ELSEVIER

Journal of Chromatography B, 734 (1999) 91–99

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Field- and flow-dependent trapping of red blood cells on polycarbonate accumulation wall in sedimentation field-flow fractionation

T. Chianéa[a,b,c], P.J.P. Cardot[a,*], E. Assidjo[a], J. Monteil[b], I. Clarot[a], P. Krausz[c]

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

[b]*Laboratoire de Biophysique, Université de Limoges, Faculté de Médecine 2 Rue du Docteur Marcland, 87025 Limoges Cedex, France*

[c]*Laboratoire de Chimie des Substances Naturelles, Université de Limoges, Faculté des Sciences, 2 Rue Albert Thomas, 87025 Limoges Cedex, France*

Received 3 February 1999; received in revised form 4 June 1999; accepted 12 July 1999

Abstract

Sedimentation field-flow fractionation (SdFFF) instrumentation is now mature. Methodological procedure and particle separation development rules are well established even in the case of biological species. However, in some biological applications, retention properties of samples not predicted by any field-flow fractionation (FFF) elution models are observed. It is demonstrated that the trapping of cellular material in the separation system is not related to geometrical instrumentation features but to channel wall characteristics. The physicochemical particle–wall attractive interactions are different depending on the flow-rate and field intensity applied. Separation power in SdFFF for biological species is therefore limited by the intensity of these interactions. In terms of separation, a balance is to be found between external field and flow intensity to limit particle–wall interactions.

Keywords: Sedimentation field-flow fractionation; Polycarbonate accumulation wall; Trapping of red blood cells

1. Introduction

Field-flow fractionation (FFF) is an analytical separation concept initiated in the late 1960s by Giddings for the separation and characterization of macromolecules, colloidal and micrometer-sized species of different origins [1,2]. Principles, methodological approaches, instrumental development and applications have already been extensively reviewed [2,3]. In the early 1980s, the pioneering work of Caldwell et al. [4] opened a wide investigation domain in cell separations using sedimentation FFF sub techniques. In these methods the external field can be either gravity (GFFF) [5,6] or centrifugation (SdFFF) [7,8]. Other field-flow fractionation techniques were also employed [9,10]. Micrometer-sized species retained and separated under FFF principles were observed to follow an elution mechanism described as ''steric-hyperlayer'' [2,7,9,11]. According to that model, a large set of elution conditions

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

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

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00339-4

(external field, flow-rate, channel geometry) [2,11], as well as particle characteristics (size, density, shape) [2,12], modulate retention and resolution. Moreover, injections in the established flow (i.e., injection without relaxation step, described in this report as ''E.F.'' injection) were demonstrated to be preferable to reduce particle–wall interactions and to enhance recovery and viability [13–15] at the cost of some retention ratio and resolution loss. However, in some experimental cases, non-elution of species at the outlet of the FFF channel is observed. Such experimental observations, unexplained by the ''steric-hyperlayer'' elution model are interpreted in term of particle–wall attractive interaction [16].

The main difference of the SdFFF system described in this report with all the others classical devices [3,17,18], is that the inlet tubing is connected to the accumulation wall, simulating an elutriation chamber and leading to a possible elutriation process depending on the flow-rate/external-field balance [19,20].

The hyperlayer distribution of the species provoked by the balance of field induced forces and ones of hydrodynamic origin acting on the particles predicts that the sample components will not be in close contact with the channel accumulation wall [2]. This hypothesis is emphasized in the case of injection into the established flow [13,21]. However, for a given external field, a low flow-rate is associated to reduced hydrodynamic lifting force intensities. The species under elution are driving to a close vicinity of the channel accumulation wall, whose limit is described by the size dependent ''steric'' elution model [2,7].

One major and preliminary goal of FFF separation development is the proper choice of the channel accumulation wall material. In some cases particle/wall interactions were employed for cell selectivity enhancement [10]. To fit with the ''steric-hyperlayer'' elution model, the channel wall material has to be chosen to limit or avoid attractive interactions with the species being eluted.

Another method to limit particle–wall interactions and particle–particle ones is to use appropriate mobile phase modifier [22] like surfactants or to modify the carrier ionic composition [23]. An extreme development of particle–wall interactions depending on the carrier phase composition was achieved in a particular FFF subtechnique described as ''Potential barrier FFF'' [24]. In terms of biological materials, these mobile phase modifiers are, so far, limited to proteins (albumin), which enhance the interest for an inert and biocompatible wall material, as already described in FFF for red blood cells (RBCs) [5,7,13,25] and *Pneumocistis carinii* [26]. Carrier phase modifiers may also interact with the channel wall: such an effect is not negligible in the case of albumin–polycarbonate polymer [26].

Particle–wall interactions are particularly critical when living materials are used as they can provoke metabolic modifications, reduce viability and even destroy cells. Extensive descriptions of biocompatible materials are available [27,28]. In FFF, experiments using PTFE [4], silicone [5,6], and polycarbonate [29] coating of the accumulation wall were performed. Polycarbonate polymers are considerably used in biomedical applications [28] and appear to be relatively successful in FFF [7,13,15]. This material is known to be suitable to enhance recovery and viability compared to classical channel walls [2–4,6,30,18]. Protein–polymer interactions are mainly of hydrophobic nature and to a smaller extent electrostatic.

Therefore, material hydrophobicity and surface free energy can determine the choice of the appropriate material [28]. Polycarbonates are hydrophobic with a low free energy surface; albumin adsorption on their surfaces is limited [26]. The glass-like surface quality of this material transforms this polymer into an appropriate FFF accumulation wall. Red blood cells present an external membrane surface (partially made of proteins) of hydrophilic characteristics [31]. Consequently, both RBC–polycarbonate hydrophobic and electrostatic interactions are limited because of the low free energy surface of that polymer. Extensive descriptions of cell–organic polymer interactions have been published so far [28,32]: they guide the choice of the channel wall material in relation to the particle surface characteristics.

The purpose of this report is first to demonstrate that, when no elution occurs, the cells enter into the channel, whatever the FFF experimental conditions. In a second step, a methodological procedure is

developed to differentiate reversible from irreversible attractive cell–wall interactions.

2. Experimental

2.1. Red blood cell samples

Sample handling procedures and RBC suspension storing were already described [13]. Before SdFFF elution, blood samples were diluted 100-fold with the isotonic carrier phase, and red blood cells labelled with radioactive technetium (^{99m}Tc) by means of the ''TCK 11 Kit'' (Cis Bio, Gif sur Yvette, France). The labelling procedure is a pre-tinning method by the stannous pyrophosphate, which penetrates the cells and reduced the ^{99m}Tc added. The ^{99m}Tc reduced is fixed by the hemoglobin, or more precisely by the globin moiety. Consequently all the ^{99m}Tc added is fixed inside the erythrocyte. The labelling intensity (740 MBq/ml of RBC suspension) was chosen to achieve significant elution signal even in high dilution cases. The ^{99m}Tc RBC binding is stable in vitro with a 99% recovery. All the experiments described below were performed during a negligible period relative to the ^{99m}Tc half-life time (6.02 h).

2.2. FFF system

2.2.1. FFF channel

The SdFFF system used in this report has already been described [7] and was validated for RBC elutions [13]. In the configuration used in this report, both channel walls were made from 2.0 mm thick polycarbonate polymer. A 250-μm thick Mylar spacer sealed the channel whose dimensions were: 78.5×1×0.025 cm. An associated void volume of 2.11±0.09 ml (2σ, $n=4$) was measured by means of 0.1% (w/w) sodium benzoate (Darasse, Paris, France) water solution.

To ensure a constant and reliable external field, the rotation of the centrifuge basket encircling the channel was generated with a M71B4B32 motor associated with a Mininvert 370 (Richards Systems, Les Ullis, France) pilot unit. Face views of the centrifuge basket sealing the channel in the SdFFF used in these experiments are given in Fig. 1.

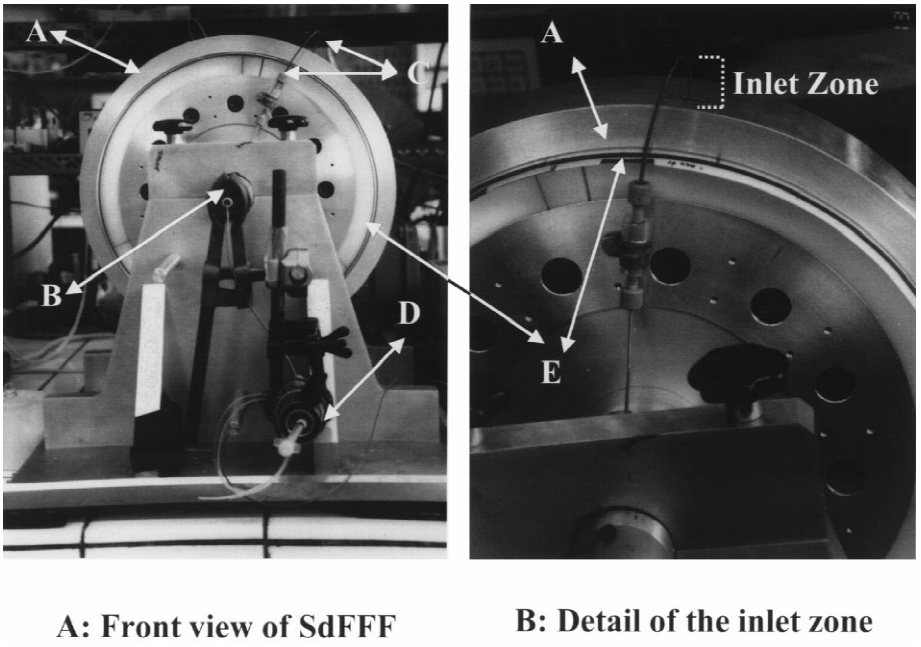

Fig. 1. The SdFFF system. (A) the rotating basket, (B) the rotating seal, (C) the inlet tubing, (D) the injection device, (E) the FFF channel.

2.2.2. Carrier phase

Phosphate-buffered saline (PBS) solution, pH 7.4 (Biomerieux, Marcy-l'Etoile, France), supplemented with 1 g/l of bovine serum albumin (A-4503, Sigma, St. Louis, MO, USA) in sterile double distilled water (Biosedra Pharma, Louvier, France) was used as the carrier phase. Its characteristics were measured at $\eta = 5 \cdot 10^{-3}$ Pa s (Rheomat 15 Contraves) viscosity, and $\rho = 1.007$ (Portable densitometer, DA 110, Kyoto Electronics, Minauri, Kyoto, Japan) density. Flow-rates were set up and controlled via a Gilson pump Model 302 (Gilson Medical Electronics, Middletown, WI, USA).

2.2.3. Sample injection photometric on-line detection and extracolumn connections

The sample suspensions (50 μl) were inserted into the SdFFF system by means of a 7125i Rheodyne valve (Rheodyne, Cotati, CA, USA). The elution signals were monitored at 365 nm with a Waters 440 (Waters, Milford, MA, USA) liquid chromatography photometric detector. Signals were systematically recorded on an IBM compatible Daewoo computer (Daewoo Europe, Roissy Charles De Gaulle, France). All connecting tubings were from Upchurch (Upchurch, Oak Harbor, NJ, USA) with a 0.0512 cm internal diameter.

2.3. Scintigraphic detection of radiolabelled RBCs

Whole channel imaging was performed using a HELIX, large field of view dual head gamma camera (Elscint, Haifa, Israel), with a low-energy all-purpose parallel hole collimator. The large area covered by this type of camera allowed to monitor the radiolabelled cells all over the channel. Fig. 2 describes the instrumental set-up.

Labelled red blood cells were introduced in the SdFFF system using the ''E.F.'' injection procedure; data digital frames (600 s, 256×256 pixels, magnitude 1.3) were acquired 15 cm away from the great surface of the rotating basket. Obtained scintigraphic signal can be cumulated for signal enhancement.

If the cells are trapped in the channel during elution, a circled signal due to the γ rays of ^{99m}Tc is observed with a radius corresponding to the one of the channel. If the cells are trapped in the inlet tubing, the signal observed is a circle of higher radius. The scintigraphic image of the basket bowl in

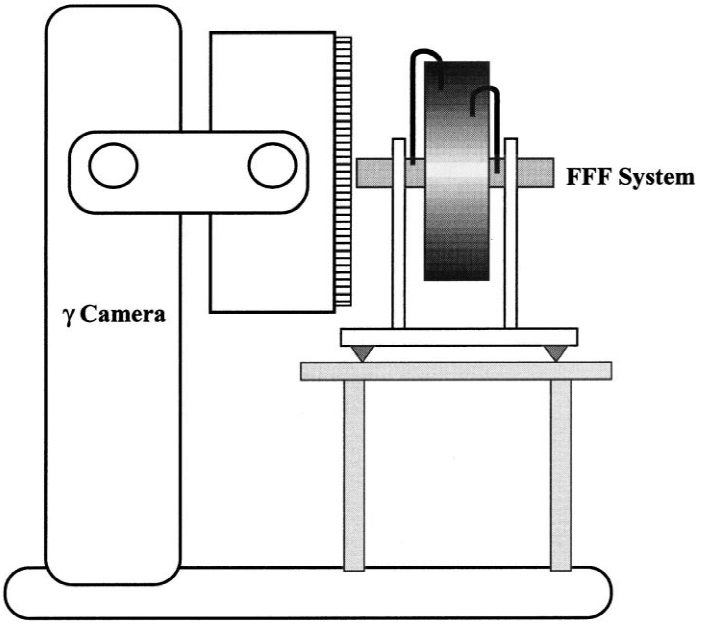

Fig. 2. Schematic representation of the whole system scintigraphic detection of RBCs in the SdFFF system.

rotation of the SdFFF device will make it possible to locate the trapping of the cells.

3. Results and discussion

Systematic RBC suspension elutions were performed using the ''E.F.'' injection procedure. Time dependent fractograms, obtained after complete elution, are displayed in Fig. 3. Field intensity dependent fractogram series A, B, C, D correspond to elutions performed at increased flow-rates. Retention ratios were calculated for all these fractograms, and the corresponding data are plotted in Fig. 4. For the same samples, retention ratios decreased with field intensity increase at constant flow-rate. At constant external field, with increasing flow-rate, retention ratios increased, which is consistent with the ''steric-hyperlayer'' model. These data match closely those obtained by Caldwell et al. [4], Metreau et al. [7], and Assidjo et al. [13]. Moreover, as shown in Fig. 4, in some experimental conditions the RBC sample can be eluted with retention ratio as low as 0.07. This value corresponds to the hydrodynamic radius of a sphere of equivalent RBC volume, demonstrating a possible size dependent ''steric'' elution mode for RBCs [7] where hydrodynamic lifting forces are overpowered by the ones provoked by the external field. Such a conclusion is in accordance with the ''steric'' and ''steric-hyperlayer'' definition developed in the Introduction.

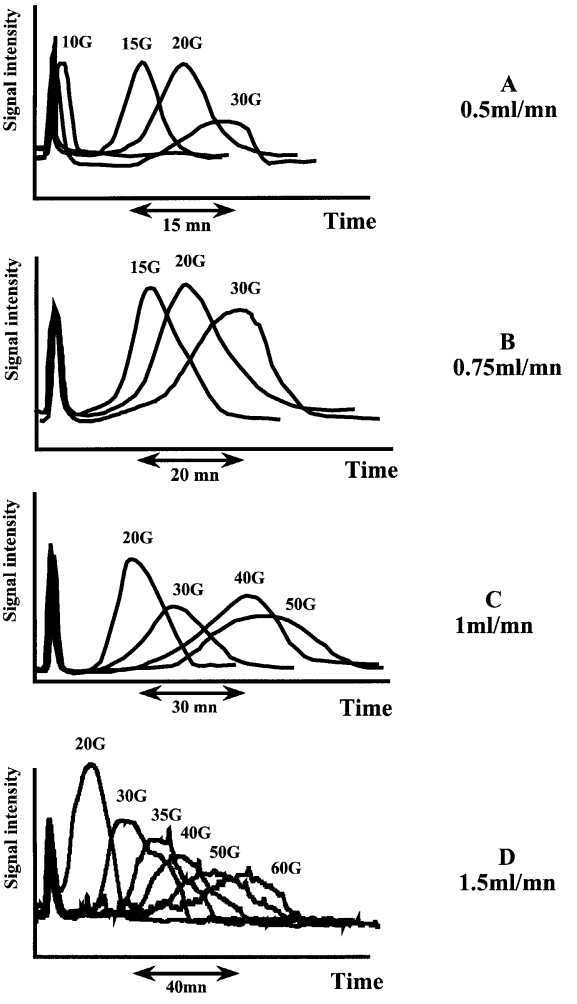

Fig. 3. Red blood cells fractograms. Flow injection, carrier phase: isotonic phosphate buffer saline solution with 0.2% (w/w) bovine albumin. (A) Flow-rate: 0.5 ml/min, field strength: 10 to 30 G. (B) Flow-rate: 0.75 ml/min, field strength: 15 to 30 G. (C) Flow-rate: 1 ml/min, field strength: 20 to 50 G. (D) Flow-rate: 1.5 ml/min, field strength: 20 to 60 G.

3.1. Evidence of sample trapping

Keeping in mind the idea to experimentally demonstrate the steric elution mode of RBCs, elutions were performed at high external fields. In such a configuration, the lifting hydrodynamic forces are overpowered by the external field driving to a size (volume) dependent retention. Time-dependent signals obtained in these cases are shown in Fig. 5A–D. In any case, no RBC elution photometric signal was observed as long as the external field was applied. This indicated clearly that RBC were trapped in the SdFFF apparatus.

However, the position of the ''cell population'' in the SdFFF system is not located. To define precisely the location of the trapping, a specific experimental set up was performed by means of a scintigraphic camera placed in front of the channel bowl as already described in Experimental.

The trapping of RBC in the SdFFF system can be originated by two different mechanisms. The first one, already described in the Introduction and schematically illustrated in Fig. 6, is an eventual elutriation process in the inlet tubing at the vicinity of the channel inlet zone. The second one, also detailed above, is of physico-chemical nature linked

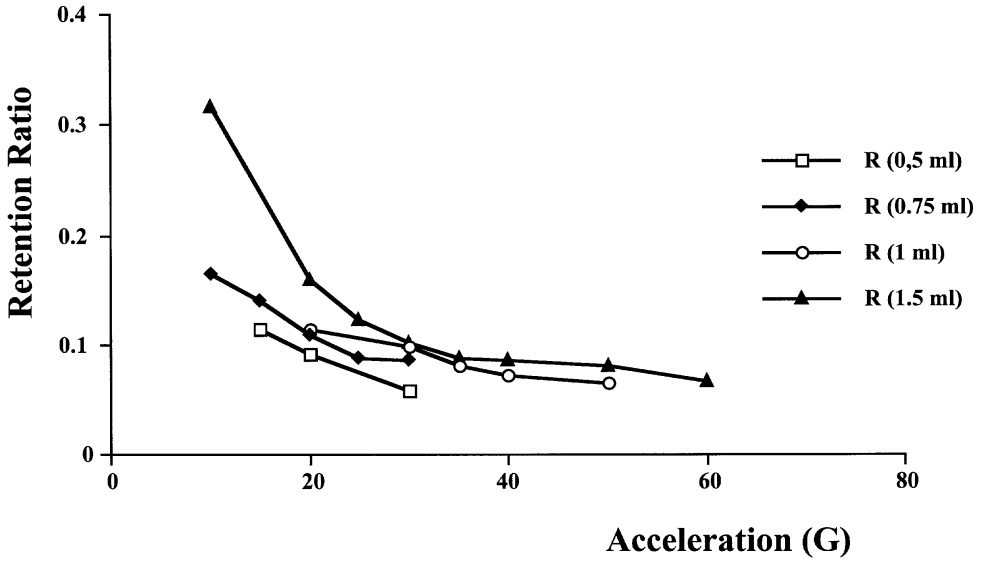

Fig. 4. Effect of flow velocity and field intensity on retention ratio of red blood cells. Flow-rate ranging from 0.5 to 1.5 ml/min.

to particle–wall interactions. Scintigraphic tracking of radiolabelled RBCs during and after elution in the whole SdFFF system was therefore necessary to determine the spatial position of the trapped RBCs in the rotating basket.

3.2. Sample trapping is not an elutriation dependent process

3.2.1. Scintigraphic reference image of the SdFFF system

In a first experiment, two populations of radiolabelled RBCs were positioned simultaneously in the inlet tubing 2.5 cm from the channel entrance and, in the channel inlet zone. In the absence of flow, scintigraphic images were acquired at 40 G as external field and cumulated data are shown in Fig. 7, slide A. Two circled bands are observed. The external one corresponded to the RBC population in the inlet tubing and the internal one to the RBC population positioned in the channel. Slide A was set up to define a reference image of the SdFFF system.

3.2.2. RBC sample flows into the SdFFF channel

"E.F." injection of radiolabelled RBCs corresponding to a flow-rate/field balance associated with sample trapping was performed (0.5 ml/min; 60 G external field) and monitored with the scintigraphic camera. The cumulated images obtained during elution are shown in Fig. 7, slide B. As the circle in slide B corresponds to the internal circle of slide A, the RBCs introduced into the FFF system were located in the channel. The immediate conclusion is: no elutriation process occurred although the inlet tubing was connected to the accumulation wall. RBC sample trapping is therefore conditioned by possible attractive particle–wall interactions.

3.3. Sample trapping is a particle–wall interaction process

Radiolabelled RBC samples were injected into the SdFFF system under two different experimental conditions. In a first series of experiments, the flow/field couple corresponded to the RBC trapping described in Fig. 5A (0.5 ml/min; 35 G); in a second series of experiments, conditions were those described in Fig. 5D (1.5 ml/min; 70 G). In both cases scintigraphic cumulated signals were identical and are shown in Fig. 7, slide C. Slides B and C showed a scintigraphic signal of the same diameter indicating the presence of RBCs in the channel. Therefore under both experimental conditions RBC trapping

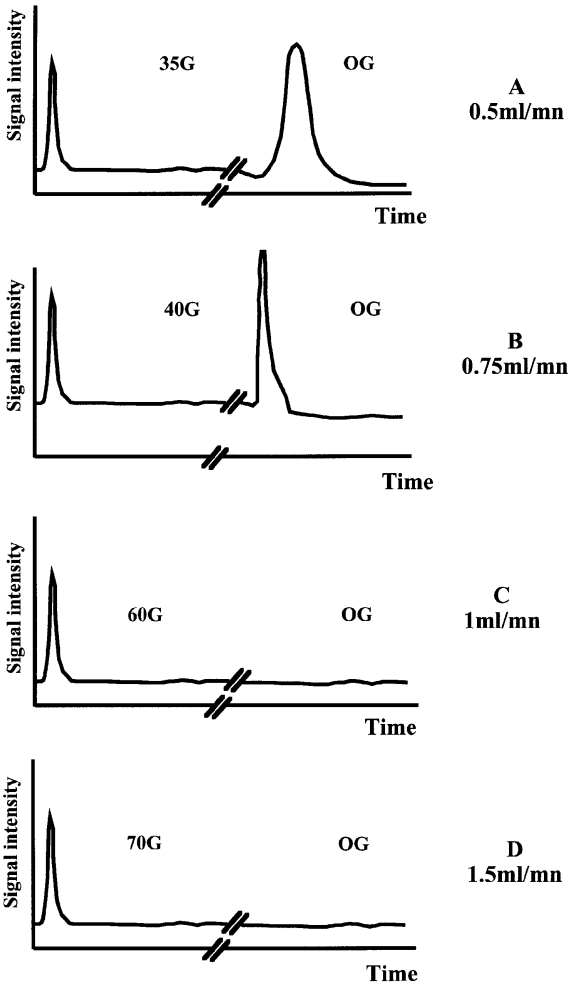

Fig. 5. Red blood cells fractograms. Flow injection, carrier phase: isotonic phosphate buffer saline solution with 0.2% (w/w) bovine albumin. (A) Flow-rate: 0.5 ml/min, field strength: first step 35 G, second step 0 G. (B) Flow-rate: 0.75 ml/min, field strength: first step 40, second step 0 G. (C) Flow-rate: 1 ml/min, field strength: first step 60 G, second step 0 G. (D) Flow-rate: 1.5 ml/min, field strength: first step 70 G, second step 0 G.

was caused by particle–wall interactions. Are these interactions reversible or not?

3.3.1. Reversible sample trapping is observed

A series of experiments corresponding to Fig. 5A and B (0.5 ml/min, 35 G and 0.75 ml/min, 40 G), were performed and the elution signal recorded. No RBC signal was obtained as long as the external field was applied. However, when the channel rotation

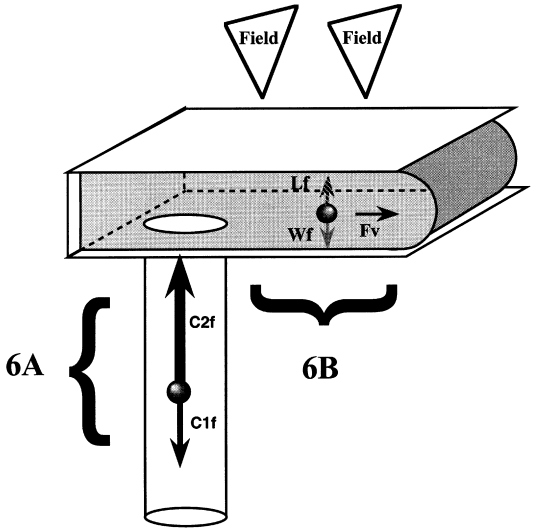

Fig. 6. Schematic representation of induced force acting on the cells. (A) Inlet tubing: elutriation process. C1f is the centrifugal force, C2f is the centripetal force (flow-rate and sedimentation velocity). (B) ''Steric-Hyperlayer'' model. Lf is the lifting force, Wf is the field induced force acting on the particle, Fv is the particle flow-velocity.

was stopped, eliminating the external field a photometric signal was observed as shown in Fig. 5A and B. This is confirmed by the scintigraphic signal which disappeared rapidly. Microscopic observation of the eluted samples showed RBCs in their integrity with an 80% recovery. It can be concluded that, under these experimental conditions, cell trapping was reversible.

3.3.2. Irreversible sample trapping may occur

A series of experiments corresponding to Fig. 5C and D (1.0 ml/min, 60 G and 1.5 ml/min, 70 G) were performed with the above procedure. The cumulative scintigraphic signal was identical to the ones of Fig. 7B and C. However, when the external field was stopped and the flow-rate maintained, no photometric elution signal was observed as seen in Fig. 5C and D. If scintigraphic frames were acquired with the rotating basket immobilized it was observed, as shown in Fig. 7D, that the cells were trapped in the first quarter of the channel length, indicating that their integrity was respected. The scintigraphic signal remained even with a flushing carrier phase procedure of 3.0 ml/min. The cells

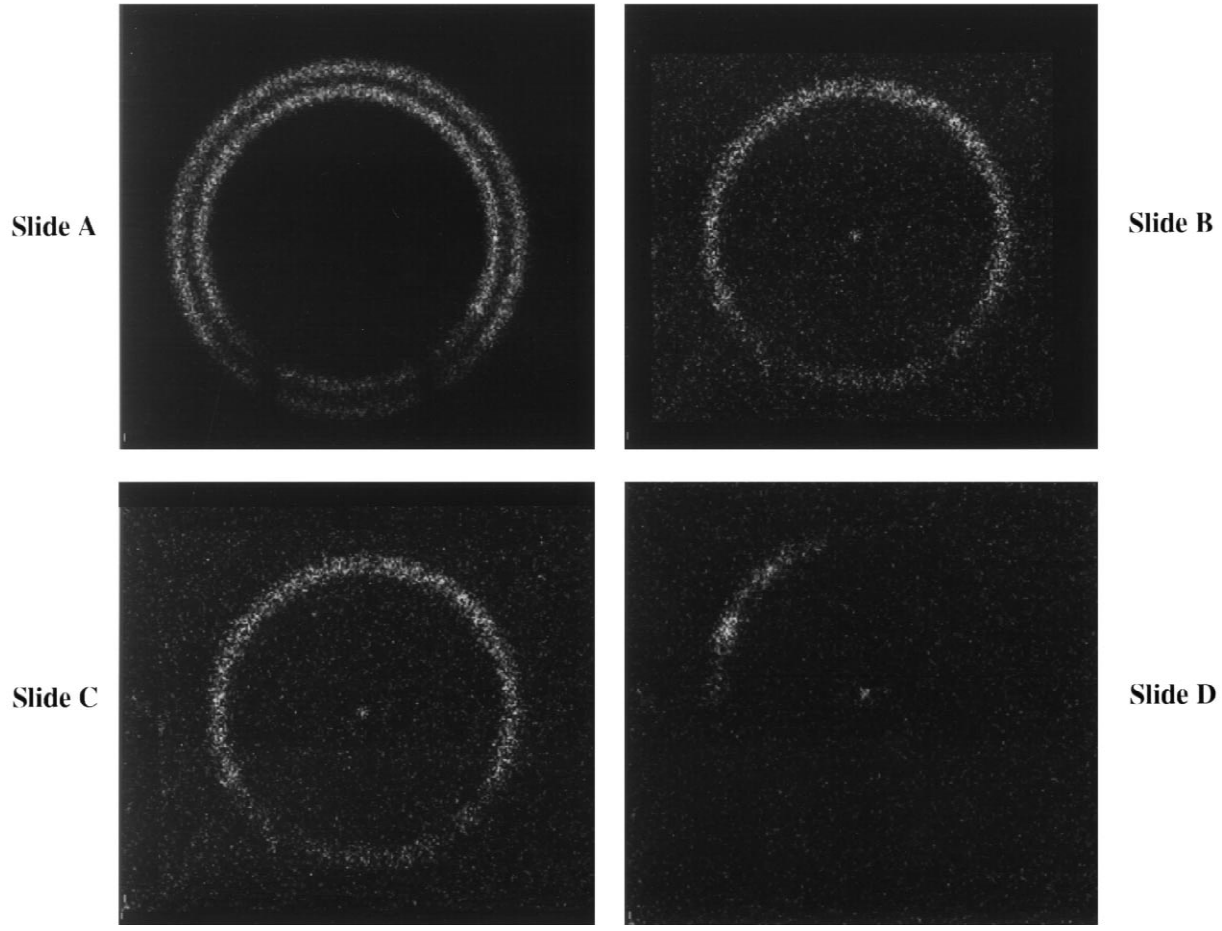

Fig. 7. Scintigraphic images obtained with ^{99m}Tc-labelled RBC. Slide A: the internal circle is the channel, the external is the inlet tubing in rotation. Slide B: flow-rate of 0.5 ml/min and external field of 60 G. Slide C: flow-rate of 0.5 ml/min and external field of 35 G. Slide D: image obtained when the external field is stopped before a run with a flow-rate of 1 ml/min and an external field of 60 G.

appeared strongly adsorbed on the polycarbonate accumulation plate. The only way to eliminate radio signal was to wash off the channel with a hypo-osmotic solution, which destroyed the cells and released the radiolabelled-hemoglobin, driving to a flow-rate dependent extinction of the scintigraphic signal.

4. Conclusion

Increasing retention and selectivity in FFF is generally obtained via the so-called ''primary relaxation stop-flow'' injection procedure [2]. Such a methodology, mainly operated in SdFFF at high external field, drives the micrometer-sized species in the close vicinity of the accumulation wall, increasing the particle–wall interaction probability and reducing channel outlet recovery. This process drives to the degradation of the channel surface, leading to complicated flushing or washing procedure [8]. The ''E.F.'' injection mode of RBCs has demonstrated that high selectivity was preserved [33,34]. ''E.F.'' injection through the accumulation wall reduced the retention loss as shown by Assidjo et al. [13,15]. Therefore, flow injections in SdFFF associated with biocompatible channel wall materials allowed to obtain high recovery of living cellular samples

[7,13]. However, SdFFF technical performances (external field, flow-rates) are to be limited according to retention and separation purposes. Living cells cannot be eluted according to the elution mode classically described as ''steric'', as strong attractive particle–wall interactions may occur, which can be reversible or not, as demonstrated by a specific experimental step, using radiolabelled RBCs monitored by scintigraphic detection.

The choice of channel wall material is of uppermost importance and depends on the sample to be separated. Rough guidelines are given in this report. Polycarbonate polymer is useful as long as the flow-rate versus external field intensities is low. Systematic recovery procedures will quantify the intensity and the characteristics of these interactions as well as the analysis of reversible interactions.

Acknowledgements

J.M. Marbouty, head of the Department of English for Specific Purpose, College of Pharmacy, Limoges University, is fully acknowledged for English language corrections.

References

[1] J.C. Giddings, Sep. Sci. 1 (1966) 123–125.
[2] J.C. Giddings, Science 260 (1993) 1456–1465.
[3] J. Janca, K. Kleparnik, U. Jahnova, J. Chmelik, J. Liq. Chromatogr. 7 (1984) 1–10.
[4] K.D. Caldwell, Z.Q. Cheng, P. Hradecky, J.C. Giddings, Cell Biophys. 6 (1984) 233–251.
[5] Ph.J.P. Cardot, J. Gerota, M. Martin, J. Chromatogr. 568 (1991) 93–103.
[6] E. Urbankova, A. Vacek, N. Novakova, F. Matulik, J. Chmelik, J. Chromatogr. 583 (1992) 27–34.
[7] J.M. Metreau, S. Gallet, Ph.J.P. Cardot, V. Lemaire, F. Dumas, A. Hernvann, S. Loric, Anal. Biochem. 251 (1997) 178–186.
[8] S. Hoffstetter-Khun, T. Rosler, M. Ehrat, H.M. Widmer, Anal. Biochem. 206 (1992) 300–308.
[9] B.N. Barman, E.R. Ashwood, J.C. Giddings, Anal. Biochem. 212 (1993) 35–42.
[10] J.C. Bigelow, J.C. Giddings, Y. Nabeshima, T. Tsuruta, K. Kataoka, T. Okano, N. Yui, Y. Sakurai, J. Immunol. Methods 117 (1989) 289–293.
[11] P.S. Williams, T. Koch, J.C. Giddings, Chem. Eng. Commun. 111 (1992) 121–147.
[12] M. Martin, P.S. Williams, in: F. Dondi, G. Guiochon (Eds.), Theoretical Advancement in Chromatography and Related Separation Techniques, NATO ASI Series C: Mathematical and Physical Sciences, Vol. 383, Kluwer, Dordrecht, 1992, pp. 513–580.
[13] E. Assidjo, T. Chianea, M.F. Dreyfuss, Ph.J.P. Cardot, J. Chromatogr. B 709 (1998) 197–207.
[14] J.P. Andreux, A. Merino, M. Renard, M. Forestier, Ph.J.P. Cardot, Exp. Hematol. 21 (1993) 326–330.
[15] E. Assidjo, Ph.J.P. Cardot, J. Liq. Chromatogr 20 (1997) 2579–2597.
[16] B. Kusserow, R.W. Larrow, Annual Report, PB 252 720/As, National Technical Information Service, Springfield, VA, 1976.
[17] J.C. Giddings, F.J.F. Yang, M.N. Myers, Anal. Chem. 46 (1974) 1917–1924.
[18] S.M. Mozersky, H.M. Farrell Jr., R.A. Barford, Sep. Sci. Technol. 26 (1991) 1467–1479.
[19] P.E. Lindhal, Nature 161 (1948) 648–649.
[20] P.E. Lindhal, Biochim. Biophys. Acta 21 (1956) 411–415.
[21] F.J. Yang, M.N. Myers, J.C. Giddings, J. Colloid Interface Sci. 60 (1977) 574–577.
[22] S.G. Stevenson, K.R. Preston, J. Liq. Chromatogr. 20 (1997) 2835–2842.
[23] Y. Mori, B. Scarlett, H.G. Merkus, J. Chromatogr. 515 (1990) 27–35.
[24] G. Karaiskakis, A. Koliadima, Chromatographia 28 (1989) 31–32.
[25] R. Parsons, V. Yue, X. Tong, P. Cardot, A. Bernard, J.P. Andreux, K. Caldwell, J. Chromatogr. B 686 (1996) 177–188.
[26] C. Bories, Ph.J.P. Cardot, V. Abramowski, C. Poüs, A. Merino-Dugay, B. Baron, J. Chromatogr. 579 (1992) 143–152.
[27] J.L. Brash, S. Uniyal, J. Polymer Sci. C66 (1979) 377–391.
[28] G. Chang, D.R. Absolom, A.B. Stong, G.D. Stubley, W. Zingg, J. Biomed. Mater. Res 22 (1988) 13–29.
[29] A. Bernard, B. Paulet, V. Colin, Ph.J.P. Cardot, Trend Anal. Chem. 14 (1995) 266–273.
[30] J.J. Kirkland, W.W. Yau, W.A. Doerner, J.W. Grant, Anal. Chem. 52 (1980) 1944–1954.
[31] C.M. Cohen, Semin. Hematol. 20 (1983) 141–158.
[32] B.D. Ratner, Biosens. Bioelectron. 10 (1995) 797–804.
[33] A. Merino-Dugay, Ph.J.P. Cardot, M. Czok, M. Guernet, J.P. Andreux, J. Chromatogr. 579 (1992) 73–83.
[34] Ph.J.P. Cardot, J.M. Launay, M. Martin, J. Liq. Chromatogr. Rel. Technol. 20 (1997) 2543–2553.