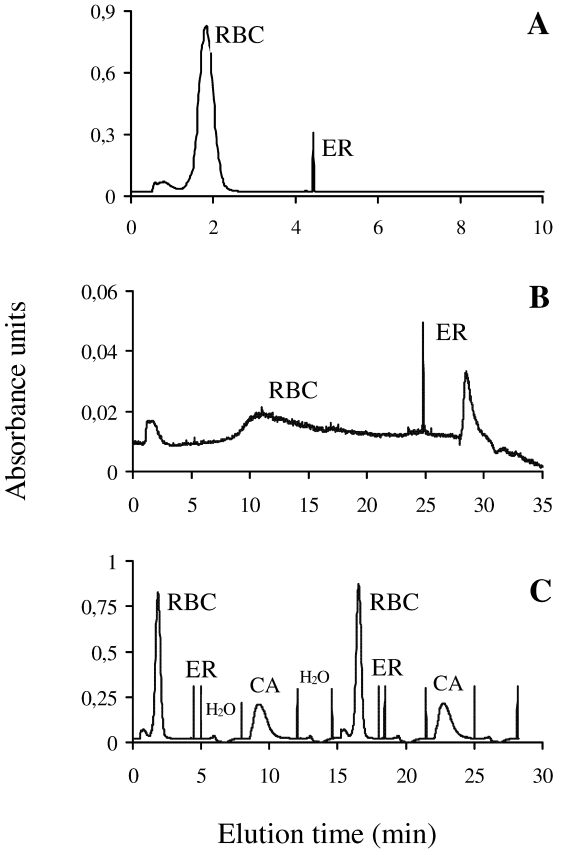

Fig. 2. SdFFF RBC elution and cleaning procedure efficiency. Fractograms were obtained with a SdFFF apparatus (785×10×0.125 mm). **A:** RBC elution profile on a new or properly washed FFF channel. Elution conditions: flow injection of 2.5×10^6 RBC (10 μl of 1/20 dilution of total blood in phosphate buffer saline pH 7.4/0.1% of bovine albumin), external field 12.9 *G* ($1G=9.81$ cm.s^{-2}), flow-rate: 1.0 ml/min, photometric detection at $\lambda=313$ nm. **B:** Channel poisoning effect observed after 47 identical injections (described in **A)**. **C:** Two sequences of RBC elution (described in **A**) and channel cleaning procedure. Each sequence is: RBC fractogram, End of Run (ER:external field stopped), hypo-osmotic shock with doubly distilled water (H_2O), cleaning agent (C. A.) signal and second water washing.

wall surface modifications, causing channel aging and poisoning which enhanced cell sticking. In the absence of cleaning procedure, the systematic injection of RBC sample led to a progressive and massive channel poisoning (after 47 injections) as shown in Fig. 2B which represented the last RBC elution of the series. This short term channel poisoning led to an important decrease in cell recovery and

elution signal repeatability and reproducibility, as measured by ratio peak area/peak height for new or clean channel versus old channel after 47 injections, and by the large peak shape modification as observed by the comparison between Fig. 2A and 2B. To overcome this inconvenience, after each sample elution a cleaning procedure was performed. Fig. 2C showed a representative fractogram of two successive RBC elutions separated by a cleaning procedure injection. We observed that cleaning conserved high recovery and repeatability, without cell release signal post-elution. Moreover, these results demonstrated that a systematic cleaning procedure allowed a long term channel use without decrease in reproducibility, recovery and very limited channel aging. Thus, more than a hundred elutions followed by cleaning procedures were performed without significant modifications of channel characteristics, reduces channel maintenance and allows use of the same channel for analysis of various cell populations without sample cross-contamination.

4.2. Decontamination procedure

As previously described, SdFFF demonstrated a great potential for cell separation and purification in order to develop biomedical applications [4,16,17,19,20]. Nevertheless, these applications depended, particularly, on the possibility of cell culture after SdFFF elution. For this, a good recovery, repeatability, reproducibility and cell viability (goal of cleaning procedure) associated to a strict sterility are necessary. To achieve this goal, the SdFFF separator and all associated devices must be stored under a laminar flow hood if possible. All injected solutions and the mobile phase must be sterilized before use. To ensure total sterility of the separation device, decontamination was performed using sodium hypochloride solution (3°) to eliminate microorganism contamination. This procedure must take into account the toxic properties of sodium hypochloride for cells, and a time-consuming sterile distilled water rinsing was required.

To improve our decontamination procedure efficiency, we artificially contaminated the SdFFF device by injection of *Staphylococcus aureus*. These bacteria are known to express a high surface adhesion potential, and are particularly involved in nosocomial diseases [29]. The study of *S. aureus* surface properties, demonstrated an important hydrophobicity as shown by the lower affinity for the aqueous phase compared to the organic phase [39], as shown in Fig. 3.

Results in Table 1 show *S. aureus* recovery after injection of a constant amount of bacteria. The increase in external field strength led to a decrease in recovery due to an increase in particle–channel wall interactions and an increase in bacteria adhesion to the channel wall. In the light of Table 1 and Fig. 3 results, the low recovery percentage could be explained by the large degree of hydrophobicity of *S. aureus* strain which is involved in the, reversible or not, bacteria sticking to the accumulation wall, even in the presence of surfactant.

Nevertheless, results of Table 1 show that whatever SdFFF conditions of *S. aureus* elution, no bacterial development was observed on Trypto–Casein–Soybean agar after 48 h at 37°C. This clearly indicated the effectiveness of decontamination which eliminated bacterial contamination even after a 100*G* SdFFF separation coupled with considerable cell adhesion (23% recovery, Table 1).

Thus by associating cleaning and decontamination, cell retention and collection can be achieved with high reproducibility, recovery, viability, under sterile conditions. The validity of these procedures are demonstrated even in the case of drastic elution conditions such as high external field strength, low flow-rate, channel pollution, or elution of hydrophobic particles.

4.3. Elution mode of S. aureus

As previously described by Giddings [3], the general elution mode of bacteria or related species (small unicellular organisms) can be ''Brownian''. In particular, Giddings [3] described a SdFFF *Escherichia coli* separation based on mobility difference. Mobile bacteria are eluted under a ''Brownian'' elution mode because high mobility properties were assimilated with a large experimental diffusion coefficient *D*. Analogous results were obtained by Berg and Tuner [33]. On the other hand Hoffstetter-Kuhn et al. [17], described the SdFFF elution mode of *Saccharomyces cerevisiae* as ''Hyperlayer''. Gao et al. [36], showed that the SdFFF elution mode of

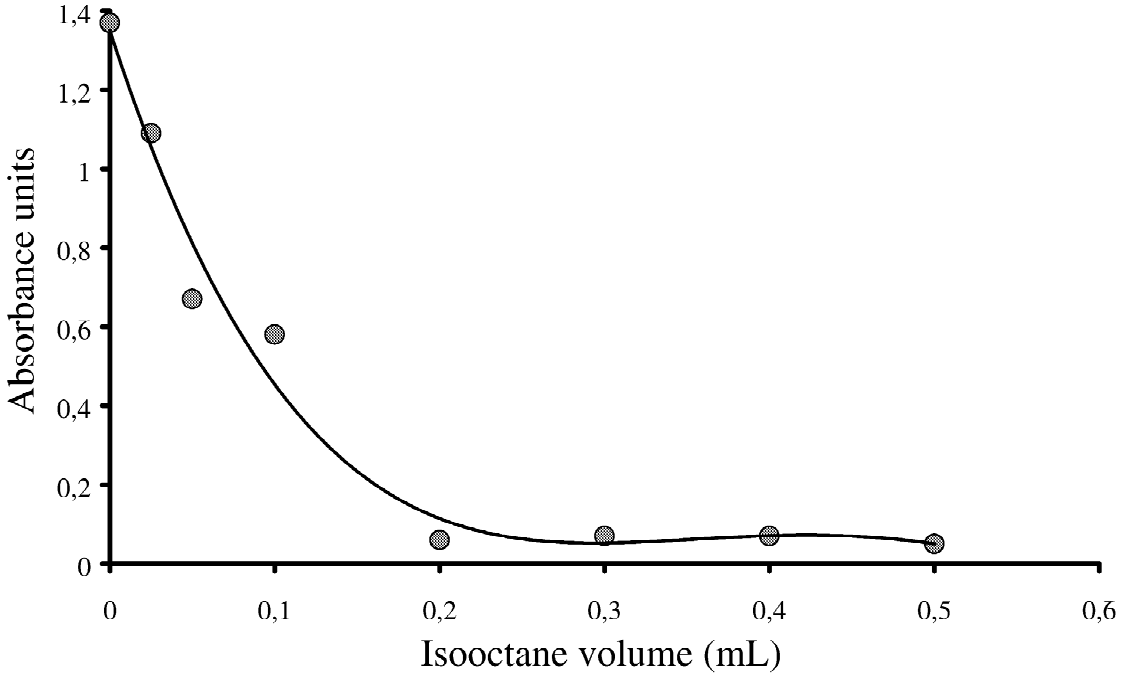

Fig. 3. Hydrophobic properties of *S. aureus*. It was evaluated by using a previously described method [40], based on the selective distribution of bacteria suspension between an aqueous phase and a non-miscible organic phase (isooctane). Into sterile culture tubes, we added to 1.2 ml of bacteria suspension (Absorbance≈1.5 at 550 nm), an increasing volume of isooctane. After overnight incubation, the aqueous phase absorbance was measured at 550 nm and compared to the standard suspension (without isooctane). The hydrophobic characteristic of bacteria surface was demonstrated by the decrease in aqueous phase absorbance.

free individual *Stenotrophomas maltophilia* (0.9–1.2 μm equivalent sphere diameter soil bacterium) was ''Hyperlayer'' because larger particles eluted first and the retention factor decreased when the applied field strength increase [36]. Sharma et al. [34] have previously used *S. aureus* as a cocci probe to characterize and quantify the relationship between the cell number and the biomass by using SdFFF. The elution mode was in that case ''Brownian'' [34]. However, fractionation was obtained for formaldehyde fixed bacteria which were sonicated before injection to resuspended cells to eliminate clumping and particle–wall interactions [34].

The actual elution mode of the living *S. aureus* appeared thus particularly interesting because of its pathological importance being its surface adhesion properties linked to the slime production that causes aggregate formation and enhances the onset of human diseases such as nosocomial infection [27,28]. Aggregate formation can determine the actual elution mode of a 1–2 μm diameter Gram positive cocci liquid culture, as shown in Fig. 4. Gao et al. [36] already described the presence of such aggregates, although, they were easily dissociated by increasing flow-rate. Nevertheless, in the experiments described in this report, these aggregates were not easily disrupted, even after ultrasonication.

Analysis of SdFFF elution mechanism of *S. aureus* was realized under various external field strengths. Stop-flow injections were performed using 5 min as a relaxation time (τ) [7,11]. Fractograms displayed in Fig. 5 corresponded to the number of UFC collected at the outlet versus the elution time. They represent the mean UFC corresponding to five experiments (*S. aureus* injection at a given field strength), for each collected fraction. All fractograms were similar in shape, with the presence of two separated peaks that are defined as peaks I and II

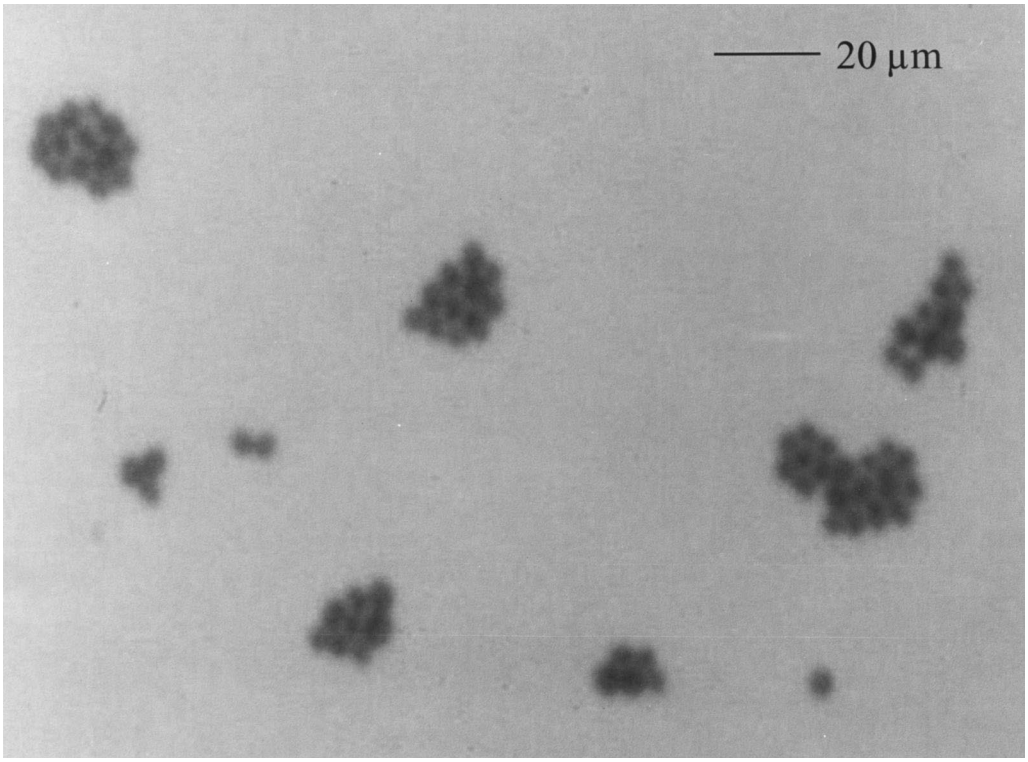

Fig. 4. Morphology of *S. aureus* strain. Representative picture of *S. aureus* strain GRAM coloration (Leica DM LB optical microscope, Leica microsystèmes, Reuil-Malmaison, France, magnification: ×1000), obtained before or after SdFFF elution of sonicated or not bacteria suspension.

(Fig. 5). Because off-line detection was used, we know that bacteria are eluted either in peak I (elution time corresponds to channel void time) or in peak II. Specific off-line detection showed a correlation between SdFFF elution and the nature of eluted particles by: (1) checking the viability of eluted bacteria and thus their capacities to develop contamination process during cellular materials elution; (2) the specificity of eluted bacteria by the use of Chapman agar which represented a selective *S. aureus* culture medium.

The measured retention factor R of particle eluted in peaks I and II (Fig. 5), was respectively $R = 1.01 \pm 0.02$ (σ, $n = 5$); and $R = 0.24 \pm 0.02$ (σ, $n = 5$). Retention ratio appeared constant whatever the applied external field strength (40–100 G). Moreover, Gram staining coloration demonstrated the presence of clusters, either in injected suspensions (before SdFFF injection and separation) or in peak I and II collected fractions. Clusters eluted in peak I appeared larger (30–40 bacteria, data not shown) than those eluted in peak II which are composed of 5–15 bacteria as shown in Fig. 4. These smaller aggregates are not dissociated by SdFFF separation (Figs. 4 and 5) or by sonication (data not shown). The large clusters eluted in peak I appeared to be more easily dissociated, as shown experimentally in Fig. 5 by the decrease in peak I signal correlated to the increase of peak II intensity consecutive to the increase in external field strength. A similar result was obtained after sonication where peak I height decreased at the same external field strength (data not shown).

As described in the ''Theory'' section, the elution order, the size of eluted particles and the independence of R from the external field strength results would indicate that living *S. aureus* clusters could be

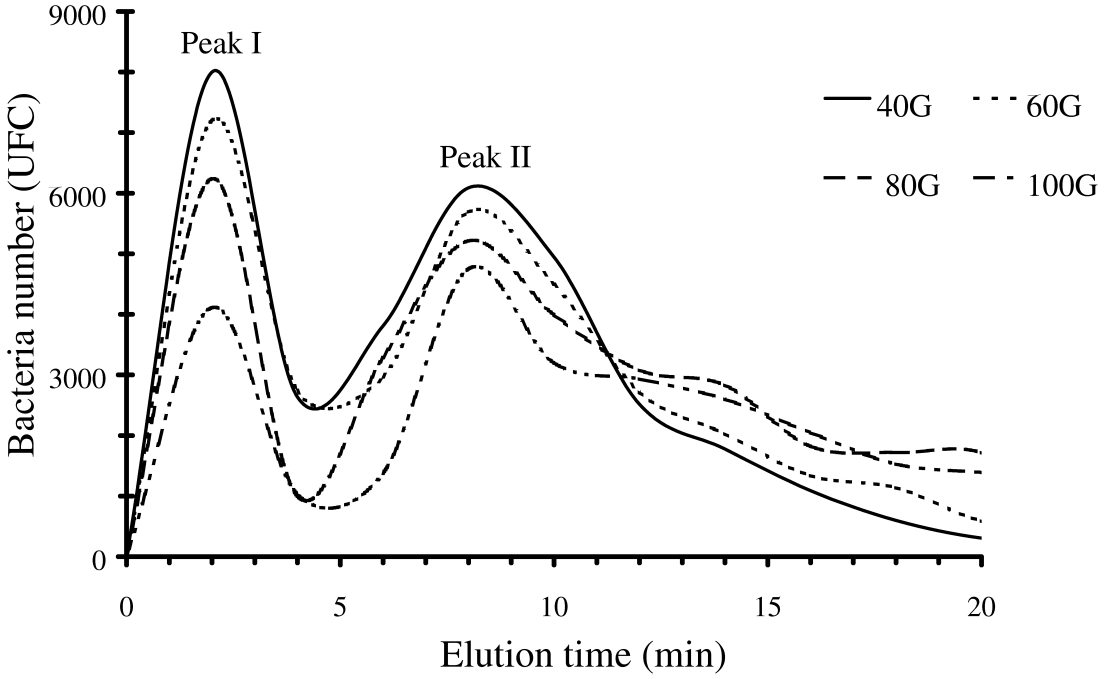

Fig. 5. SdFFF elution of *S. aureus*. Fractograms were obtained through the SdFFF apparatus: channel dimensions 785×10×0.250 mm; stop-flow injection (5 min) of 50 μl of a *S. aureus* 9.10^7 UFC/ml suspension; multigravitational external field: 40–100 *G*; mobile phase: sterile isotonic solution of 9.0 g/l NaCl, 0.2% m/v BSA; flow-rate: 1 ml/min; Off-line detection (counting) of eluted bacteria.

eluted according to the ''Steric'' mode. However, as recalled by Chmelik [12], pure ''Steric'' elution has been experimentally observed in few cases [8–11,40] and further work on the SdFFF elution model of *S. aureus* has to be performed.

5. Conclusion

Cleaning procedures in FFF enhances cell separation reproducibility as well as recovery. Decontamination procedures demonstrated that SdFFF can be operated under sterile conditions. The possibility to elute and separate cell subpopulations under sterile conditions allows their future use in biotechnology (cell cultivation or transfection), or in transplantation. It is therefore possible to introduce SdFFF as a mature technology for cellular engineering, biotechnology and cell transplantation. In this demonstration, the use of *S. aureus* as a contamination probe demonstrated the efficiency of decontamination procedure, as these highly surface-adhesive particles are withdrawn from the channel or destroyed. *S. aureus* is shown to be eluted and separated as clusters by ''Steric'' SdFFF elution.

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