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Sedimentation field-flow fractionation application to *Toxoplasma gondii* separation and purification

Fawzi Bouamrane [a], N. Emmanuel Assidjo [a], Bernard Bouteille [b], Marie F. Dreyfuss [a], Marie L. Dardé [b], Philippe J.P. Cardot [a,*]

[a] *Laboratoire de Chimie Analytique et de Bromatologie, Université de Limoges, Faculté de Pharmacie, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France*

[b] *Service de Parasitologie–Mycologie, Université de Limoges, Faculté de Médecine, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France*

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Abstract

Toxoplasmosis is a worldwide disease caused by *Toxoplasma gondii*, an intracellular protozoa of micronic size range (4–10 μm). Its classical purification processes are complex and often associated with low recovery. All investigation procedures concerning this parasite require its isolation and purification from at least the mouse ascitic fluid. For this purpose, a recently developed laboratory technology was used, i.e. sedimentation field-flow fractionation. This chromatographic-like separation technology was demonstrated to be particularly selective for isolation and separation of micron-sized biological particles. Sedimentation field-flow fractionation operated on the steric-hyperlayer mode was used to isolate the parasite from the remanent ascitic contaminants of different origins and from red blood cells. With this technology, 86% recovery with 97% viability was obtained in less than 30 min.

Keywords: Toxoplasma gondii; Size fractionation; Erythrocytes; Field-flow fractionation

1. Introduction

Field-flow fractionation (FFF) methods were conceived in the late 1960s by Giddings [1]. This chromatographic-like separation family appeared to be particularly well suited for the isolation and characterization of micron-sized species [2,3]. The FFF separation principle lies on a differential retention of the particles in a liquid flowing on a laminar mode through a ribbon-like channel [3]. The separated components are eluted one at a time into a detector and/or a fraction collector because of their susceptibility to an external field applied perpendicularly to the great surface of the ribbon [4]. The external field can be of very different nature: thermal [5], electric [6], flow-driven [7,8], magnetic [9], sedimentation [10]; it drives the components toward one of the great

* Corresponding author. Tel.: +33-5-5543-5857; fax: +33-5-5543-5859.

E-mail address: cardot@unilim.fr (P.J.P. Cardot)

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surfaces of the ribbon (usually described as the accumulation wall) [1–3,10]. For micron-sized particles and because of a complex balance of forces, the components settle in appropriate and specific flow streams [2,3]. Separation is therefore made possible as particles of different characteristics travel along the channel at different velocities. Such an elution mode has been extensively studied and is described so far as the 'steric-hyperlayer' [3]. Its specific feature is that retention is flow-rate dependent due to the resulting balance on the particles of the external field and forces of hydrodynamic origin. Therefore, micron-sized particles are focused in flow stream layers of given velocities. The flow profile of a Newtonian liquid in motion inside the ribbon-like channel is parabolic [3], therefore species eluting closer to the accumulation wall are more retained. For a given average flow rate and a given field, the particle position depends on its density, size, shape and rigidity [11].

During the past two decades, a considerable development of these methods was observed for mineral and organic species [3] as well as for those of biological origin [12,13]. In that application area, a group of FFF methods using gravitational or centrifugal forces to create the external field and described generically as sedimentation FFF (SdFFF) appeared to be particularly well-suited for cell separation and purification [3,13,14].

A pioneering report by Caldwell et al. in 1984 [14] demonstrated the suitability of the SdFFF method for cell isolation and separation using red blood and Hela cells. Red blood cells (RBC) of different origins were studied [15–17], and age-dependent human RBC separations obtained [18] as well as the monitoring of experimental pathologies [19]. It was also demonstrated that cell size played a key role [14,19] in the separation as well as the density [19]. Cell swelling was also studied [20], and the cell rigidity effect on retention demonstrated. Nucleated cells were successfully isolated from a complex matrix [21] and in the early 1990s, the potential of SdFFF methodology was demonstrated for protozoa isolation. Living and dead *Trichomonas vaginalis* were separated from one another [22], microfilariae were purified from blood samples [23] and *Pneumocystis carinii* extracted from broncho alveolar surfactant [24]. *Toxoplasma gondii*, which provoked toxoplasmosis, is an intracellular crescent protozoa parasite whose size varies from 4 to 5 μm in width and from 9 to 10 μm in length, with an average density estimated at 1.056 [25]. *T. gondii* mean size and density are in the same range as RBC, which are used in FFF as 'probes' to evaluate biological applications of these separation methods. These biophysical properties (shape, size, density) make *T. gondii* isolation by SdFFF methods theoretically possible. The investigation of *T. gondii* elution and purification properties with FFF is reinforced by its purification complexity using more classical methods like filtration or centrifugation [26,27]. Usually, low recovery was obtained [27,28] because of particle–wall interactions (filtration) or potential toxic action (density gradient). It is expected that *T. gondii* can be separated from RBC and nucleated cells because of size differences [3]. Recovery may be increased because of the focusing effect of the steric-hyperlayer elution mode which limits particle–wall interactions [3,11,12]. The very simple isotonic medium used as the carrier phase with no possible toxic effects, and the dilution process provoked by the elution of the sample, which limits particle–particle interactions, will also enhance recovery [3]. The most simple SdFFF technique which uses the simple gravitational field [29,30] was used to demonstrate the potential of these techniques as a biocompatible purification tool in parasitology.

2. Materials and methods

2.1. Methodology

2.1.1. Principle of SdFFF (steric-hyperlayer mode)

As in chromatography, a sample mixture quantity is set at the inlet of a parallelepipedic-like channel [3] whose exact geometry is shown in Fig. 1A. One critical dimension is the thickness of this ribbon-like channel which is usually in the 100–300 μm range [3,31]. In the absence of flow, and under the action of the external field, the different micron-sized particles settle on the accumulation

wall as schematically described in Fig. 1B. When a flow stream is established in the channel, because of the viscosity of the medium, a parabolic flow velocity profile is observed in the channel thickness. Particles of different sizes, with their gravity centre in different flow streams, are eluted at different velocities according to their size. This elution model is described as 'steric' [3], and is limited to size differences. However, it does not describe some experimental features such as flow rate dependent retention. Caldwell et al. suggested that particles were ejected from the accumulation wall under the effect of velocity-dependent forces [14]. The balance of the sedimentation force created by the external field (Wf in Fig. 1B) and this 'lifting' force (Lf in Fig. 1B) focuses particles in a thin layer within the channel thickness [2]. It was established that particles of different characteristics were therefore eluted selectively according to a complex balance of parameters not totally mastered so far, i.e. size, density and shape. In terms of cell separations, slight differences in shape, size and density can play major roles in the separation process [14]. Such a model is understandable if, during the elution process, all particles are at their equilibrium position [2,3]. This is not the case if particles are introduced into the channel with an established flow stream (flow injection process) or if the particles have settled on the accumulation wall (stop-flow injection process). In both cases, the equilibrium position kinetics of particles in the channel thickness is not totally assessed and systematic studies are required depending on the sample characteristics. However, some theoretical approaches which describe this mechanism are available and can be of use for separation optimization [2,30]. From a practical point of view, separation and elution parameters used in FFF are common to those of chromatography. In this report, retention ratio, asymmetry factor and peak dispersion parameter (height equivalent to a theoretical plate (HETP)) were calculated from elution signal according to the procedure defined by Bidlinmeyer and Warren [32].

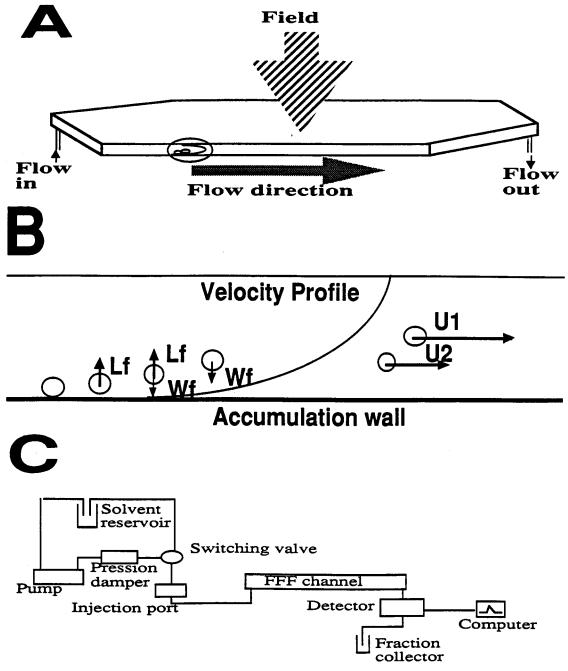

Fig. 1. (A) Principle of FFF techniques. (B) Schematic representation of the steric-hyperlayer elution mechanism: Wf, field induced force acting on the particle; Lf, lifting force. U1 and U2, channel-thickness-position particle velocity. (C) Schematic representation of gravitational field-flow fractionation apparatus.

2.1.2. *Toxoplasma gondii* separation development

According to the qualitative basis of the presented theoretical considerations, *T. gondii* may be retained whatever the injection process. In a first step, a systematic study of the injection procedure will be performed to define the retention operating conditions and general elution characteristics. In that purpose, *T. gondii* elution characteristics will be compared with FFF well-known cellular population, i.e. RBC from different origins. In a second step, eluted fraction analysis will be performed to demonstrate the suitability of the SdFFF method to purify fractions containing *T. gondii* from macromolecular and cellular contaminants. Finally, as FFF is a separation technique, demonstration of separation is given using an artificial mixture of *T. gondii* and RBC.

2.2. Experimental

2.2.1. FFF system

Using the gravitational field led to the construction of the most simple FFF device. The separator

used in this report has been technically described by Bernard et al. [33] as well as the general set-up of any FFF system. In Fig. 1C, the FFF separation system set-up is schematically described; the only differences from a classical liquid chromatography system are the nature of the separator and the switching valve to allow flow and stop-flow injection procedures.

In this report, the channel is made of two polycarbonate walls and a mylar spacer. Its dimensions are: $1 \times 50 \times 0.025$ cm^3 in width, length and thickness, respectively. The system void volume which includes tubing connections and detection volumes was measured at 1.25 ± 0.03 ml (2σ, $n = 6$) by means of a 0.1% (w/w) sodium benzoate solution (Darrasse Frères, Paris, France). The polycarbonate nature of the wall was chosen to reduce possible sample–wall interactions.

A Gilson chromatographic pump model 302 (Gilson Medical Electronics, Middleton, WI, USA) connected to a pressure damper allowed flow rates ranging from 0.05 to 5 ml/min. A switching valve V100 L (Upchurch Scientific, Oak Harbour, WA, USA) was placed after the damper. Such a device allowed to divert the flow away from the channel for stop-flow injection procedure time. Samples were introduced into the separator by means of a 20 μl loop Rheodyne valve model 7525i (Rheodyne, Cotati, CA, USA). At the outlet of the separation channel, a Waters 440 photometer (Waters, Milford, MA, USA) set up at 280 nm allowed particle detection. Data were recorded on line with a Daewoo computer (Daewoo Europe, Roissy Charles de Gaulle, France) using a 16-byte acquisition card operating at 1 Hz. The carrier medium was a physiological 0.9% NaCl isotonic aqueous solution (Biosedra Pharma, Louviers, France) supplemented with 0.2% (w/w) bovine serum albumin (No. A-4503; Sigma Chemical, St Louis, MO, USA). FFF system decontamination procedures were systematically performed prior to any elution to reduce or eliminate bacterial contamination. Twenty-five percent (w/w) perchlorate solution in bidistilled water was percolated through the system at low flow for 1 h, followed by sterile double-distilled water (Biosedra Pharma, Louviers, France) for 20 min.

2.2.2. Toxoplasma

T. gondii suspensions were obtained from mouse ascitic fluid. Toxoplasma tachyzoites, 10^4 (RH strain), in a 0.9% NaCl solution were inoculated intraperitoneally to a 30-g female Swiss mouse. Forty-eight hours later, the mouse was sacrificed and toxoplasma tachyzoites aseptically collected from the ascitic fluid. Recovery was enhanced by washing the intraperitoneal cavity with 2 ml of 0.9% NaCl. Ascitic fluid and physiological medium from three mice were pooled. After a 10 min $300 \times g$ centrifuge, numeration was performed after supernatant removing by means of a 1 μl Malassez haemocytometer (Preciss, Paris, France). An appropriate dilution in 0.9% NaCl was performed to obtain a final suspension containing $75 \times 10^5 \pm 20 \times 10^5$ parasites per μl.

2.2.3. Red blood cells

Human blood was drawn from a voluntary healthy donor. Mice blood was collected from the heart of *T. gondii*-infected sacrificed mice. Blood samples were immediately mixed with potassium ethylene diamine tetracetic acid (K_3EDTA) salt in a sterile Vacutainer® system (Becton Dickinson, Meylan, France) and stored at 4°C. Prior to analysis, and depending on the blood sample, 200- to 300-fold blood dilutions in 0.9% NaCl were performed to obtain RBC suspensions of 25 000 cells/μl.

3. Results and discussion

3.1. Toxoplasma tachyzoite purification

As a result of size and density characteristics of *T. gondii*, its retention in FFF is expected. However, an injection procedure choice was needed. Therefore, in a first attempt and to reduce possible parasite–channel wall interactions, the flow injection method was chosen. For that purpose, 20 μl of a toxoplasma tachyzoite suspension were injected into the channel exactly like a molecular sample in chromatography, i.e. in a given established flow. *T. gondii* retention was obtained for linear flow velocities lower than 0.07 cm/s (flow

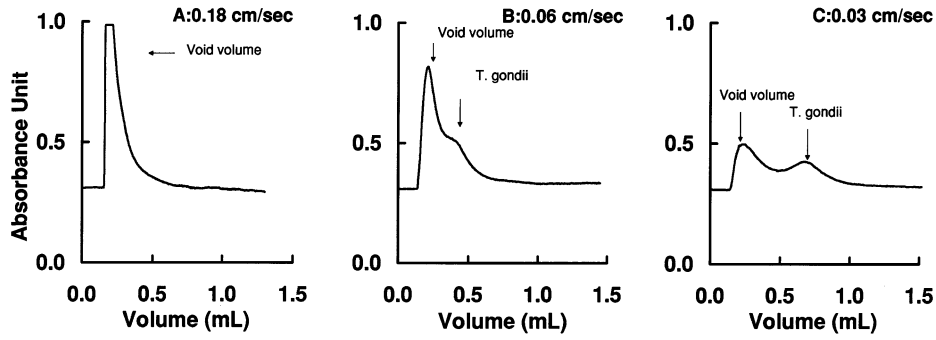

Fig. 2. Fractograms of toxoplasma tachyzoites in gravitational field-flow fractionation, flow injection, 20 μl loop, 15×10^6 toxoplasma injected; carrier phase, 0.9% NaCl solution with 0.2% (w/w) bovine serum albumin. Factograms A, B and C were obtained at, respectively, 0.18; 0.06 and 0.03 cm/s average flow velocity.

rate = 0.12 ml/min). When identical elution was performed at reduced flow velocity, the retention ratio increased as predicted by the 'steric-hyperlayer' elution model. The obtained elution signals, described in FFF as fractograms, of *T. gondii* are shown in Fig. 2. In Fig. 2A, *T. gondii* are eluted in the void volume of the system and are therefore not retained. At lower flow rate, the elution signal is modified, and microscopical analysis of collected fraction signed the retention of *T. gondii*, although its resolution from the void volume was low, as shown on Fig. 2B. With a flow rate of 0.05 ml/min, i.e. a flow linear velocity of 0.03 cm/s, the *T. gondii* suspension was completely resolved from the void volume peak. Fig. 2C shows a typical fractogram of retained *T. gondii* suspension. The particles eluting in the void volume are not affected by the external field and elute at the average flow velocity. On the contrary, *T. gondii* population settles in the channel thickness during the elution process and is therefore eluted at lower average speed. It can be understood that the injected *T. gondii* quantity was chosen to obtain a significant signal and not to overload the channel. Whatever the retention obtained, the very low flow required led to a long residence time of the species, which might limit their viability or recovery. Reducing elution time was not possible by simply increasing the flow rate because of the specific features of the steric-hyperlayer elution mode. In a second step, stop-flow injections were performed at different flow rates, whose fractograms are shown in Fig. 3. In that case, *T. gondii* retention and resolution from the void volume peak were obtained at much higher flow velocities (fivefold magnitude), considerably reducing the elution time. Such a result is concordant with the 'relaxation' process occurring during the stop-flow time, where *T. gondii* can settle on the accumulation wall and therefore be eluted directly in low velocity flow streams. The advantage of the stop-flow procedure compared to the flow injection one is to increase considerably *T. gondii* retention at identical flow rate or to obtain identical retention values at increased flow velocity, as observed when Figs. 2 and 3 are compared. It is observed that with the stop-flow procedure a considerable signal intensity enhancement is obtained, authorizing much more diluted samples. The main limitation of the stop-flow procedure in FFF resides in the possible development, during the sedimentation process, of strong particle–channel wall interactions which may lead to particle destruction, sticking or viability variation. On the instrumentation point of view, the channel wall material was empirically chosen to limit such interactions as already observed with *T. vaginalis* [22]. Such possible effect can be observed when fractogram peak shapes of Fig. 3 are compared. The Fig. 3A–C sequence is in accordance with the steric hyperlayer elution mode. However, at very low flow velocity (1.17 cm/s), *T. gondii* resolution from void volume signal is decreased as shown in Fig. 3D, with a complete modification in

the *T. gondii* peak shape, which can be interpreted as a constant release of stuck cells during the elution process.

To confirm that hypothesis, the fractogram of Fig. 3D can be interestingly compared to that of Fig. 2B, where signal intensity and peak shape are in the same range. It is therefore possible to elute selectively *T. gondii* in FFF with a stop-flow injection procedure in a flow velocity range of 0.20–0.35 cm/s. Whatever these preliminary results, injected samples are produced from ascitic fluids and contain contaminants of different types: dead and living *T. gondii* as well as nucleated cells. There is therefore a need to assess the purification process by fraction collection and microscopic analysis. Systematic elution of *T. gondii* samples were performed in the experimental conditions of Fig. 3C and the eluted fraction analysed. Microscopic observation of a crude *T. gondii* sample and the fractions described in Fig. 3C were performed: results are shown in Fig. 4. The initial ascitic fluid dilution is shown in Fig. 4A, and contains toxoplasma tachyzoites with other constituents of the peritoneal fluid. Slides of Fig. 3C, fractions I and II, are shown in Fig. 4B,C. Compared to the initial ascitic fluid (Fig. 4A), fraction I which corresponds to non-retained species contains all peritoneal fluid constituents, whereas fraction II corresponding to the retained peak is only made of toxoplasma tachyzoites. Such separation of tachyzoites from contaminants, associated with a retained fraction of pure tachyzoites, demonstrated that the separation hypothesis of *T. gondii* with FFF methods is valid. However, no information at that time could be drawn about *T. gondii* recovery and viability. The general process of fraction collection is therefore repeated to investigate these two points.

3.2. Recovery

From 100 μl of toxoplasma tachyzoites suspension, two 20 μl samples were taken. The first 20 μl were diluted in 5 ml of carrier phase and stored at 4°C; this *T. gondii* suspension is described in the following as the 'reference suspension'. The second 20 μl of toxoplasma tachyzoites suspension was eluted by FFF. The toxoplasma tachyzoite peak fraction was collected and diluted with 0.9% NaCl to 5 ml. Haemacytometer counting was therefore performed on both samples and recovery calculated (Table 1). A mean recovery of $86.45 \pm 2.28\%$ (2σ, $n = 6$) was observed, which appears to compete with other techniques [26].

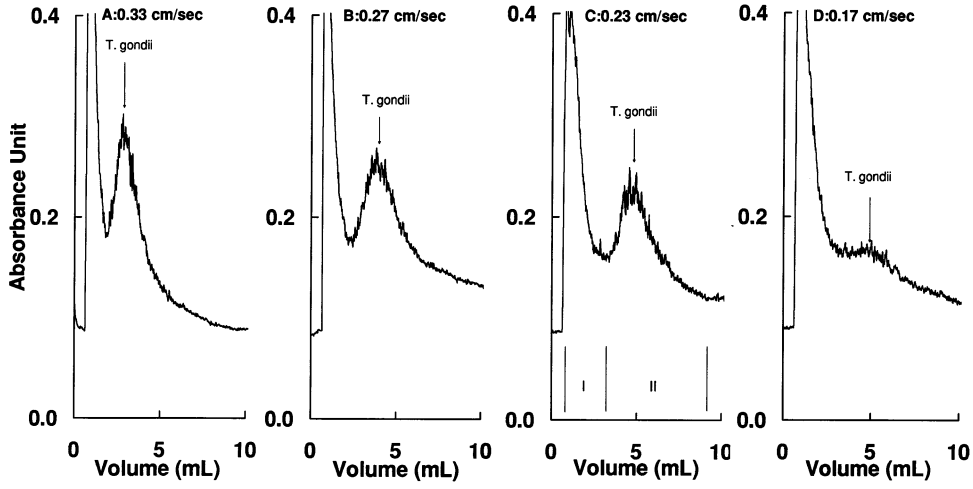

Fig. 3. Fractograms of toxoplasma tachyzoites in gravitational field-flow fractionation, stop-flow injection: relaxation time, 4 min; 20 μl loop; 15×10^6 toxoplasma tachyzoites injected; carrier phase composition described in caption to Fig. 2. Fractograms A, B, C and D were obtained at different average flow velocities. Fractions I and II of fractogram C were collected under time control.

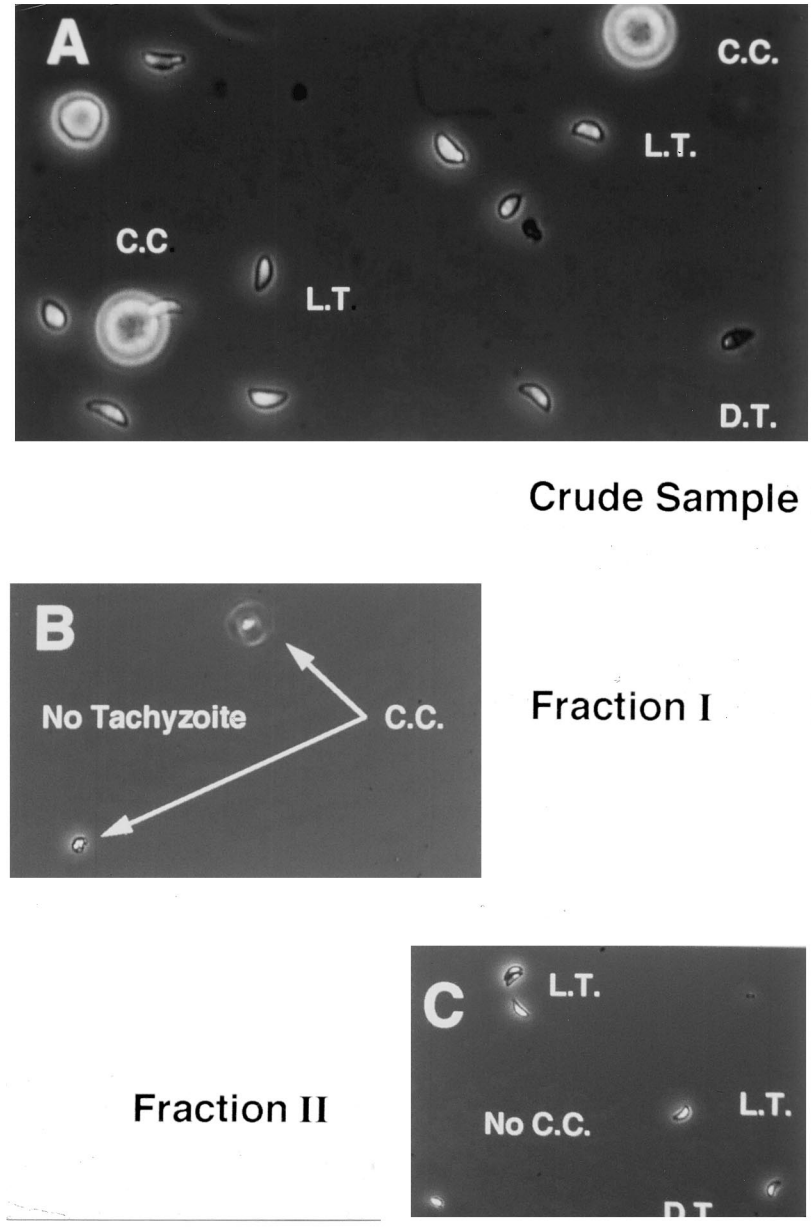

Fig. 4. Crude sample and collected fractions slides. Fractions I and II are described in Fig. 3. (A) crude sample, (B) and (C) slides of collected fractions I and II. Microscopic observation, magnification $\times 400$; slide abbreviation: C.C., contaminant cells; L.T., living tachyzoites; D.T., dead tachyzoites.

3.3. Viability

Although, FFF appears to be a very simple method to purify toxoplasma tachyzoites from suspension contaminants, and as the purified suspension is easily collected, no information can be drawn concerning tachyzoites viability. By means of a phase contrast microscopy, it is possible to observe living and dead toxoplasma tachyzoites [34]. Living tachyzoites appeared to be refringent,

whereas dead ones contained dark granulations [34]. A procedure analogous to that used for recovery analysis was performed and viability calculations showed that $97.06 \pm 1.78\%$ (2σ, $n = 6$) of the injected living toxoplasma tachyzoites were eluted alive at the FFF channel outlet.

3.4. *T. gondii*/RBC separation by FFF

To compare *T. gondii* elution characteristics with cellular material, systematic retention studies were performed using mice and human RBC. Using a Coulter counter TAII (Coulter Electronics, Luton, UK), mice RBC, human RBC and toxoplasma tachyzoites sizes (diameter of a theoretical sphere of identical volume) were determined at 4.6 ± 0.2, 5.8 ± 0.3 and 10.3 ± 1.0 μm, respectively. Densities were, respectively, 1.085 ± 0.015, 1.051 ± 0.009 and 1.056 ± 0.011 [25]. Out of cell shape differences, one can observe that mice RBC are the smallest and the most dense; according to the steric-hyperlayer mechanism, they are expected to be the most easily retained. Size differences between human RBC and toxoplasma tachyzoites are in a twofold range and, according to data of Figs. 2 and 3, compared to previously published human RBC fractograms [35], toxoplasma tachyzoites are expected to elute first. Using RBC and toxoplasma tachyzoites suspensions, systematic elution characteristics of each population were studied (Fig. 5). In all cases, a 4-min stop-flow injection procedure was performed. Retention ratio is defined as the ratio of the elution time of a non-retained species to the elution time of the retained one. Its values as a function of the average flow velocity are plotted in Fig. 5A. As expected, mice RBC are the most retained, and toxoplasma tachyzoites, the least. Statistical comparison showed significant retention ratio differences for human and mice RBC. Elution peak variance was classically assessed using the HETP parameter. The toxoplasma tachyzoite elution peak spread much more than that of RBC (Fig. 5B). Such a feature may be linked to the tachyzoite population heterogeneity in terms of biophysical parameters.

As in chromatography, an additional parameter is studied which describes the elution peak shape, i.e. asymmetry factor. Although its signification in FFF is not physically established so far, the data shown in Fig. 5C demonstrated that all cells behaved similarly. When Fig. 5A–C are compared, an optimum flow rate region emerged, centred around 0.25 cm/s.

To confirm the versatility of FFF methods in cell and parasite separation, an artificial mixture of toxoplasma tachyzoites and mice RBC was prepared. Respective concentrations of RBC and toxoplasma tachyzoites were chosen to obtain an elution signal of equivalent intensities: 15×10^6 toxoplasma tachyzoites and 5×10^5 mice RBC were injected via the 20 μl loop into the FFF channel with a 4-min stop-flow time at a 0.24 cm/s flow velocity. The obtained fractogram is shown in Fig. 6. Three regions, designated as I, II and III, can be observed. Region I corresponds to the void volume and contains proteins as well as subcellular contaminants or nucleated cells components. Region II corresponds to the *T. gondii* elution characteristics. No RBC were found by microscopic observation of region II fraction collection. In Region III, all the RBC of the sample mixture are eluted. A highly satisfactory resolution (1.31) of toxoplasma versus RBC is obtained which demonstrates the separation versatility and power of FFF techniques. In the light of these

Table 1
Toxoplasma gondii recovery and viability after elution in the GrFFF channel

	Toxoplasma tachyzoite recovery and viability experiments								
	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	Mean (%)	S.D.	R.S.D.
Recovery	83.54	84.91	88.89	87.91	86.32	87.1	86.45	1.97	2.28
Viability	98.72	94.25	97.53	95.74	97.23	98.87	97.06	1.73	1.78

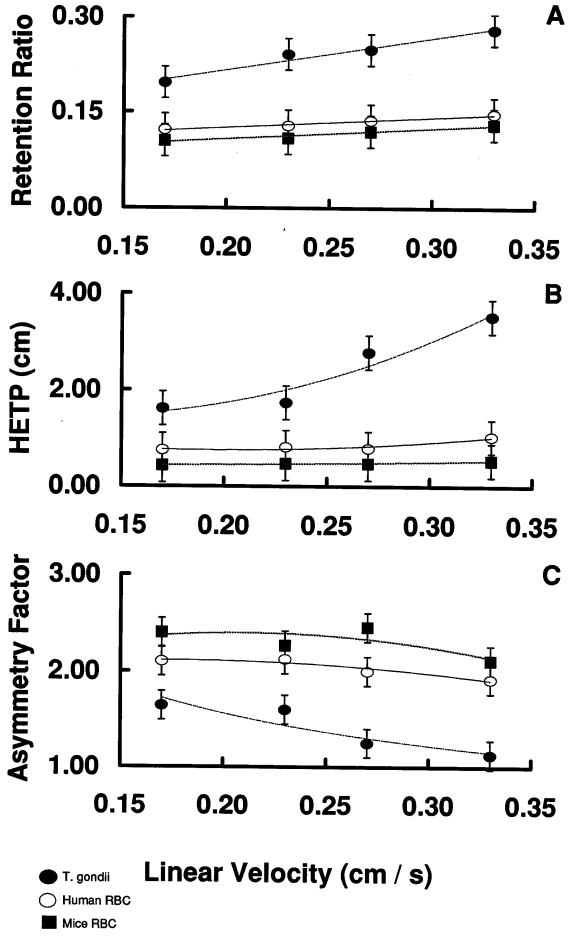

Fig. 5. Effect of flow linear velocity on toxoplasma tachyzoites, Human and mice red blood cell fractogram characteristics. Flow rate varying from 0.17 to 0.33 cm/s (0.17, 0.23, 0.27, 0.33), $n = 3$; stop-flow, 4 min; 20 μl loop; 15×10^6 toxoplasma injected; carrier phase described in caption to Fig. 2. (A) retention ratio, (B) HETP, (C) asymmetry factor.

results, it may be therefore possible to isolate toxoplasma tachyzoites from more complex samples, allowing FFF methods to enter biological methodology as a purification tool in the future.

4. Conclusion

The FFF system used in this report is the most simple one, both in design and operating process. It can be constructed at low cost without mechanical skills. Such a separator will simply replace the column of any liquid chromatographic system. Light instrumental modifications (a switching valve) will allow larger injection procedures. Such simplicity is in favour of its development, even in non-specialized laboratories. The use of polycarbonate plates to build the channel allows high recovery. Methodologically, stop-flow injection procedures are possible when *T. gondii* is involved and no tachyzoite staining is necessary for elution detection allowing high viability. The low interaction possibilities of the eluted species with the separator material also enhance recovery and viability performances. It appeared experimentally that, from a qualitative point of view, the steric-hyperlayer model is valid for micron-sized species of biological origin. Moreover, separation development based on that model are successful. The separation process is therefore based on cell biophysical properties. Although density differences or effective mass correlation were not evidenced with the experiments described herein, size appeared to be the major separation parameter. The separation of *T. gondii* from RBC predicts the future use of more sophisticated SdFFF systems for diagnostic purposes. In that case, external field can be increased using centrifugal forces and/or reduced channel thickness to enhance selectivity. In terms of detection, all on line systems used for elution and chromatography can be used as well

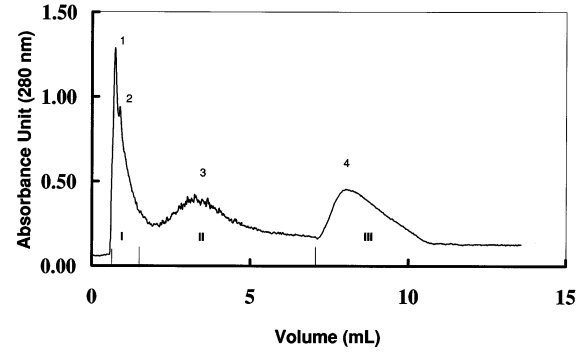

Fig. 6. Fractogram of a mixture of toxoplasma and mice red blood cells. Flow rate: 0.24 cm/s, 4 min relaxation time, 20 μl loop, carrier phase described in caption to Fig. 2, a mixture of 15×10^6 toxoplasma tachyzoites and 5×10^5 mice blood cells was injected. Fractions I–III were collected under time control. 1, 2, void volume; 3, *T. gondii*; 4, mice RBC.

as sequential ones like flow cytometry. Detection and separation selectivity can be enhanced using specific fluorescent probes of immunological origin.

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