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Instrumentation of hollow fiber flow field flow fractionation for selective cell elution

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ABSTRACT

Hollow fiber flow field flow fractionation (HF5) columns can be built with minimized cost and instrumental skills by incorporation of commercial hollow fibers into holders made of classical chromatographic tubing and connectors. The proposed design leads to differential elution of human cells of different origin. Suspensions of red blood cells (RBC) and adherent human colorectal cancer (CRC) cell lines were used. These CRC nucleated population have been linked to cell-to-cell and cell to instrument interactions that are limiting factors in terms of recovery and viability. These interactions can be limited depending on injection/elution conditions. By using RBC we observed that the focalization/relaxation step played a major role in the elution process. However, HF5 opens a large potential, which completes the diversity shown by SdFFF in cell sorting methodologies and technologies.

1. Introduction

Recent developments in hollow fiber flow field flow fractionation (HF5) techniques have used simple manufactured fibers of commercial origin or designed specifically to minimize cell–wall interactions [1–4]. Concerning cell sorting and general separation sciences for biological applications, the pioneering studies of Moon and Reschiglian groups have opened new fields with the development of HF5 instrumentation [1–4]. Cell sorting with field flow fractionation is for the time being a rather discreet story [5–10]. It emerged when FFF Sedimentation techniques (GFFF and SdFFF) achieved maturity with the ground breaking work of Caldwell [11]. It continues its path via laboratories associated with strong instrumental capabilities [12]. Nevertheless, the absence of commercial interest, the lack of a large scientific application market associated with a high level of interfacing competences linking cell biology, instrumentation, chemistry, and material sciences limit the wide spread use of cell sorting by SdFFF. However, the success of all FFF techniques using flow as an external field has positioned the FFF concept in some key positions [13] for environmental studies [14] or biotechnologies [15–20]. Expansion of FFF cell sorting methodologies by means of an adapted and simplified instrumentation, which could be easily used by unspecialized laboratories, opens a new trend. The aim of this report was to show that with ordinary chromatographic skills it was possible to set up an HF5 column in place of a conventional column in any modern HPLC system and perform cell separations at limited cost.

2. Materials and methods

2.1. HF5 instrumentation

2.1.1. Commercial hollow fiber

The HF5 system described in this report was developed in our laboratory. It starts with the choice of its essential component, i.e., the hollow fiber (HF), that is composed of polysulfone with a molecular cut off centered around 30 kDa (UFB-30, Romicon, Koch International, Lyon, France). Individual fibers were isolated from a Romicon UFB-30-5A cartridge. The fiber used for cell elution was chosen with a manufacturer claimed inner diameter of 0.9 mm and a length of 18.0 cm. The associated void volume extrapolated from measured length (dry conditions) was calculated at 114.4 ± 0.6 μL. In contrast to other groups [4,16–18,21], the fiber holder tube is made of standard HPLC tubings coupled to fitting equipment (Upchurch, C.I.L, Ste Foy La Grande, France) as described in Fig. 1.

2.1.2. HF5 dry columns

As described [2], glue free HF5 design is possible with minimized void volume. In our setup, HF was immobilized within an exterior housing (the holder) made of 1/8 in. O.D. PEEK tubing (Upchurch).

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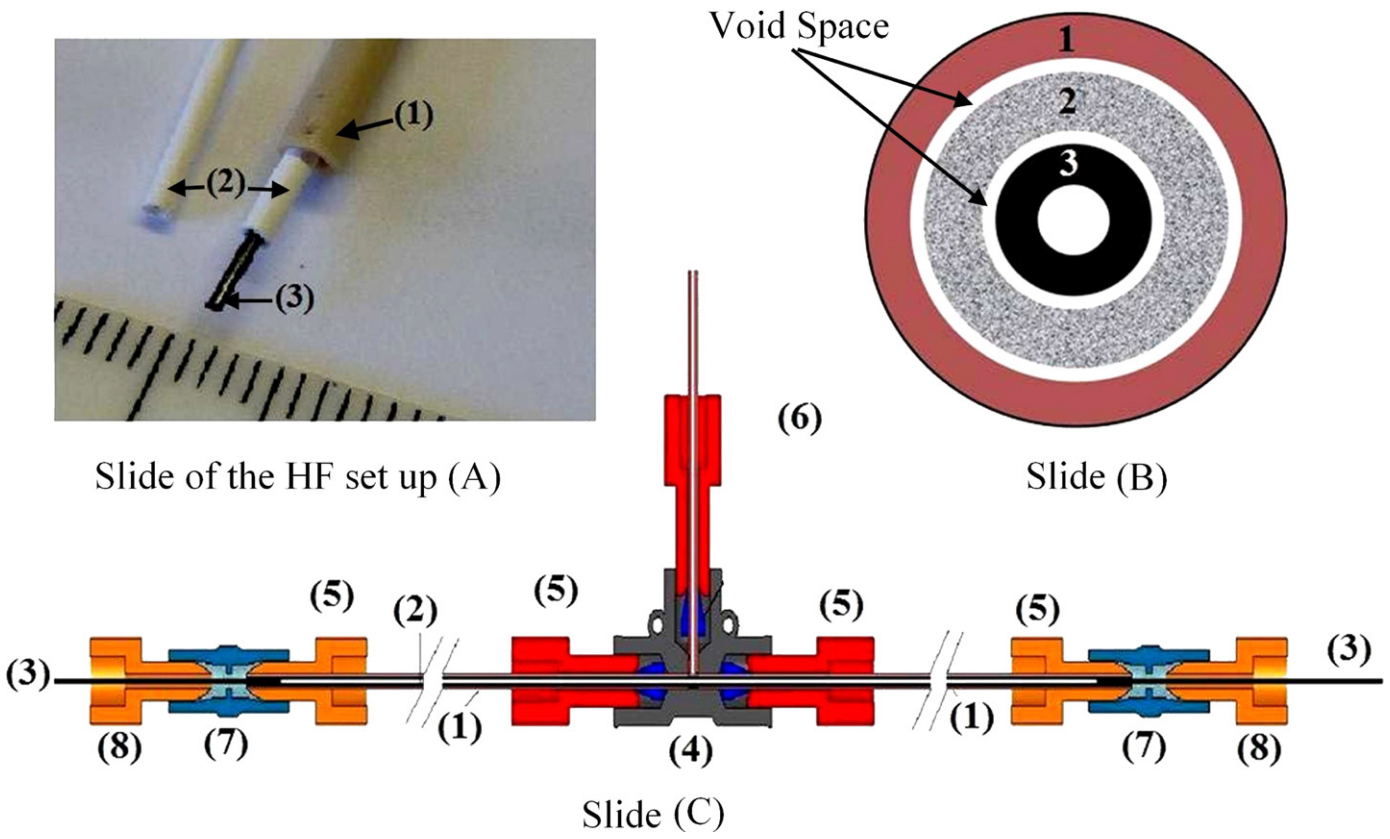

Fig. 1. HF FIFFF component and column design. (A) The holder tube made of PEEK tubing (1/8 in. OD × 0.080 ID) (1) contains the hollow fiber from Romicon (1.2 mm OD, 0.9 mm ID) (2) in which the connecting capillary tubing (1/32 in. OD × 0.0035 in. ID) (3) are inserted. (B) Transverse cross section of the HF5 column. (C) The longitudinal cross-section centered on a T-Cross PEEK device, laboratory modified for 1/8 in. OD tubes (4), sealing is made possible via PEEK 1/8 in. short flangeless nuts (5) and PEEK 1/16 in. short flangeless nuts (6). Upchurch 0.050 in. female adapter thru-hole for 1/16–1/8 in. PEEK tubings (7) and fingertight fitting for 1/16 in. PEEK tubings (8) complete the design.

In practice, the 1.2 mm O.D. commercial dry fiber can be inserted into 1/8 in. O.D. tubing with an inner diameter of 2 mm as shown in Fig. 1A. Fig. 1B shows the relative position of the fiber in the holder section; the dry fiber was cut exactly to the holder dimension. Then, capillary tubing was inserted into the fiber inlet and outlet as shown in Fig. 1A. Sealing of the connecting tubing within the fiber and holder shortened fiber length as shown in Fig. 1C. It is therefore possible, by carefully measuring the capillary tubing length before and after sealing, to determine the HF length available for separation and the geometrical volume of the dry fiber as described in Table 1. One interesting feature of this instrumentation was that sealing of PEEK tubing led to deformation of both the holder and the connecting capillary tubing. Then, the fiber was sealed and compressed at fitting locations in order to avoid leakage under flow pressure. The second advantage of PEEK tubing holders was the possibility to collect the radial flow at selected points of column length by means of a simple hole drilled in the holder as shown in Fig. 1C. Sealing a connecting T equipped with appropriate flow restrictors at that position controlled the radial flow rate. Obviously, and in contrast to a HF5 glass holder or asymmetrical flow FFF (AFFFF), it was not possible to visualize the possible focusing position [13,15,16]. However, some blind focalization processes appeared effective as previously demonstrated [21] and have been systematically applied in this report. Nevertheless, this HF5 device was built with very limited instrumentation skills and at low cost, with most of the components being re-used when HF replacements were necessary.

2.1.3. HF5 design and operation

We setup a single pump system configuration shown in Fig. 2, as previously described [1,4]. Two-step run cycles were used: (a) classical injection (with relaxation/focalization, Fig. 2A); and (b) direct injection/elution (no focalization step, Fig. 2B).

When classical injections were processed, the HF5 system was set in configuration A (Fig. 2A) with primary relaxation and focalization of the sample. After a programmed time (relaxation/focalization time), the switch between configuration A and the elution configuration (configuration B) required simultaneous action of both valves A and B as shown in Fig. 2. These 6-port valves

Table 1
Determination of the hollow fiber characteristics.

Flow rate	0.15 mL/min		0.2 mL/min
Manufacturer claimed inner diameter		900 μm	
Geometrical volume of the dry fiber		114.5 μL	
Dextran elution volume (peak summit)	93 ± 14.7 (n = 4) μL		71 ± 16.5 (n = 6) μL
Effective fiber diameter (peak summit)	881 ± 88 μm		744 ± 78 μm
Dextran peak first moment	122 ± 25 μL		114 ± 35 μL
Effective fiber diameter (first moment)	929 ± 96 μm		897 ± 135 μm

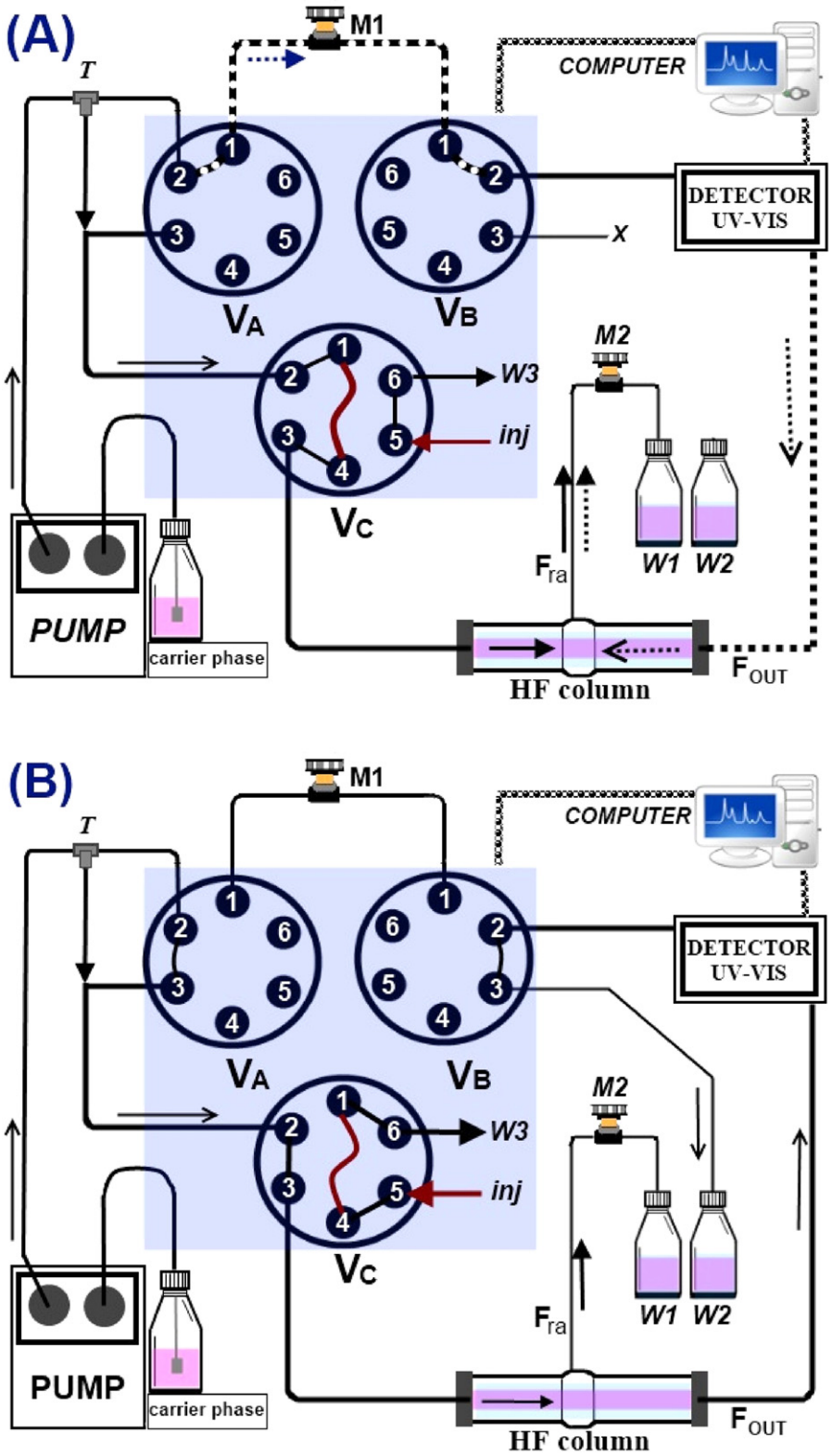

Fig. 2. Single Pump HF5 system design. Three 6-port valves V_A–V_B–V_C are used to select configurations (A and B) and sample injection (V_C). (A) Focalization/relaxation configuration: circuit operative when both A and B valves are in position 1–2. (B) Elution configuration, circuit operative when both V_A and V_B valves are in position 2–3. The other equipment include: connecting tee (T); metering valves (M1 and M2). W1, W2, and W3 describe flow waste respectively from radial flow, axial flow, and injection valve. F_{out} is the measured axial flow rate at column outlet and F_{rad} is the measured radial flow rate.

Model 7060 (Rheodyne, Cotati, CA, USA) were electronically controlled by means of an EPS 130 actuator (Eurosas, Le Blanc Mesnil, France) whose purpose was also to considerably reduce switching pressure shocks during configuration change. To complete the system a metering valve SS-31RS4 (Nupro, Willoughby, OH, USA) was inserted between valves A and B in order to produce axial flow split regulations (Fig. 2, M1).

Fiber inlet flow rates were produced using a classical HPLC instrumentation consisting of a Waters pump model 590 and Waters 481 UV detector was installed at the axial outlet (Waters, Guyancourt, France) (Fig. 2).

Samples were introduced by means of model 7161 injection valve (Rheodyne, Cotati, CA, USA) with 0.7–1.2 μL injection loops (Fig. 2, injection valve V_C). A second metering valve model SS-SS1 VH (Nupro, Willoughby, OH, USA) was used to control radial flow (Fig. 2, M2). Because of the setup, when both 6-port switching valves A and B were in the outlet 1 position, the focusing relaxation configuration is setup (Fig. 2A). When both outlets 2 were selected, the elution configuration was established (Fig. 2B). Tubing characteristics (length and internal diameter) were chosen in order to minimize connecting volumes and possible pressure modifications at configuration change. Flow rates were systematically measured and controlled.

For cell species eluted in the "Steric Hyperlayer" mode [11] it is possible to avoid the "relaxation/focalization step" of the sample. This elution procedure proved effective in cell sorting with SdFFF [6–9,12] but has not been studied in flow FFF. Specific column washing was established in order to avoid fiber poisoning. First, the radial flow was stopped. Secondly, a series of alternate (1 min) carrier phase high (0.3 mL/min) and low flow rates (0.03 mL/min) [22] were implemented for a cumulative volume of ten times the system void volume.

2.2. Carrier phase and samples

The carrier phases were freshly distilled water, Phosphate Buffered Saline solution (PBS) (Gibco Inc., Grand Island, NY, USA), or 9.0 g/L NaCl isotonic solutions (Prolabo, Fontenay sous Bois, France). Dextran solutions in bidistilled water were obtained using 2.5 g/L polydispersed Blue dextran MW = 2000 kDa (Aldrich, ST Quentin-Falavier, France). Benzoate solutions in bidistilled water were prepared using 10 g/L sodium benzoate (Merck, Fontenay Sous bois, France). Human red blood cells (RBC) were collected from blood samples under EDTA as anticoagulant. Blood samples were diluted to 1/100 ($5{\cdot}10^9$ RBC/mL) prior to HF5 analyses. Nucleated cells from colorectal cancer cell lines (CRC) were obtained from the American Type Culture Collection (ATCC). Colo205 cells were cultured in RPMI (Invitrogen, Gibco) supplemented with 10% inactivated fetal calf serum (FCS, Invitrogen), 100 UI/mL penicillin, 100 μg/mL streptomycin, and incubated at 37 °C in a humidified 5% CO_2 environment. Cell suspensions were obtained through trypsinization with 0.5% trypsin for 5 min. Coulter counter particle size analyses [12] showed a 12 μm average diameter of spherical cells in suspension. All samples to be assayed were used at 10^6 cells/mL.

3. Results and discussion

3.1. HF5 column void volume determination

Experimentally, connecting and flowing water or aqueous buffers through a dry HF5 column does not allow establishing a radial flow. Therefore, a specific "column" conditioning procedure was needed. According to the manufacturer indications, this consists of flowing 10 mL of ethanol/water mixture (50/50, v/v) at low flow rate (0.1 mL/min) through the dry column then decreasing the ethanol proportion by 10% every 10 mL until reaching pure water. When the column was equilibrated, any type of aqueous carrier phase could be used with appropriate axial and radial flows. As described [1], a diameter increase of 2.5% was measured between dried and wet membranes consequently modifying the geometrical volume. In addition, Moon and Reschiglian groups [1–3,16] described a flow pressure-dependant fiber volume. All these converging data required careful attention in the characterization process of the void volume. We observed that the fiber wetting procedure described above, increased fiber length by 0.5 ± 0.1% (15–20 cm range, $n = 5$). Therefore, we measured the fiber internal volume in operating conditions, which means after insertion into holder and wetting procedure and under pressure produced by the flow rate. The wet, ready to use HF column, was flowed by the appropriate carrier phase in the absence of radial flow.

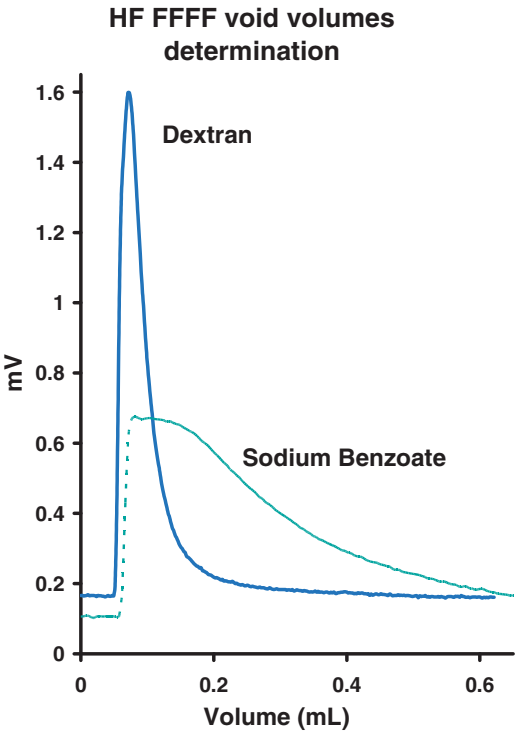

Fig. 3. Experimental void volume determination. Elution conditions: mobile phase: PBS, 0.15 mL/min; HF5 system configuration: see Fig. 2B; spectrophotometric detection at λ = 400 nm (Dextran T2000) or λ = 254 nm (sodium benzoate (10 mV = 0.01 A.U.F.S).

The elution of appropriate probes resulted in two very different void volumes. The first was the fiber inner volume and the second the holder volume free for solvent, i.e., the holder geometrical volume minus the fiber volume. The fiber inner volume was determined with a sample having molecular weight greater than the membrane cut-off. Polydispersed blue dextran aqueous solution was eluted at 0.15 mL/min inlet flow rate with no radial flow as shown in Fig. 3; elution was also performed at 0.2 mL/min. The dextran asymmetrical peak profile shown in Fig. 3; was analyzed by means of peak moment measurements [23], which calculated the column volume and consequently an effective inner wet fiber radius as recorded in Table 1. No statistical difference between both flow rates was shown, which gave an average fiber diameter of 913 ± 100 μm ($n = 10$, 2σ) of wet fiber, a result very close to that claimed for dry ones.

In contrast, eluting a molecule that can diffuse through the fiber will determine the volume free for solvent of the holder. For that purpose, sodium benzoate solution was eluted in the same conditions. Compared to dextran, we observed a very different

elution profile (Fig. 3). The profile appeared complex at first, a sharp front shape at an elution volume similar to dextran, and secondly, a broad trailing region. The first moment calculated volume of 269 μL indicated an intermediate value between the inner fiber (114.5 μL) and the calculated geometric holder inner space (565 μL), which was compatible with the volume taken up by the fiber. Such peak profile analysis indicated the existence of a dual physical phenomenon. First, part of the benzoate sample was eluted in the lumen of the fiber and appeared in the front portion of the elution profile. In the absence of radial field (flow rate), an other benzoate fraction diffuses through the membrane which is at the origin of the tailing part of the profile. It appears obvious that in the presence of a radial field this benzoate peak shape would be considerably modified (data not shown). Nevertheless, that comparative procedure (dextran/benzoate) may be a potential tool to evaluate the "barrier" performance of the membrane in the future.

3.2. HF5 for cell separation

The very low geometrical volume of hollow fibers compared to those for elutriation or SdFFF gives HF5 techniques access to the domain of micro analytical methods [8]. In that regard, there is a major interest in developing cell-sorting techniques applicable to micro sized samples such as tumor fragments [8]. These tumor samples are usually comprised of small cell numbers that can be suspended in sub-μL volumes to be analyzed by HF5 at concentrations less than 10^6/mL. HF5 separation leads to eluted material that can be collected at appropriate concentrations, allowing the tracking of rare cell types. This trend is particularly critical in cancer research where complex tumor populations are often encountered [24,25].

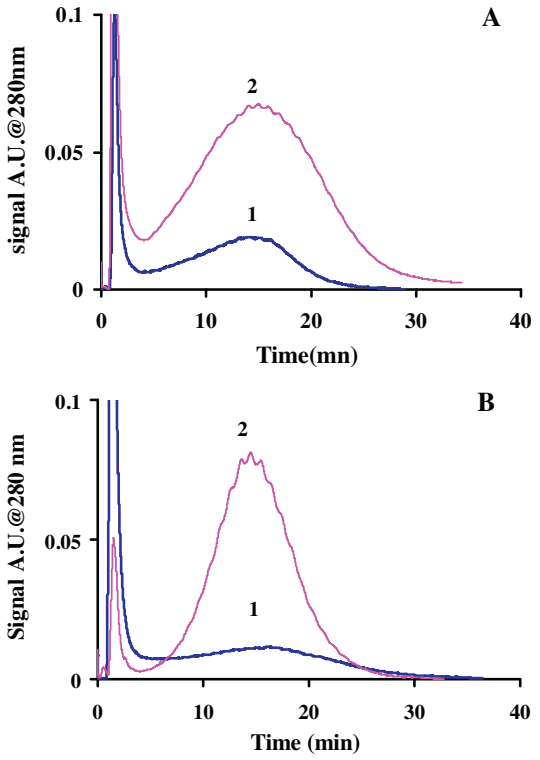

Fig. 4. Elution profile of human red blood cells. Experimental conditions: sample = 10^6 RBC in 1.2 μL; carrier phase: A = Isotonic PBS/B = Isotonic 9 g/L NaCl solution; focalization position: 50% HFL; inlet flow = 0.175 mL/min; radial flow: 0.14 mL/min; axial flow: 0.035 mL/min. 1 = flow injection and 2 = 1 min relaxation.

3.2.1. Red blood cell elution by HF5

Injection procedure and recovery. Historically, any instrumental FFF cell sorting investigation starts using RBC or a specific cell lineage as demonstrated by the pioneering works of Caldwell [11], Chmelik's group [26–28] and our group [5,29,30] in SdFFF. More recently, it was demonstrated that HF5 was able to elute RBC using membranes specifically designed for that purpose [31]. RBC suspensions are of major interest because of low cost, sturdiness, and capacity to mimic characteristics specific of any cell elution by FFF. Furthermore, it was also demonstrated that cells follow a retention mechanism described in the literature as "Steric Hyperlayer" or "Inertial" [8,11,12]. In the latter case, our group has shown [6,8,11,12] that cell elution, retention and separation can be performed without any stop flow time or primary relaxation process. Consequently, we tested our HF5 column design in its most simple configuration, by simply replacing an HPLC column by the HF5 column. However, in practice, to take advantage of HF5 versatility, the operating instrumentation configuration used is described in Fig. 2B.

First, RBC have been considered as an homogeneous population with an average size, shape, and density. For the first set of experiments, the carrier phase and dilution media were a PBS isotonic solution. Fig. 4A(1) shows representative fractograms. Profiles were characteristics of RBC elution in FFF as already demonstrated with GrFFF, SdFFF, and HF5 [5,11,28,31]. Quantitative analysis showed that 30–35% of injected cells were eluted between 3 and 28 min. Eluted fractions collected between injection time and 2 min corresponded to 60–65% of injected cells. Classical relaxation focalization injections [1–4] with 1 min focalization and focalization position at 50% of the fiber length (50% HFL) led to the profile shown in Fig. 4A(2). In that case, fractions collected between 3 and 28 min comprised 72–75% of injected cells, while only 13–15% were eluted in the void volume. The recovery difference between both injection protocols was 30%. The washing procedure systematically applied after every elution released around 5–10% of the injected material.

In terms of peak profile comparison, cell peaks in Fig. 4A showed analogous patterns, with retention ratios of 0.10 and 0.08 respectively for profile 1 and 2. Band spreading characteristics, expressed in time dependent standard deviation, were also of the same order with 3.9 and 4.2 min respectively. The main difference between both injection conditions was linked to the "retained cell" quantity, which was strongly increased when primary relaxation/focalization step was performed leading to increased peak intensity, area or counting (Fig. 4A). This result was strongly different from those obtained with SdFFF [5,32]; for which during the primary relaxation step RBC were positioned close to the accumulation wall by injection via the accumulation wall, avoiding the focalization–relaxation step [33]. In contrast, with HF5, it was not possible to directly lay down the cells at the fiber inner wall. Such differences explain the needs of sample analyses with focalization/relaxation procedures in HF5. These results were confirmed in analogous field and flow conditions but using NaCl (9 g/L) as a carrier phase, as shown in Fig. 4B. Flow injection (Fig. 4B(1)) led to low cell recovery (25%) while stop flow resulted in a 85% recovery (Fig. 4B(2)). All these profiles (Fig. 4) showed great similarities with the ones obtained in Bologna [3,4].

3.2.2. Steric Hyperlayer elution mode

If Fig. 4 elution profiles are analyzed with the hypothesis of a "Steric Hyperlayer" elution mode of the RBC, then it is possible to calculate the retention ratio [23] from void volume elution time and the retention time of the cellular population.

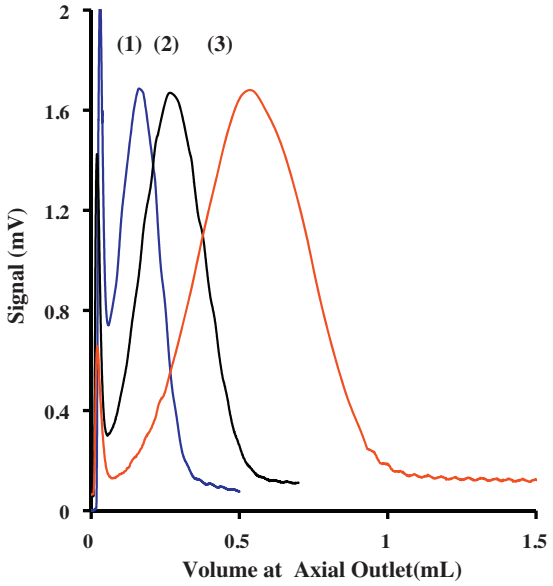

Fig. 5. RBC Hyperlayer elution mode in HF5. Elution conditions: Sample of $2.5{\cdot}10^6$ RBC in 2.45 μL carrier phase (isotonic PBS); focalization: 50% HFL; relaxation time: 1 min. (1) V_{out} = 0.090 mL/min, V_{rad} = 0.230 mL/min. (2) V_{out} = 0.050 mL/min, V_{rad} = 0.140 mL/min. (3) V_{out} = 0.028 mL/min, V_{rad} = 0.080 mL/min.

As a consequence the average cell population position, centered at "pseudo equilibrium position" (**a**) from the inner fiber wall, can be calculated [34,35] from the retention ratio R according to Eq. (1)

$$R = \frac{4\mathbf{a}}{\omega} \tag{1}$$

where ω is the fiber lumen diameter. The position **a**, the "average cell" distance from the fiber wall is calculated according to the following equation:

$$\mathbf{a} = \frac{R\omega}{4} \tag{2}$$

An average position (**a**) centered on 10.2 ± 0.6 μm was calculated depending on the injection conditions and carrier phase. Therefore and considering an average RBC size of 5 μm, the cells seemed to be eluted in conditions very close to those of Steric Hyperlayer mode. As RBC showed a discoid shape (length: 6–8 μm/thickness: 2–3 μm), this mean that whatever the RBC position in the flow profile (vertical or horizontal), the average position was always greater than particle radius ($a = 10.2 > 1.5$ or 4 μm). However, it is essential to demonstrate the retention dependence of R on the axial flow rate at almost constant field, which identifies the "Hyperlayer effect" as demonstrated by Caldwell et al. [11]. In order to demonstrate this effect, elution profiles should be shown as functions of the outlet elution volume. However, this is based on a fundamental basic hypothesis, i.e., a linear decrease of the axial flow velocity (flow rate) along the channel, so far largely accepted [1–4,31]. Systematic retention increased while axial flow decreased at almost constant external field intensity (flow ratio variations less than 5%) confirming the dependence of retention on the axial flow rate (Fig. 5). In order to obtain these constant conditions, inlet/axial flow rate ratio was maintained constant at 1.37 ± 0.02, while outlet flow rate decreased. These observations demonstrated the "Hyperlayer effect" [11,23,34]. Another way to analyze the Steric Hyperlayer elution mode of RBC was to modify carrier phase osmolarity [5]. Cell size, shape, and density will be modified and consequently their elution characteristics. Cells eluted in hyper-osmolarity conditions will be smaller than those eluted in iso- or hypo-osmolar ones. Therefore, the Steric

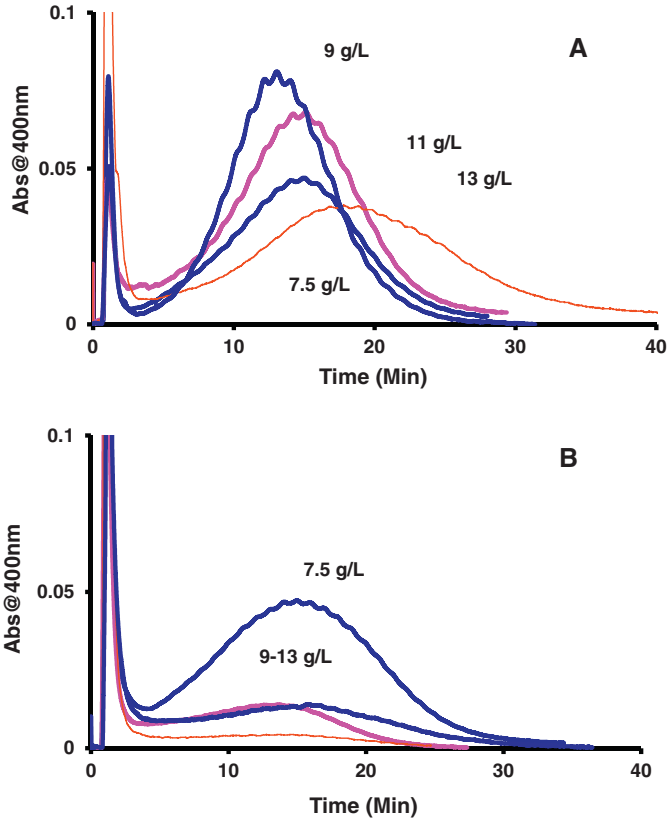

Fig. 6. Osmolar dependent elution of RBC. Elution conditions: sample: 10^6 RBC in 1.2 μL of carrier phase of osmolarity from 7 to 13 g/L NaCl; inlet flow = 0.175 mL/min; radial flow = 0.140 ml/min; axial flow = 0.035 mL/min. (A) Focalization at 50% HFL with 1 min relaxation; (B) flow injection.

Hyperlayer elution of RBC with different osmolarities can be predicted [5].

3.2.3. *Effects of carrier phase osmolarity on RBC elution in HF5*

Elution in analogous physical conditions (flow, field, and stop flow time) was performed and fractograms are shown in Fig. 6A. The only differences were in the osmolarity characteristics of the NaCl carrier phase which ranged from 7.5 to 13 g/L. Not surprisingly, the osmolarity conditions where RBC were retained the most corresponded to 13 g/L. In these very hyperosmolar conditions, RBC were smallest and most dense. When 11 g/L NaCl solutions were used, the RBC were larger and appeared less retained. This was confirmed using iso-osmolar carrier phase solutions. It can be concluded, from iso- to hyper-osmolar conditions, that retention and size are correlated in terms of the Steric Hyperlayer model, an effect already demonstrated [4]. In contrast, elution in hypo-osmolar conditions (NaCl 7.5 g/L) did not confirm this model (Fig. 6A). In order to interpret this discrepancy, flow elution were performed and fractograms are shown in Fig. 6B. Clearly, two elutions patterns emerged: the group of iso- and hyperosmolar elution conditions with a low percentage of injected cells effectively retained; and that of hypo-osmolar conditions giving a fractogram profile almost analogous to the one obtained with a stop flow procedure. RBCs diluted in (NaCl 7.5 g/L), showed an increased cell volume and sphericity while density decreased. Therefore, RBCs eluted similarly regardless of the injection/elution procedure as shown in Fig. 6A and B. If both profiles were compared, the stop-flow/focalization procedures led to 80% recovery (Fig. 6A) compared to 60% in Fig. 6B obtained by direct injection.

In both cases, release at washing reached 5%. It can be explained that, under the hydrodynamic field, cells are driven differently

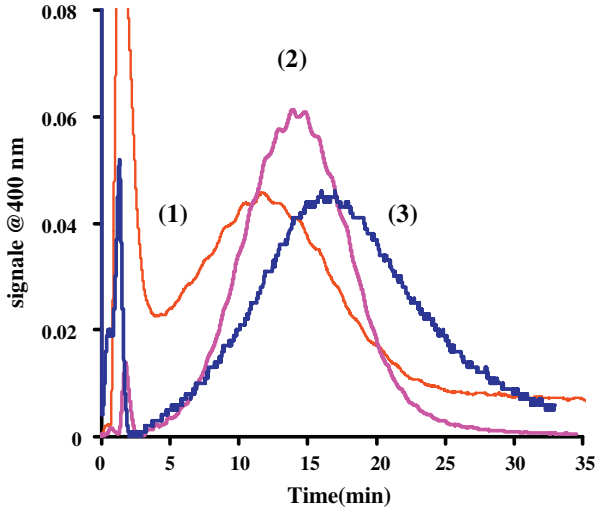

Fig. 7. Osmolar dependent elution of RBC. Elution conditions: Sample: 10^6 RBC in 1.2 μL of carrier phase of osmolarity from 7 to 13 g/L NaCl; inlet flow = 0.175 mL/min; radial flow = 0.14 mL/min; axial flow = 0.035 mL/min; focalization = 50% HFL. (1) Hypotonic 7.5 g/L NaCl with 30 s relaxation; (2) Isotonic 9 g/L NaCl with 1 min relaxation; (3) Hypertonic 11 g/L NaCl with 2 min relaxation.

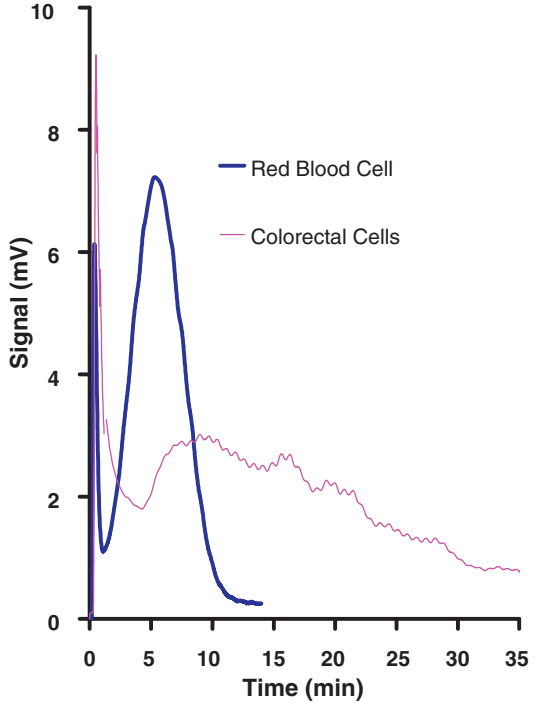

Fig. 8. Elution profiles of HRBC and CRC lineage. Samples: 10^6 RBC in 2.4 μL (injection loop); isotonic PBS as carrier phase, 10^6 CRC in 10 μL (injection loop). Focalization time and position: 2 min, 50% HFL. Inlet flow rate: 0.19 ml/min; radial flow: 0.14 ml/min; axial flow: 0.05 ml/min.

toward the fiber inner wall depending on the osmolarity of the carrier phase. We observed (Fig. 6B) that the larger the particle size and the smaller the density difference with the carrier phase (hypoosmolar conditions), the more effective was the primary relaxation step [13,36].

Therefore the data shown in Fig. 6A and B confirm the hypothesis that cell populations containing large cells compared to the channel lumen (hyposmolar conditions), have faster radial flow kinetics during elution or focalization. Whatever the origin of this effect or its complexity, it is possible to draw immediate consequences in order to adapt relaxation/focalization time for cell separation or elution characteristic comparison. In FFF, the Steric Hyperlayer elution mode predicts that the larger and less dense cells are eluted with a higher retention ratio than the denser and smaller ones [36]. Therefore, it is necessary to adapt the focalization time depending at least, on cell size. Practically, this means increasing focalization time as the cell size decreases (hyperosmolar medium) and inversely. Size-dependent elution order of cells eluted in different osmotic conditions and adapted focalization time is shown in Fig. 7. The elution pattern of RBC already observed in SdFFF [5] was also obtained, and it can be concluded that RBCs in HF5 systems are eluted according to the "Steric Hyperlayer model" with the restriction that considerable attention must be taken paid to "stop flow relaxation step" conditions.

3.2.4. Mammalian cells

In the above section, we confirmed that HF5 was suitable for RBC elution as pioneered by Moon's and Reschiglian's group [1–4]. A simple and low cost instrumentation was developed. However 21st century cell sorting trends are directed toward nucleated mammalian cell populations [6–10,17,24,25]. Therefore, elution of nucleated cells will demonstrate the high potential of HF5 for cell sorting. At present, ecological characterization of tumors is a major trend in cancer research. For this reason nucleated cells originating from a colorectal carcinoma (CRC) were used [12,24,25]. They are adherent cells, which means that they have the capacity to generate strong interactions upon contact with any materials. Therefore, and prior to any elution, the cultured sample was transformed into an individual cell suspension taking care to eliminate doublet and triplet or higher aggregates [12,24,25]. If cells are improperly washed or diluted, adhesion reappears destroying the suspension and creating both aggregates and irreversible cell/wall interactions with cell lysis as a consequence. Most of these adherent-cell suspension problems have been overcome for SdFFF separations. However no equivalent studies have been performed for HF5 so far [6–10,12,17,24,25]. Therefore, CRC cells were used to evaluate the potential of HF5 for cells separation. CRC elution profiles were compared to those of RBCs (Fig. 8). A large peak profile of these cells compared to RBC was observed. This is generally accepted in FFF to be due to large polydispersities, even if their extent has not yet been completely assessed [37]. Nevertheless, recovery greater than 70% was measured. However, and in contrast to SdFFF, the elution order of RBC and colorectal cells did not match with the size dependent hyperlayer model despite analogous densities [5,31,36]. In Fig. 8, at equivalent relaxation/focalization time, the larger (on size and volume) cells are eluted after the smaller ones (12 μm colorectal cell diameter compared to 5 μm for RBCs). The origin of this observation, demonstrated in the previous section may rely on the complexity of the primary relaxation/focalization process. Again larger cells seem to be more effectively relaxed (close to the accumulation wall) than smaller ones. Close examination of SdFFF versus HF5 primary relaxation reveals that the multi-gravitational field induced sedimentation of cells (SdFFF) is very different from radial flow induced one (HF5). Nevertheless, the possibility that HF5 performs cell sorting appears to be confirmed with rather simple instrumental and methodological efforts. Information obtained from separation patterns as well as their comparison with SdFFF or elutriation processes may generate a complete original position of HF5 methods for cell sorting. The small dead volume of the hollow fiber generates paradoxical situations. On one hand, it can limit the sample loading capacity and consequently the amount of collectable material at the outlet. On the other hand the limited dilution imposed by the small void volume makes analysis of reduced size samples like tumors possible. Whatever the final verdict, the specificity of the injection relaxation process in HF5 implies an excellent potential for selective cell elution.

4. Conclusions

The instrumental development of an HF5 column at low cost generates a strong potential for this technique compared to the highly sophisticated design, knowledge, and assembly required for SdFFF. So far, most cell sorting applications in FFF are linked to Sedimentation sub techniques. HF5 columns are simple to develop. For the first time in HF5 literature, mammalian nucleated cells were eluted with a recovery ratio compatible with cell sorting requirements. Surprisingly, and in contrast to Sedimentation FFF, primary relaxation/focalization appears to play a major role in HF5 cell sorting methodologies which will open a specific separation domain. Most cell separation development strategies acquired with SdFFF and described under the concept of "functional selectivity" should be applicable to HF5. In addition, the very low column volume, places HF5 in "micro scale" cell sorting technologies while focalization makes it possible to download large suspension volumes.

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