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Elution mode of *Pneumocystis carinii* cysts in gravitational field-flow fractionation

Christian Bories, Philippe J. P. Cardot, Vincent Abramowski, Christian Poüs, Annabelle Merino-Dugay and Bruno Baron

G-FFF Research Group, Laboratoire de Chimie Analytique et d'Electrochimie Organique, Université Paris-Sud, Centre Pharmaceutique de Châtenay-Malabry, Rue J. B. Clément, F-92296 Châtenay-Malabry Cedex (France)

Geneviève Mougeot

Laboratoire de Parasitologie, Centre Hospitalier Universitaire St. Antoine, Boulevard St. Antoine, F-75571 Paris Cedex 12 (France)

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ABSTRACT

The simplest field-flow fractionation technique, *i.e.* gravitational, was used in an attempt to purify a *Pneumocystis carinii* cyst suspension. This parasite is an opportunistic invader in immunocompromised patients, especially those suffering from AIDS. The cyst stage is spherical and 5 μm in diameter. Unexpected retention times, not systematically related to the size and the density of the parasite, were obtained under various experimental conditions. When silicone-coated walls were used, *Pneumocystis carinii* cysts were eluted in the void volume, whereas when uncoated walls were used with a sodium dodecyl sulphate-enriched carrier phase, retention was observed. These phenomena are probably related to the high degree of hydrophobicity of these micrometre-sized biological particles; this degree can be easily determined. The use of the gravitational field-flow fractionation technique can be of a great interest for the development of new methods for diagnostic purposes. Particle wall interactions and their modifications due to the carrier phase or to the wall treatment can be employed in the search for new bronchoalveolar lavage solutions.

INTRODUCTION

Field-flow fractionation (FFF) is a recent tool for the separation and purification of biological particles [1–5]. The simplest of such techniques, which is based on the gravitational field (G-FFF), is particularly well suited for micrometre-sized particles. An example of its application in parasitology with blood-dwelling microfilariae has already been published [2].

Correspondence to: Dr. C. Bories, G-FFF Research Group, Laboratoire de Chimie Analytique et d'Electrochimie Organique, Université Paris Sud, Centre Pharmaceutique de Châtenay-Malabry, Rue J. B. Clément, F-92296 Châtenay-Malabry Cedex, France.

During the last decade, *Pneumocystis carinii* has gained in importance as an opportunistic invader in subjects who suffer from a deficiency of the immune system, *e.g.*, some patients with AIDS die from a dysfunction of the lung [6] due to complete coverage of the respiratory surface by parasites at different stages, namely cysts and trophozoites. Disseminated pneumocystosis can occasionally occur in the blood circulation [7].

P. carinii is an extracellular eukaryote which develops extracellularly. It undergoes encystment during one phase of its life cycle and settles within the alveoli of infected lungs. Trophozoites and cysts live stuck to the pneumocystes in lung exudate fluids. In the final stage of the disease, they

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live in a characteristic eosinophilic exudate which fills the alveoli. When the lung imprint is stained, encysted forms of the parasites are found embedded within a foamy matrix or situated along the alveolar wall [8]. This clustering causes alveolar capillary blockage, which results in a disturbance of the blood–gas exchange processes in the lungs.

The biological diagnosis of this alveolar disease is difficult to carry out by the observation of trophozoites and cysts in the centrifugation sediment of bronchoalveolar lavage (BAL) performed with sterile physiological saline. The first aim of this study was to improve the detection of *P. carinii* cysts (PCC) in BAL by means of G-FFF. The second aim was to prepare purified PCC for immunological assays.

EXPERIMENTAL

Pneumocystis carinii cyst

PCC enriched suspension was obtained from the lungs of immuno-depressed rats as previously described [9]. *P. carinii* pneumonia was induced in 200-g white Wistar female rats (IFFA-Credo, Lyon, France) by immunosuppression with bi-weekly cortisone acetate injections (125 mg/kg subcutaneously for eight weeks). Rats were killed by Nembutal anaesthesia overdose (Clin-Midy, Fresnes, France) and bled; lungs were removed intact, pooled and cut into 6–18 mm^3 pieces in Hanks balanced salt solution without Ca^{2+} and Mg^{2+}. Then gentle stirring was performed for 2 h. Large particles were removed by pouring the homogenate through a sterile gauze. The filtrate was centrifuged at 1400 g for 4 min. The supernatant was then centrifuged at 5000 *g* for 10 min. The red blood cells (RBC) of the sediment were haemolysed with 0.15 *M* NH_4Cl–Tris buffer (pH 7.2) for 10 min at 4°C. After centrifugation (5000 *g* for 10 min), the pellet was sieved using a stainless-steel screen (60 μm mesh). The homogenate was then centrifuged again. Cystic forms in the sediment were counted in 2-μl air-dried smears (Hamilton 801 RNE 5-μl syringe; Touzard et Matignon, Vitry, France) stained with toluidine blue O. The number of cysts in the suspension was $8.8 \cdot 10^8 \pm 2.1 \cdot 10^8$ ml^{-1} (five determinations).

Cyst diameter size was measured using a Microsizer, Coulter Counter Model ZM, set up for 64 channels (Coultronic, Margency, France), and the results were 4.5 ± 0.3 μm. The buoyant density of the cysts was determined by means of isopycnic centrifugation in a preformed linear saccharose gradient ranging from 0.8 to 1.3 *M* [10]. A cyst suspension in 1.8 *M* saccharose was loaded at the bottom of the gradient. Ultracentrifugation was carried out using a Beckman SW 41 rotor at 90 000 *g* for 18 h. The density of the saccharose solution in which the cysts are equilibrated was measured by refractometry and the presence of the cysts was checked by microscopic observation. The experimentally determined density was 1.177 for the PCC population used in these experiments.

Red blood cells

Blood samples from a healthy volunteer were used for experiments. One volume of 3.6% sodium citrate solution was mixed with nine volumes of blood to avoid clotting. The RBC size was measured using a Model ZM Coulter counter (Coultronic).

Field-flow fractionation

A G-FFF apparatus analogous to that described by Giddings *et al.* [11] was set up with sulphochromic acid-washed glass plates or with plates coated with a biocompatible silicone (Silbione, Rhône-Poulenc, Paris, France). The channels were 86 cm long, 2 cm wide and 0.175 mm thick with a dead volume of 3.11 ml including the connection tubes and the detection volume. Some experiments were performed with a 0.250-mm-thick channel and uncoated glass plates. Detection was performed with a UV–VIS chromatographic spectrophotometer (Shimadzu SPD 6A) at 313 nm. The carrier phase was a 0.15 *M* isotonic saline solution. Before injection, the cyst suspension was diluted with the same volume of physiological saline. A 50-μl aliquot of the dilution was injected with different stop-flow times (2–10 min) into the inlet part of the channel. The stop-flow time injection protocol was simple: the carrier phase flow was stopped and the cyst sus-

pension was injected; the flow was set after different times at different velocities with a Waters Model 6000 A chromatographic pump.

During elution, fractions of 0.5 ml were collected manually at timed intervals. They were centrifuged at 5000 *g* for 20 min and the sediment was observed microscopically with 1000-fold magnification (HM-Lux, Leitz, Weilar, Germany) for PCC. The quantitative analysis of parasites in the collected fractions was performed after concentration to 25 μl by centrifugation at 5000 *g* for 20 min. The aqueous phase was discarded and the sediment was thoroughly suspended in a 25-μl aliquot of physiological saline. Cysts were counted in 2-μl air-dried smears after staining with toluidine blue O.

The viability of the cysts was assessed by propidium iodide fluorescent staining according to the technique of Lapinsky *et al.* [12]. Propidium iodide is a positively charged fluorescent stain which does not enter intact cysts. Alteration of the cyst wall allows entry of this stain. An incubation time of 1 h with 20 μg ml^{-1} of staining die was performed prior to checking microscopically the lack of fluorescence of the nuclei of the cyst (excitation filter at 530–560 nm, barrier filter at 580 nm; Orthoplan, Leitz).

PCC surface hydrophobicity was measured using the method described by Rosenberg *et al.* [13] for bacteria. Cysts were suspended in isotonic phosphate-buffered saline solution (pH 7.2) to an initial absorbance of *ca.* 1.5 measured at 400 nm. Various volumes of the test hydrocarbons (*n*-hexadecane, isooctane and *p*-xylene) were added to acid-washed round-bottomed test-tubes (11 mm diameter) containing 1.2 ml of cyst suspension, and the phases were mixed in a vortex-mixer for 120 s. Organic and aqueous fluids were allowed to separate overnight and the absorbance of the lower aqueous fluid was measured at 400 nm according to the techniques developed by Rosenberg and Doyle [14]. Adherence of parasites to hydrocarbons was calculated as the percentage decrease in absorbance relative to that of the initial cyst suspension; this technique is an alternative measurement of hydrophobicity usually used for live micrometre-sized species such as bacteria.

RESULTS

FFF elution of PCC

Elution with coated glass plates. Using variable stop-flow times, the elution of PCC suspension in FFF does not, unlike the RBC, present a characteristic elution peak profile with a retention factor lower than 1. This retention factor is defined as the void volume of the separation system divided by the retention volume of the particle. A systematic fraction analysis was performed for twice the time required to elute particles of the same average size in a full steric elution mode. It was observed that PCC were eluted in the void volume of the channel. After elution, flushing procedures were performed at high flow-rates (3 ml min^{-1} for 10 min) to check the cyst interactions with the coated channel walls; this volume of carrier phase collected under time control was centrifuged at 5000 *g* for 20 min. The recovery of PCC after the flushing procedures was variable. For the particles collected in the void volume, their recovery was correlated with the number of injections; the first injection induced very low recoveries ($< 10\%$) and as the number of injections increased, the recovery increased to a maximum of 53% after seven injections of the diluted PCC suspension. This result is at variance with the data usually described with all the theoretical models (steric, inertial, normal) or with the many experimental results obtained for latex particles or biological material of equivalent order of size and density.

According to previous work performed with RBC, latex particles and microfilariae, micrometre-sized particles are assumed to be eluted with a retention mechanism involving both steric and inertial modes. This allows retention according to both size and density. If we consider only the steric elution mode, the expected retention factor of PCC would be 0.1 in a channel 0.175 mm deep, and lower in a channel 0.250 mm deep. The retention factor of 1 obtained in our experiments on FFF apparatus with coated glass plates is at variance form these values, as is the low recovery obtained. This result cannot be explained by the density, which is one of the highest found

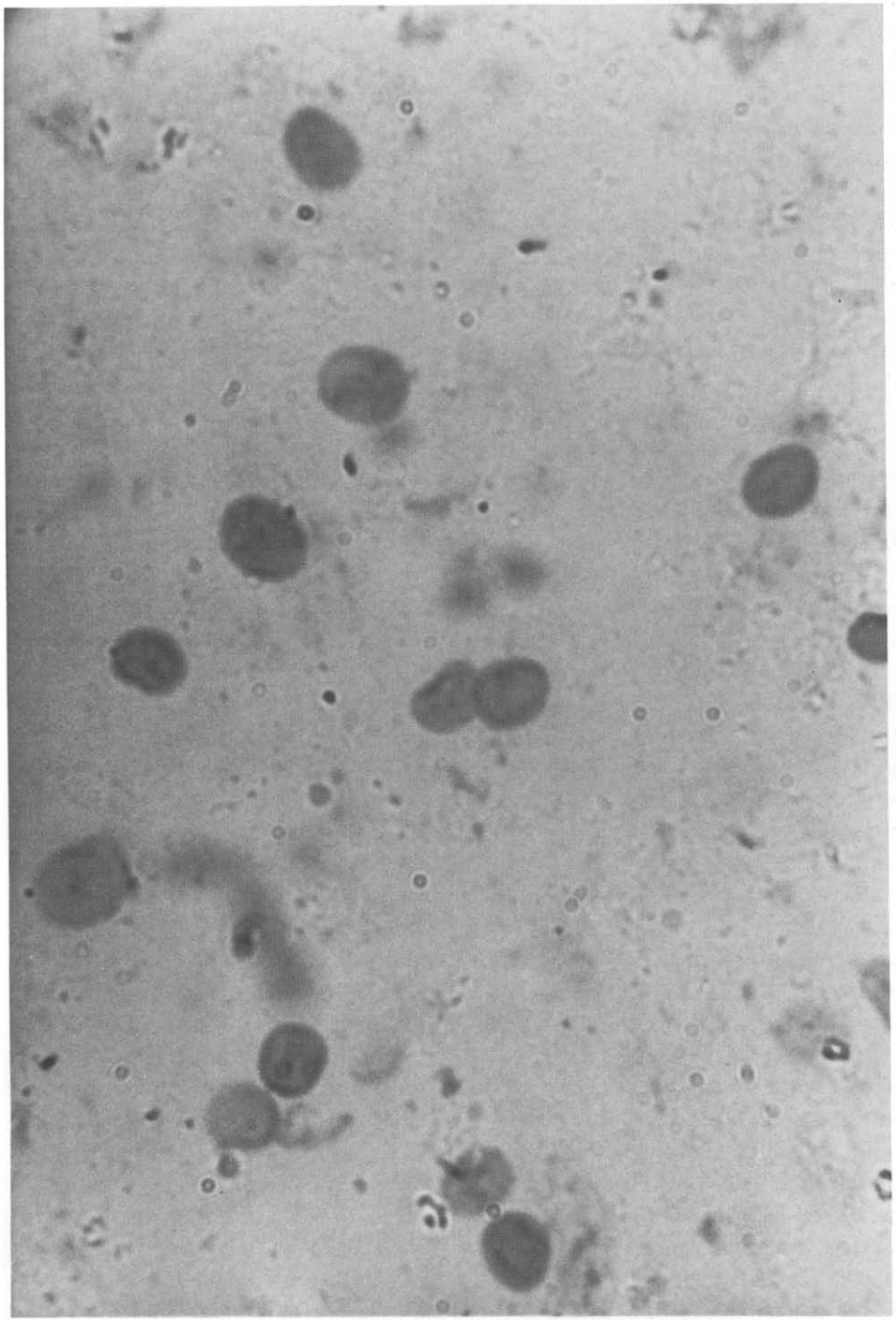

Fig. 1. Photograph of *P. carinii* cyst solution stained with toluidine blue O; magnification × 1000.

for particles of this size. It cannot be attributed to the shape of PCC as they are spherical parasites as shown in Fig. 1. To confirm this discordance with most of the experimental published data, systematic elutions were performed at different flow times. In all instances, with stop-flow times varying from 1 to 20 min, no PCC peak was observed. Fraction collection of the void volume showed systematically a sediment made of PCC which suggests strong particle–wall interactions. No aggregate of PCC could be observed microscopically in the eluted fractions. The percentage viability of the cyst in the collected fractions was identical with that of the injected suspension (*ca.* 95%). It must be pointed out that, in some instances, the "flushing procedure" was performed after a series of elutions and the presence of PCC in the fraction collected at that time was observed microscopically. The possibility that the PCC particles interact with the coated walls cannot be eliminated, especially because of the elution of particles in the flushing procedures (recovery ranging from 9 to 20% of the total PCC number injected).

Elution with uncoated glass plates. The same experiments were performed in a channel with uncoated walls. In this instance the presence of PCC in the void volume was also observed but a retained peak appeared. Fig. 2 shows a set of fractograms obtained under different conditions. Fig. 2A shows the elution profile of the PCC suspension supernatant obtained after centrifugation. No peak was obtained after the two system peaks. However, a flushing procedure showed the presence of some biological material from the lung extraction medium. This material obviously adhered to the channel. The fractogram in Fig. 2A also shows the absence of a peak interfering with those in Fig. 2B–D for elutions of PCC suspensions.

Fig. 2B shows the elution of PCC in an isotonic NaCl solution. To obtain a characteristic signal, the sensitivity of the detector was set at 0.64 a.u.f.s. The recovery of PCC in the peak (retention factor 0.52) was low (30%) and the amount of PCC recovered after the flushing procedure was about 52%. This experiment showed that

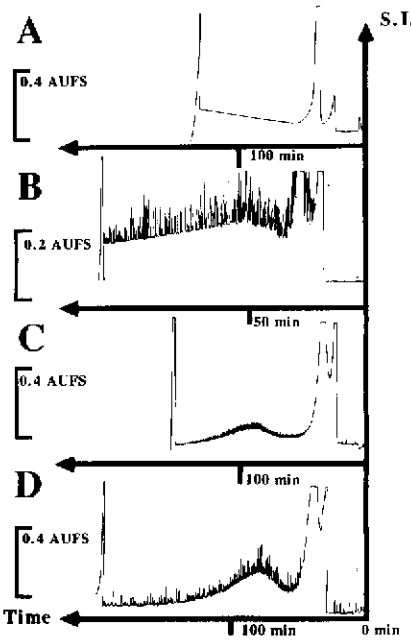

Fig. 2. Fractograms of *P. carinii* cyst suspension in G-FFF with uncoated glass walls. A 50-μl aliquot of PCC diluted suspension ($2.2 \cdot 10^7$ cysts per 50 μl) was injected without relaxation. (A) Uncoated glass plates, 0.9% (w/w) NaCl carrier phase, flow-rate 0.1 ml min^{-1}, elution of supernatant PCC suspension. (B) Elution of PCC suspension (same conditions). (C, D) As (A) and (B) with 0.05% (w/w) sodium dodecyl sulphate in isotonic saline carrier phase. Flow-rate, 0.1 ml min^{-1}.

with an uncoated channel, particle–wall interactions were still operative. These interactions seemed to be of lower strength because of the appearance of a retained peak. In Fig. 2C the same amount of PCC was injected but in an isotonic carrier phase enriched with 0.05% of sodium dodecyl sulfate (SDS), and a characteristic peak was observed with a retention factor of 0.40. The recovery of the particles in the peak was higher than in Fig. 2B (42%), but the remaining particles were eluted in the flushing procedure. In Fig. 2D, both the channel walls and the particles were incubated with the same carrier phase for 24 h before injection. Fig. 2C and D demonstrate the relative reproducibility of the FFF elution of this kind of biological material.

In Fig. 2B–D, unlike the RBC elution profiles [1], the PCC peak is much wider. In all instances the retention factor measured is higher than ex-

pected with the steric elution mechanism. To date and because of particle–wall interactions, no conclusions could be drawn using the "inertial" elution mechanism [15]. It is possible to state that under these elution conditions (saline isotonic carrier phase with surface-active agent and uncoated plates) the particle–channel wall interactions were reduced. In order to understand more closely these phenomena it was necessary to analyse the characteristics of the PCC wall.

PCC biophysical characteristics

As size, density, channel thickness and injection procedures did not explain the retention characteristics of PCC, other physical parameters were studied. PCC can be "seen", by the flow profile, as a particle of larger size than expected. One explanation may be provided by local aggregation on injection and during elution. The possible destruction of this aggregate at the outlet of the channel (detection cell, centrifugation procedures) cannot be ignored. To investigate these possible phenomena, a suspension of cysts diluted in the carrier phase was systematically checked under time control. No PCC aggregates were discovered microscopically. As the natural development medium of PCC is different from those for all other biological particles eluted in FFF, it is possible to imagine that the aqueous medium used for the PCC elution can substantially modify its real biophysical properties.

The particular structure of the PCC cell wall can be of interest, hence the hydrophilic nature of PCC was tested according to the extraction procedure of Rosenberg and Doyle [14]. Not surprisingly, the results (Fig. 3), demonstrate a very high hydrophobicity of these micrometre-sized particles.

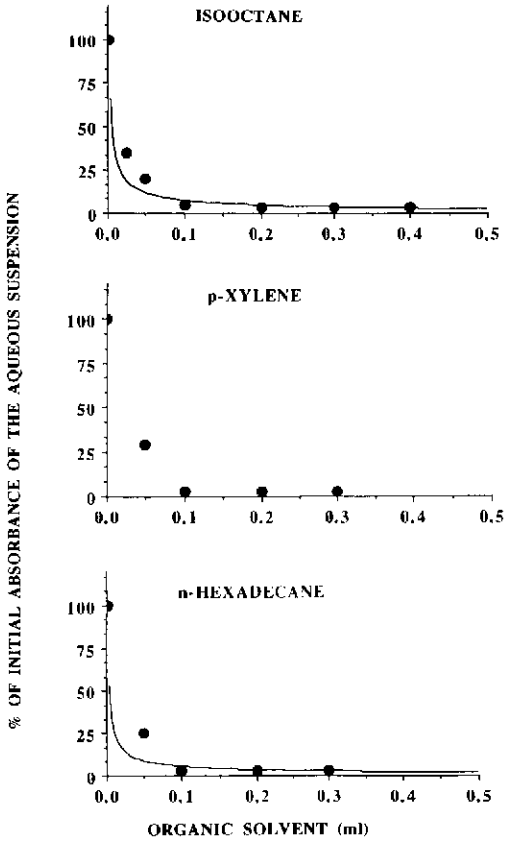

Fig. 3. Affinity of *P. carinii* cyst suspension towards hydrocarbon as a function of hydrocarbon volume. Aqueous PCC suspensions were mixed with various volumes of *n*-hexadecane, isooctane and *p*-xylene as described under Experimental.

FFF elution of RBC

In Fig. 4, the elution profile of a normal pop-

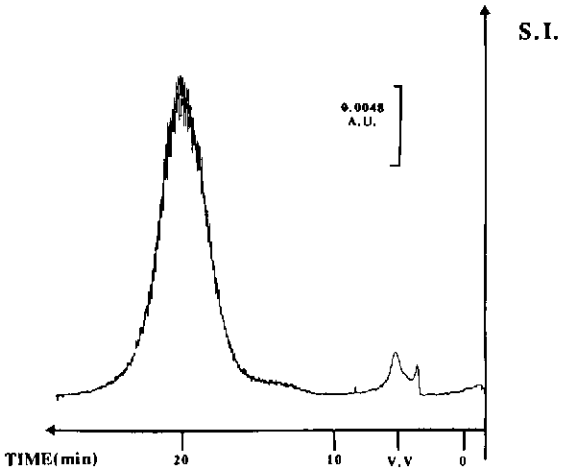

Fig. 4. Elution profile of normal human RBC in G-FFF. A 50-μl aliquot of a 100-fold dilution of total blood sample was injected under the following conditions: channel, 0.175 mm deep; coated walls; flow-rate, 0.64 min^{-1}; relaxation time, 4 min; average cell volume measured by means of a Coulter counter, 91 μm^3. Symbols V.V and S.I. indicate the void volume of the channel and the signal intensity, respectively.

ulation of human RBC retained in a coated FFF channel is shown. It was observed that the peak elution profile of particles (RBC) was much narrower, and that the retention under these elution conditions was steric or possibly inertial, as previously described by several workers [1,3,4]. With coated walls, the "flushing" procedures did not present any significant peaks relative to the wall-adsorbed cells. Using uncoated glass, similar profiles were obtained but the "flushing" procedure was the cause of a retained cell peak with an adsorption rate on the wall ranging form 5 to 12% of the injected RBCs.

DISCUSSION

PCC elution with silicone-coated glass wall

As channels made of silicone-coated walls were used in our previous studies on biological material in our laboratory, the PCC experiments were started using this material. The carrier phase was the same as that used for the elution of RBC and microfilariae. According to the results obtained previously with RBC and the closely related size of PCC, low flow-rate conditions were used. At low flow-rates a retention factor similar to that observed for RBC was expected. The experiments showed that PCC were found in the void volume peak, only a few particles were eluted. Without any further injection, a high flow-rate was set up and PCC were then observed at the outlet of the channel. This "flushing" procedure indicates that PCC were not destroyed in the channel and that interaction existed between the separation system and PCC. When injections were made at high flow-rates, PCC were eluted in the void volume peak of the channel. These results indicate that the PCC need to be eluted in the channel far from the walls to be observed at the outlet (PCC were microscopically observed in the void volume peak), that is, near the middle of the channel thickness.

At low-flow rates, and as demonstrated by the flushing procedure, the majority of the particles injected in the inlet of the channel are not destroyed, but do not elute. There is therefore a strong probability that the particles stick on the channel wall. The only particles observed travel in the channel at such a distance from the walls that they do not interact with them. At high flow-rates or during the flushing procedures, the PCC which can interact with the channel wall are hydrodynamically desorbed.

These interpretations partly explain the variation of the recovery of PCC in the void volume peak with regard to the injection number. The injections are performed with "relaxation", that is, with a stop-flow time, and the particles settle on the channel surface corresponding to the injection volume. Based on the hypothesis of particle–wall interactions, there is a progressive coating of PCC on the injection surface. The progressive saturation of the injection surface with the PCC allows, at each following injection, an increased recovery of PCC in the void volume peak. A flushing procedure will break this particle–wall interaction.

The PCC elution process can be described as follows. If PCC, during their elution through the channel, reach the accumulation wall, the cysts will stick. At low flow-rates, if PCC appear at the outlet of the channel, it means that the probability of interacting with the wall is low (near the middle of the channel, for example). At high flow-rates, PCC elute far from the channel wall and, when PCC are coated on the channel wall, PCC are hydrodynamically desorbed. In both instances, as most of the particles stick near the inlet of the channel, the retention factor will be high (*ca.* 1).

PCC elution with uncoated glass wall

With an uncoated glass wall, and with equivalent injection and elution conditions to those described above, it appeared that the PCC are retained. The elution profile is shown in Fig. 2B. Analysis of the PCC collected in the elution peak volume demonstrated a low recovery with *ca.* 50% of the injected population stuck on the channel. There is therefore a double elution mechanism; one is related to the external field, which can be considered as an FFF mechanism, and the other can be related to particle–wall interactions. This effect has been described by Han-

sen and Giddings [16] and by Mori *et al.* [17] for latex submicrometre particles in sedimentation FFF. In the experiments presented here (Fig. 2B), the elution conditions differ from those described by Hansen and Giddings [16]. The principal parameters which can be involved are the composition of the carrier phase, the nature of the channel wall and the nature of the particle surface. The ionic strength of the isotonic carrier phase is 0.15 *M* and differs from the ionic strengths used in sedimentation FFF [16]. More important is the absence of surfactant in the experiment shown in Fig. 2B, contrary to the experiments performed by Hansen and Giddings [16]. Mori *et al.* [17] have described surfactant addition in sedimentation FFF of latex beads. The gravitational FFF channel walls, with glass plates, differ from those used in sedimentation FFF by Mori *et al.* [17] or Hansen and Giddings [16], who used Teflon, polyimide and stainless steel. Moreover, the eluted particles are different. If the characteristics of the latex bead surfaces used by Hansen and Giddings [16] and Mori *et al.* [17] can be chemically described, this is not the case with the PCC wall. The only useful information presented in the results, is the strong hydrophobic characteristic of the PCC. Anyway, the results observed in this work, like those obtained by Hansen and Giddings [16], show that particle–wall interactions are observed.

If we compare the elution of PCC in uncoated and silicone-treated channels, the presence of a peak in the former instance and the strong sticking effect in the latter indicate that the particle–wall interactions are weaker with uncoated walls.

With the purpose of obtaining a more precise interpretation of the interactions involved, a surfactant was added to the carrier phase. In this instance, the elution conditions matched better those described by Mori *et al.* [17] and Hansen and Giddings [16]. Different hypotheses can be put forward to describe the mode of action of the surfactant. First, the surfactant can coat the PCC surface leading to a more hydrophilic particle; second the surfactant can coat the channel wall to lead to a more hydrophobic material. It was observed that, when the surfactant was added, the peak profile was modified as shown in Fig. 2C and D as compared with Fig. 2B. The extension of the results obtained by Hansen and Giddings [16] and by Mori *et al.* [17] to PCC and gravitational FFF has to be taken with caution. The lack of information on the PCC wall structure, unlike the well defined characteristics of latex beads, is a limiting factor. The glass channel walls in gravitational FFF cannot be compared with Teflon, polyimide or stainless steel [16,17].

In the absence of surfactant, the particle–wall interactions seem to be different in nature from those described by Hansen and Giddings [16] and Mori *et al.* [17]. This hypothesis is confirmed in the elution of the PCC in a coated-wall channel where the sticking forces act between a hydrophobic particle and a hydrophobic surface (silicone). If Fig. 2B and C are compared, the difference lies in the addition of surfactant. When surfactant is added (Fig. 2C), the PCC were more retained than without surfactant (Fig. 2B). This result is qualitatively in accordance with those of Mori *et al.* [17], that is, the retention factor of latex particles decreases when surfactant is added.

Particle–wall interactions: the case of biological materials

When a biological matrix, even prepurified, is injected into an FFF channel, some unknown compounds may interact with the channel walls. This could lead to a modification of the separator wall properties. This effect was observed by Litzén and Wahlund [5] for well retained biological compounds in asymmetric FFF, as was its influence on the retention and on the peak profile [5]. The results obtained with PCC indicate a similar tendency with variations in the recovery rate of the parasite cyst. In sedimentation FFF, Koliadima and Karaiskakis [18] demonstrated that a modification of the carrier phase composition will affect the retention of inorganic particles. Mori *et al.* [17] showed similar results in the case of a modified carrier phase with surfactant addition. The fractograms shown in Fig. 2 indicate that the retention is modified by the carrier phase composition and by the nature of the channel

wall and also depends on the particle type.

If we analyse the retention properties of hydrophilic biological particles such as those found in blood (RBC, microfilariae), there is a higher recovery when hydrophilic particles are eluted in a hydrophobic (silicone) channel, using a hydrophilic carrier phase, as described by Cardot *et al.* [1] and Merino *et al.* [2].

Biological particles taken from a relatively well known hydrophilic matrix, *e.g.*, blood, can be considered as flowing in a well defined aqueous saline and proteic medium. The characteristics of their retention times in gravitational FFF can be qualitatively evaluated on the basis of conventional FFF elution theories. This is the case if we compare, at a determined flow-rate, the retention of RBC and of microfilariae. Their elution characteristics were proved to be closely related to their volume and their density [1–3].

At the present stage of development of the steric and inertial models, it is not possible to compare PCC and RBC elution modes. When hydrophobic particles (PCC) are injected into a hydrophobic channel, strong interactions are observed. The elution of hydrophobic particles is possible in a channel with acidic walls; this can be improved in terms of recoveries, retention and peak shape by adding a surfactant to the carrier phase. The experiments described here, compared with those published elsewhere [1,5,16–18], demonstrate clearly the importance of the particle–wall interactions and may contribute to future trends in FFF in biology where a high recovery of living material is called for. Limitations to injected material and FFF wall interactions are necessary to improve reproducibility and recoveries.

Biological hydrophilic and hydrophobic particles

The elution conditions observed for the retention of both PCC and RBC indicate that the nature of both the channel wall and the biological particle wall introduce new variables into the definition of the FFF elution models. Steric and inertial models are based on hypothesized negligible or weak particle–wall interactions. The development of this method (FFF) in biology requires a modification of the existing theoretical models. The adaptation of the separation conditions and the determinaton of the particle characteristics are also necessary. This procedure is described here with the density determination of PCC, with the surface hydrophobic characteristics as defined by organic solvent extraction and with the systematic particle–channel wall interaction analysis. Another useful application of the particle–channel wall interactions in biology can be observed in the retention of the blood lymphocyte subpopulation in FFF as described by Bigelow *et al.* [19].

CONCLUSIONS

PCC invade the pulmonary alveoli surface which is covered with the pulmonary surfactant. This low pathogenic opportunist respiratory disease agent is well adapted to the amphiphilic liquid structure of the surfactant [20]. A decrease in the local immune response enhances the parasite growth and leads to respiratory distress. The knowledge of the sturcture of the PCC surface is incomplete; the cell wall contains chitin [21], a hydrophobic structure, and this could explain why cysts are not always discovered in the sediment of bronchoalveolar lavage (32%) whereas they are always found in alveoli at necropsy of the same AIDS patients [22].

We feel that the primary application of G-FFF of PCC is in research and do not advocate its use in routine diagnosis and the management of *P. carinii* pneumonia. The principal use of the described technique may reside in the study and purification of PCC from lung homogenates of laboratory animals. We foresee possible applications in the study of solutions for BAL, antipneumocystis agents acting on PCC walls or for immunological purposes.

The analysis of the PCC retention time in G-FFF using various compositions of human lung-compatible solutions (*e.g.*, physiological saline with artificial pulmonary surfactant) may lead to the development of a new bronchoalveolar lavage solution for an improved recovery of the parasite in immunocompromised patients.

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