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Micro-thermal field-flow fractionation of bacteria

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Abstract

The retention of *Staphylococcus epidermidis* bacteria cells, achieved with the use of micro-thermal field-flow fractionation and described in this paper, represents the first experimental proof that the separation and characterization of the bio-macromolecules and biological particles is possible by exploiting Ludwig–Soret effect of thermal diffusion. The experiments were carried out under gentle experimental conditions preventing the denaturation of the bacteria. Lift forces, appearing at high linear velocities of the carrier liquid, generated the focusing mechanism of the retention which resulted in high-speed and high-performance separation performed in less than 10 min.

Keywords: Micro-thermal field-flow fractionation; Bacteria; *Staphylococcus epidermidis*

1. Introduction

Separation and analytical characterization of the macromolecules and particles of the biological origin represents a permanent challenge. Many separation methods and techniques were described in the literature and are used in current laboratory practice. Field-flow fractionation (FFF) belongs to the family of the methods which are particularly suitable for the separation of the biological macromolecules and particles because the retention is due to the gentle interactions of the separated species with the physical fields. Consequently, the separations can be performed under the conditions preserving the viability of the solutes. Various cells, bacteria, proteins, nucleic acids, viruses, etc., have already been separated and characterized by sedimentation FFF (SFFF), flow FFF (FFFF), and electrical FFF (EFFF). These separations discriminate the solutes according to the differences in their sizes, densities, diffusivities, electrical charges, etc., correspondingly to the nature of the applied field.

All three mentioned FFF methods were used also for the separation and analysis of various bacteria [1–16]. The first paper, published already in 1985 by Fox et al. [1], concerned the application of SFFF which, in the meantime, became the most frequently used FFF technique when taking into account the number of published papers [1–11]. However, it seems that the use of FFFF, firstly applied in 1996 by Ross et al. [3], is more intensively studied in the last 10 years [1,3,7,10,12–16]. The EFFF was only exceptionally used by Saenton et al. [7] who compared it with SFFF and FFFF. The list of the cited papers on the applications of FFF to the analysis of the bio-macromolecules and biological particles is not exhaustive, however, these works represent well the potentials of FFF.

At our best knowledge, thermal FFF (TFFF) has never been applied to the separation of the species of the biological origin. This is probably due to the fact that standard size channels for TFFF need relatively high energy power (of the order of kilowatts) so as the total heat flow across the channel thickness is high enough to generate the sufficient thermal diffusion flux and thus the efficient separation. Unfortunately, the evacuation of such an enormous heat flow from the cold wall represents a difficult problem. Consequently, the control of the important cold wall temperature is very delicate or impossible at all if high temperature drop across the channel must be applied. However, this problem is resolved by the miniaturization of the TFFF channel proposed and realized recently [17]. The micro-TFFF, already successfully applied to the separation of synthetic polymers, various nanoparticles, and micron-sized particles [18,19],

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has the potential to become a new high-performance tool for the separation and characterization of the bio-macromolecules and biological particles. In this paper, we describe the first successful retention of *Staphylococcus epidermidis* cells in micro-TFFF experiments carried out under the conditions preventing the denaturation of the bacteria.

S. epidermidis are Gram-positive spherical bacteria cells that occur in microscopic clusters resembling grapes and are common members of the normal skin and mucous membranes microflorae. Generally, staphylococci are facultative anaerobes that can grow by aerobic respiration or by fermentation that yields principally lactic acid. Most strains of *S. epidermidis* are nonpathogenic and may even play a protective role in their host as normal flora. On the other hand, some strains of *S. epidermidis* may be a pathogen in the hospital environment. A characteristic of the pathogenic strains is the production of a slime resulting in biofilm formation. The slime is predominantly a secreted teichoic acid, normally found in the cell wall of the staphylococci. This ability to form a biofilm on the surface is probably a significant determinant of virulence for these bacterial strains. Obviously, a possibility to study the characteristics and behaviour of *S. epidermidis* cells or generally the bacteria with the use of a new analytical tool, micro-TFFF, is challenging.

Micro-TFFF has the potentials to become very competitive method for the separation and analysis of biological macromolecules and particles, in comparison with SFFF, FFFF, and EFFF, for several reasons:

- The range of molecular weights and particle sizes that are retained in micro-TFFF is much more extended in comparison with the SFFF that cannot be used for the macromolecules whose molecular weights are lower than roughly 10^6.
- The use of various water-based or organic solvents as carrier liquids is without any problem in micro-TFFF which is not always the case for FFFF.
- Periodic or systematic cleaning of the micro-TFFF channel is much easier compared to the change of a plugged membrane in FFFF channel.
- Whereas only electrically charged species are retained in EFFF, the electrically neutral and charged macromolecules (such as polyelectrolytes) or particles may be retained in micro-TFFF.

Moreover, a simultaneous use of temperature gradient and electrical field is also possible with micro-TFFF and it may produce more efficient separations. Such a separation of synthetic particles was already performed with the use of standard size TFFF channel [20] and resulted in an interesting and potentially useful tuning of the retention. Therefore, such an experimental setup can also be realized by using micro-TFFF channel.

2. Theoretical bases

In *polarization* FFF, each retained uniform species develops a nearly exponential concentration profile across the channel due to the field generated flux and the opposed diffusion flux. Larger species exhibiting lower diffusion coefficients are usually compressed closer to the accumulation wall in a zone of lower longitudinal velocity within a nearly parabolic flow velocity profile of the carrier liquid. The elution order is thus from the small to the large size retained species. If the field strength is high enough so that all retained solutes are in contact with the accumulation wall, the elution order is inverted because the solutes are eluted correspondingly to the average velocity of the carrier liquid streamline in which they move. This mechanism is called *steric exclusion* mode. On the other hand, if the linear velocity of the carrier liquid increases, the retained species can undergo the effect of lift forces and the steric exclusion mechanism is no more effective but the *focusing* mechanism dominates the separation. In such a case, the micro-thermal focusing FFF provides high-performance and high-speed separations of the large size particles. It has to be stressed, however, that purely "steric" FFF represents rather an exceptional case of separation mechanism.

The retention ratio describing the action of polarization and steric exclusion or focusing mechanisms is [21]:

$$R=\frac{V_0}{V_R} = 6(\delta - \delta^2) + 6\lambda(1-2\delta)\left[\coth\left(\frac{1-2\delta}{2\lambda}\right) - \frac{2\lambda}{1-2\delta}\right] \quad (1)$$

where V_0 is the elution volume of an unretained solute (marker) which is equal to the void volume of the channel, V_R the elution volume of the retained species, $\delta = r/w$ is either the ratio of the radius r of the retained species to the thickness w of the separation channel in the case of steric exclusion mechanism or the ratio $\delta = r_{cg}/w$ of the distance of the centre of gravity r_{cg} of the focused zone to the channel thickness w, in the case of the effective focusing mechanism, and λ is a dimensionless retention parameter defined below. Eq. (1) is rigorously valid only if the flow velocity profile formed inside the channel is parabolic. This is not the case of micro-TFFF because the viscosity varies with the temperature across the channel and thus the flow velocity profile is not strictly parabolic and also the coefficients of molecular diffusion, D, and thermal diffusion, D_T (see below) are temperature dependent. Nevertheless, the use of the approximate Eq. (1) is acceptable for a semi-quantitative evaluation of the experimental results obtained in this work by using micro-thermal focusing FFF.

Retention parameter λ in Eq. (1), valid for hard spherical particles exhibiting the thermal diffusion, is given by [22]:

$$\lambda = \frac{k_B T}{6\pi\mu r D_T \Delta T} \quad (2)$$

where k_B is Boltzmann constant, T the temperature, μ the kinematic viscosity of the carrier liquid, D_T the coefficient of thermal diffusion, and ΔT is temperature drop across the micro-TFFF channel. The retention parameter λ can also be related to the force F of an external field acting on the retained species [23]:

$$\lambda = \frac{k_B T}{Fw} \quad (3)$$

Fig. 1 represents the dependence of the retention ratio on the particle radius calculated from the Eq. (1). The curve a corresponds rigorously to the Eq. (1) and demonstrates the domains

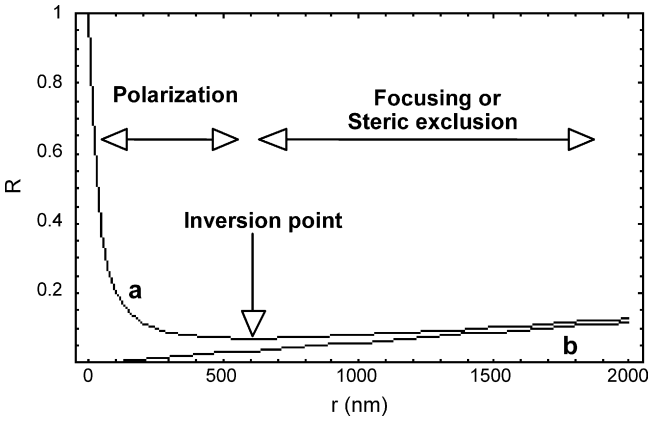

Fig. 1. Theoretical dependence of the retention ratio R on the particle radius r calculated from Eq. (1) by using $w = 0.01$ and Eq. (2) to calculate the dependence of λ on r under the experimental conditions close to those applied in this work by using D_T for polystyrene latex particles determined previously [28]. Curve a corresponds to original Eq. (1), and the apparent straight-line b corresponds to the truncated Eq. (1) with $\lambda = 0$ (only steric mechanism is effective).

of the prevailing polarization and steric exclusion or focusing mechanisms with the inversion point. The apparent straight-line b corresponds to the case when $\lambda = 0$ in Eq. (1) and then either steric exclusion or focusing mechanism is operational. If the focusing mechanism generated by lift forces is operational, the experimentally found dependence $R = f(r)$ should lie above the theoretical straight-line b or above the curve a in the range of particle radius above the inversion point.

3. Experimental

3.1. Micro-TFFF

The experimental setup for micro-TFFF consisted of a syringe pump model IPC 2050 (Linet Compact, Czech Republic) equipped with a special stainless-steel syringe (Institute of Scientific Instruments, Academy of Sciences of the Czech Republic), a commercialized micro-TFFF channel unit (MicroFrac Laboratory, http://www.watrex.cz, Czech Republic), equipped with an electronic device (MicroFrac Laboratory, Czech Republic) regulating the electric power for heating cartridge and controlling the temperature of the hot wall. The dimensions of the micro-TFFF channel used in this work were 0.1 mm × 3.2 mm × 76 mm. The compact micro-TFFF unit was equipped with an injection valve model 7413 (Rheodyne, USA) with a 1 μl loop and with a system of a graduated micro-splitter valve, model P 470, and a micro-metering valve, model P 446 (Upchurch Scientific, USA) allowing the splitting of the carrier liquid flow into two separated entries of the channel and also the casual splitting of the outgoing liquid between the detector and the waste [24].

A variable wavelength detector (Watrex UVD 250, Czech Republic) equipped with 1 μl measuring cell and a UVD 250A Data Monitor Software enabling the PC data collection were used to record the fractograms. The wavelength of 260 nm was chosen. A low temperature thermostat model Ministat 125 (Huber, Germany) was used to control the temperature of the cold wall of the channel. The temperatures at very close proximities of the cold and hot walls were measured also independently by digital thermometer (GMH 3230, Greisinger) equipped with two thermocouples.

3.2. Isopycnic focusing

The densities of the bacterial cells and polystyrene latex (PSL) were determined by using the isopycnic focusing centrifugation. Density gradient was formed in Percoll (Pharmacia, Sweden) which is a suspension of colloidal silica particles covered by poly(*N*-vinyl pyrrolidone). The original Percoll (1.131 g/mL) was diluted with distilled water and a concentrated solution of NaCl so as to obtain density gradient forming medium of the average density of 1.080 g/mL and the concentration of 150 mM/L NaCl. The coloured Density Marker Beads (Pharmacia, Sweden) having well defined and stable buoyant densities were added as markers to monitor density gradient formed during the centrifugation and thus to calculate the densities of the bacteria and PSL from their respective positions in density gradient.

The rectangular centrifugal cuvette containing bacteria and Density Marker Beads suspended in density gradient forming medium described above was centrifuged (MR 23i, Jouan, France) at 7700 × *g* and 20 °C for 2 h. After the run time was completed, the centrifugal cuvette was carefully withdrawn from the centrifuge and photographed.

3.3. Microscopy

Photomicrographs of both PSL and bacteria were recorded using a light microscope with phase contrast (Olympus CX41, Olympus, USA) at a magnification of 1000× and equipped with a digital camera. The mean size of the cells was determined by QuickFOTO PRO 2.0 system (OLYMPUS).

3.4. Chemicals and samples

Isotonic solution (150 mM/L aqueous NaCl) was used as a carrier liquid in micro-TFFF experiments. Spherical PSL particles (Duke Scientific, CA, USA) of narrow particle size distribution were used in this study. The certified mean particle diameter of the PSL sample was 2.062 ± 0.025 μm. The PSL was used as a reference sample.

S. epidermidis cells isolated from human skin were used in all experiments. The culture was identified on the base of its basal properties and with the aid of biochemical micro-test STAPHYTEST 16 (PLIVA-LACHEMA, Czech Republic). The bacterial strain was enriched in 20 mL of liquid medium containing (in $g\,L^{-1}$) tryptone 10, soya peptone 2, yeast extract 2 (Himedia, India), NaCl 2, and K_2HPO_4 0.5 (Penta, Czech Republic) for 22 h at 25 °C. The chosen period of cultivation time gave rise to the prevailing single cells suspension with the average diameter of 0.8 μm (ranging from 0.6 to 1.0 μm). However, the pairs of the cells (average length of 1.5 μm, ranging from 1.2 to 1.7 μm) and the short chains or small cell clusters (ranging from 1.6 to 3.8 μm) were seldom present too. The cells were

harvested by centrifugation (10,000 × *g*, 4 °C, 10 min), washed trice with isotonic saline solution (8.5 g L^{-1} aqueous NaCl) and then suspended in 5 mL of the same saline to obtain approximately 10^8 cells/mL. Prior to use, a nutrient medium and saline solution were sterile filtered using a MILLEX® GP 0.22 μm sterile filter (Millipore) for foreign cells removal.

4. Results and discussion

The picture of a section of centrifugation cuvette in Fig. 2 shows the result of isopycnic focusing experiments aimed to determine the densities of the *S. epidermidis* cells and PSL particles. It can be seen in Fig. 2 that the bacterial cells were focused in two distinct zones of slightly different densities while PSL particles formed only one focused zone. The calibration curve in Fig. 3 represents the dependence of the density on the distance from the bottom of the centrifugal cuvette calculated from the positions at the end of centrifugation of the individual Density Marker Beads. The densities corresponding to two zones of the *S. epidermidis* cells and PSL particles calculated by using this calibration plot are 1.129 g/mL, 1.125 g/mL, and 1.030 g/mL, respectively. Photomicrograph in Fig. 4a confirms the spherical shape of the bacterial cells and, by comparison with the photomicrograph of the PSL sample in Fig.4b, also the diameter of the bacteria which is close to 1 μm.

The optimized injection-stop-flow-time (ISFT) procedure was applied in micro-TFFF experiments. The injection at the low flow-rate of 0.005 mL/min was carried out during 25 s. In this manner, the whole sample volume was introduced into the channel with a minimum zone broadening. Thereafter, the flow rate was stopped for 5 min to allow the establishment of a steady-state concentration distribution (relaxation) of the sample across the channel thickness. Finally, the flow rate was restarted at different higher values. This ISFT procedure was found previously [25,26] as optimal, minimizing the time of the analysis and keeping high the resolution of the separation. The temperature of

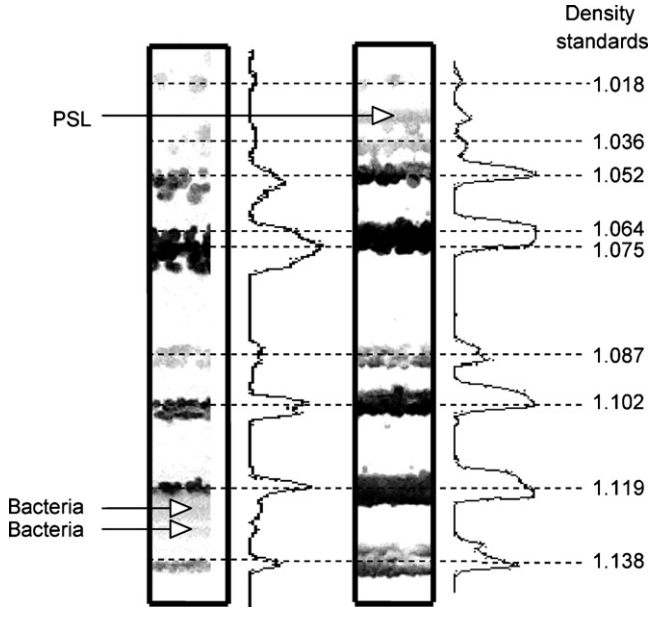

Fig. 2. Picture of the sections of centrifugal cuvettes showing the focused zones of Density Marker Beads (density standards) and the focused zones of the bacteria cells and polystyrene latex particles (PSL). The densitograms on the RHS of the cuvette pictures were obtained by scanning the pictures and data treatment using SCAN-IT-GEL software.

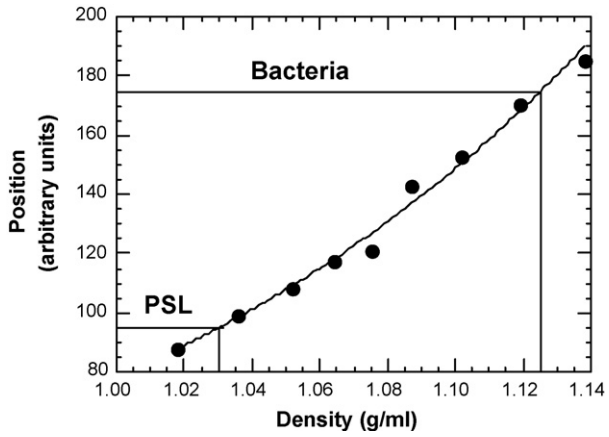

Fig. 3. Calibration plot of the density gradient (formed by centrifugation in density forming medium) showing the dependence of the density as a function of the position (measured from the bottom of the centrifugal cuvette in arbitrary units) of Density Marker Beads. The figure indicates also the positions of the focused zones of the bacteria cells and polystyrene latex particles (PSL).

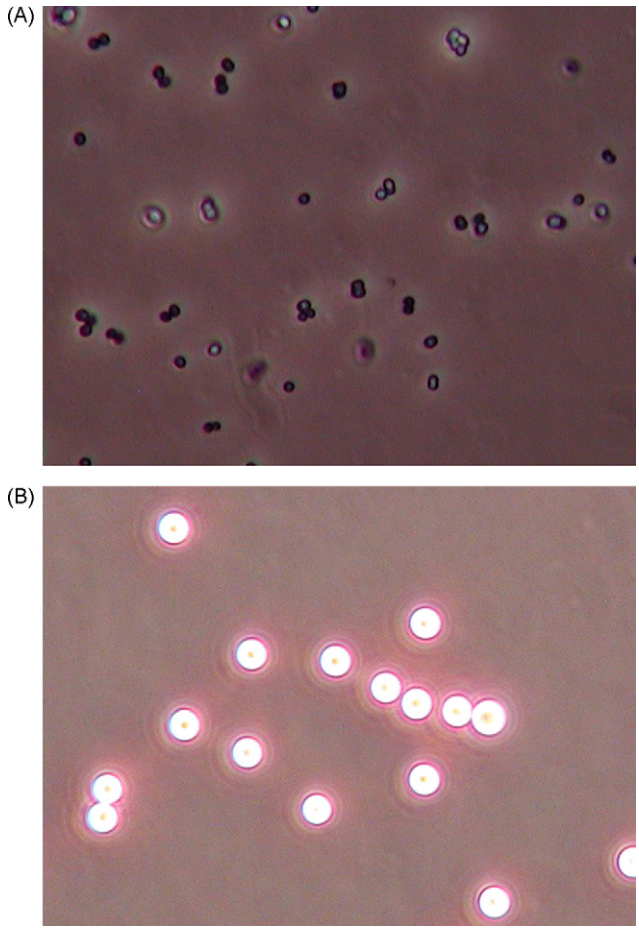

Fig. 4. Photomicrographs of the bacteria cells (A) and polystyrene latex particles (PSL) (B) obtained by light microscopy.

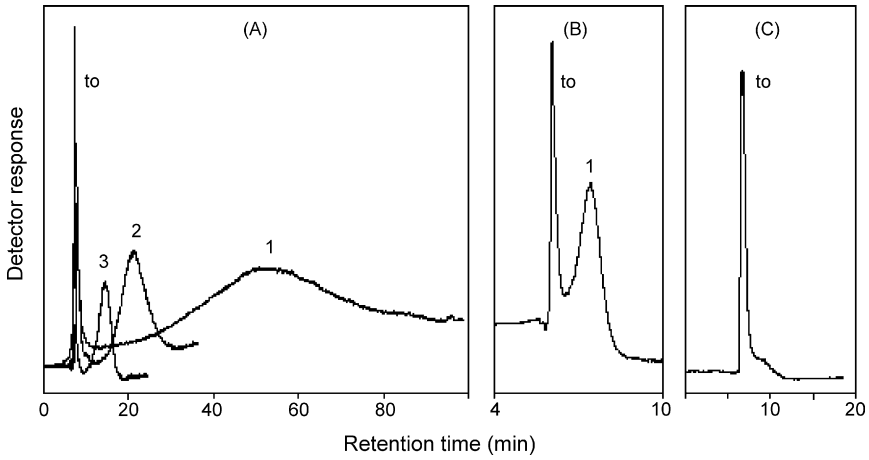

Fig. 5. Fractograms of the bacteria cells obtained at various experimental conditions. (A) Fractogram 1; temperature drop $\Delta T = 33\,^{\circ}C$, constant flow rate was 0.005 mL/min during the whole run without stop-flow period for relaxation. Fractograms 2 and 3; temperature drop $\Delta T = 33\,^{\circ}C$, flow rate during the injections was 0.005 mL/min, stop-flow period for relaxation 5 min, flow rate applied after the relaxation period was 0.02 mL/min (fractogram 2) and 0.04 mL/min (fractogram 3). (B) Fractogram 1; temperature drop $\Delta T = 33\,^{\circ}C$, flow rate during the injection was 0.005 mL/min, stop-flow period for relaxation 5 min, flow rate applied after the relaxation period was 0.1666 mL/min. (C) Temperature drop $\Delta T = 0\,^{\circ}C$, flow rate during the injection was 0.005 mL/min, stop-flow period for relaxation 5 min, flow rate applied after the relaxation period was 0.04 mL/min.

the cold wall, $T_c = 21\,^{\circ}C$, was kept constant in all experiments. Relatively low temperature drop $\Delta T = 33\,^{\circ}C$ was chosen to prevent bacteria denaturation. On the other hand, temperature drop $\Delta T = 0\,^{\circ}C$ applied in some micro-TFFF experiments allowed to determine the casual contribution of bacteria sedimentation in gravitational field during the run.

Some of the results of micro-TFFF experiments with bacteria are shown in Fig. 5. The fractogram 1 in Fig. 5A was obtained at a flow rate of 0.005 mL/min kept constant from the injection to the complete elution without the application of stop-flow for relaxation. Bacteria cells were obviously retained and the corresponding peak is well resolved from the "void peak" eluting at t_0 of an unretained component of the sample. This unretained sample component was not identified, however, it is certainly not a product of a casual thermal decomposition of the bacteria because an identical peak was observed when the experiment was carried out at $\Delta T = 0\,^{\circ}C$ (see Fig. 5C). The "void peak" cannot, no more, correspond to a product of shear degradation of the bacteria in flow velocity gradient because peak area does not change with the flow rate. It means that the amount of this unidentified component is independent of various shear stresses to which bacterial cells are exposed at various flow rates. Micro-TFFF of the filtrate of bacterial suspension (filtered on 0.22 μm filter) resulted in the only unretained peak that might correspond to the metabolic bacterial products which are inherently present in the sample. The fractograms 2 and 3 in Fig. 5A and the fractogram 1 in Fig. 5B were obtained at different, higher post-ISFT flow rates. Obviously, the analysis time has substantially been reduced when applying such an optimized elution protocol. The micro-TFFF experiment whose result is shown in Fig. 5B was as short as 8 min. Although the resolution between the "void" and bacteria peaks is lower, coherently with the theory, still their separation is good. Somewhat longer analysis time of 18 min (fractogram 3 in Fig. 5A obtained at lower post-ISFT flow rate in comparison with the fractogram 1 in Fig. 5B), resulted in perfect resolution between the mentioned peaks.

The fractogram in Fig. 5C was obtained by applying the identical ISFT procedure and the same final flow rate as those for the fractogram 3 in Fig. 5A but with $\Delta T = 0\,^{\circ}C$. It means that the gravitation alone cannot generate an important retention of the particles. Evidently, most of the bacterial cells eluted at t_0, thus unretained. A small shoulder on the fractogram in Fig. 5C may correspond to a part of weakly retained bacterial cells with the retention ratio approximately three times higher than that of the fractogram 3 in Fig. 5A.

Since the isotonic solution of NaCl was used as a carrier liquid, it seemed interesting to determine whether NaCl may form a concentration gradient across the channel, generated by thermal diffusion and thus whether it may form the gradient of the chemical potential casually influencing the retention of the bacterial cells. The outlet splitting option of the micro-TFFF unit allowed such an analysis. The concentrations of NaCl in the original sample of the carrier liquid and in the samples taken from the outlets at the hot and cold walls with a splitting ratio 50/50% were determined by potentiometric titration with 50 mM/L $AgNO_3$ and with the use of AgCl measuring electrode. The determined NaCl concentrations were 147.9 mM/L, 148.3 mM/L, and 145.6 mM/L for the original, "hot wall", and "cold wall" samples, respectively. Such a weak NaCl concentration gradient cannot influence the retention of the studied bacteria and PSL samples. All the results of micro-TFFF study of the behaviour of bacteria are shown in Fig. 6 as a plot of the retention ratio R versus average linear velocity $\langle v \rangle$ of the carrier liquid.

The above mentioned ISFT procedure has to be taken into account when calculating the retention ratio R from the experimental fractograms. The elution volumes V_0 and V_R are calculated from the corresponding retention times t_0 and t_R:

$$V_0 = q_{inj} t_{inj} + q_{el}(t_0 - t_{stop} - t_{inj}) \quad (4a)$$

$$V_R = q_{inj} t_{inj} + q_{el}(t_R - t_{stop} - t_{inj}) \quad (4b)$$

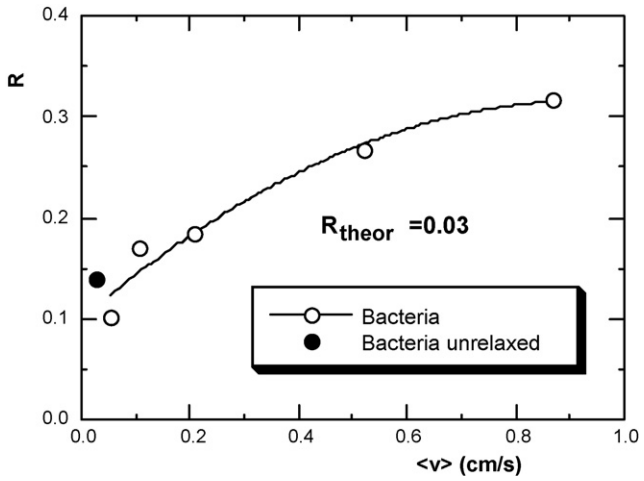

Fig. 6. Dependence of the retention ratio R on the mean linear velocity $\langle v \rangle$ of the carrier liquid in micro-TFFF experiments with bacteria. Calculated constant R_{theor} value corresponds to purely steric exclusion mechanism.

where q_{inj} and q_{el} are the flow rates during the injection and elution, respectively, and t_{inj} and t_{stop} are injection and stop-flow-rate times, respectively.

The theoretical retention ratio R_{theor}, given in Fig. 6, was calculated from Eq. (1) for $\lambda = 0$, it means by supposing the pure steric exclusion mechanism for the hypothetical particles of the diameter of 1 μm. Significantly lower value of $R_{theor} = 0.03$, in comparison with all experimental data obtained by applying ISFT procedure and shown in Fig. 6, indicates that steric exclusion mechanism was never effective but focusing. Intentionally, larger diameter of the hypothetical 1 μm particles compared to the studied bacterial cells was chosen for the theoretical calculation because lower diameter value obviously results in even much lower R_{theor} value. An important increase in retention ratio with increasing linear velocity of the carrier liquid when the bacterial cells were studied, obtained at various relatively high flow rates and with the application of ISFT procedure, proves clearly the action of the focusing mechanism caused by lift forces. The only experimental point which is out of the monotonic dependence R versus $\langle v \rangle$ is that obtained for the bacteria at the lowest flow rate of 0.005 mL/min during the whole run and without the application of ISFT procedure. It means that, in this case, lift forces were too weak, the polarization mechanism prevailed and, as a result, the retention ratio increased.

It has to be stressed that the application of the Eqs. (4a) and (4b) to calculate correct retention volumes V_0 and V_R requires an ideal functioning of the pump. It means that the high flow rate following the stop-flow period should be achieved immediately. Such a condition is difficult to fulfil with the reciprocating pumps equipped with dumpers. Syringe pump is more appropriate provided that no dumping elements (such as bubbles) are present in the separation system. Nevertheless, even if for any reason the start and stop of the flow is not ideally immediate, the retention ratio $R = V_0/V_R$ calculated using the Eqs. (4a) and (4b) is practically uninfluenced. Thus, for example, if the actual mean flow rate during the elution following stop-flow period in the experiment resulting in fractogram 2 in Fig. 5A and in corresponding point in Fig. 6 would be 0.03 mL/min instead of the supposed 0.04 mL/min (which is quite enormous and improbable difference), the resulting retention ratio should be $R = 0.187$ instead of $R = 0.184$ shown in Fig. 6, thus a minor and negligible difference.

5. Conclusion

Our investigation of the applicability of micro-TFFF to the analysis of the samples of biological origin has definitely confirmed this possibility. *S. epidermidis* bacteria cells were sufficiently affected by a "soft" temperature gradient and the important retention in micro-TFFF was thus obtained. Although both polarization and focusing mechanisms can be exploited under the appropriate experimental conditions, the micro-thermal focusing FFF provides more rapid analysis of the order of few minutes without any loss of the resolution compared with the conditions under which polarization mechanism is effective.

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