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Tandem hollow-fiber flow field-flow fractionation

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ABSTRACT

Reinjection of one ore more collected fractions of eluted samples is recognized as a useful procedure in analytical separation techniques, among which field-flow fractionation (FFF), to improve the actual separation of complex samples. Hollow-fiber flow FFF (HF5) is a micro-channel subset of flow FFF (F4), which has recently reached a performance comparable to that of standard, flat-channel F4. To further improve HF5 of complex protein samples, we present a new device and method for in-line, reinjection HF5 that we call tandem HF5 (HF5/HF5). HF5 is ideally suited for tandem operation because (1) small channel volume and low operation flow rates allow reducing dilution and volume of the collected fractions, and (2) the relaxation/focusing step that takes place between the 1st and 2nd run (refocusing) allows reestablishing the volume and concentration of the sample plug before the 2nd elution. HF5/HF5 proves particularly effective in the case of oligomeric proteins since it allows collecting and reinjecting the bands that correspond to each separated oligomeric form. This provides information on the dynamic equilibria between the different oligomers. For HF5/HF5 operations, a modified, prototype HF5 instrumentation is presented which includes a "trap" constituted of a four-port, two-way valve positioned downstream the UV detector and a collection loop. The effect of refocusing conditions on HF5/HF5 performance is investigated by varying refocusing time. With a complex protein samples such as blood serum, HF5/HF5 can improve detectability of the low abundance components since overloading effects due to high-abundance components are reduced. This is shown for serum lipoproteins: while after the 1st run high density lipoproteins (HDLs) are not separated from high-abundance serum proteins, after the 2nd run it is shown possible to separate the HDL subclasses.

1. Introduction

In analytical separations reinjection into the separation system of one or more collected fractions of the eluted sample is a procedure to increase the separation efficiency. This is because the number of theoretical plates is increased by increasing the separation space under which the selected sample fractions are re-run. Field-flow fractionation (FFF) is a family of separation techniques suited for the analysis of macromolecular, nanosized, and micron-sized analytes [1,2]. Over 25 years the theoretical basis of the reinjection mode in FFF was given [3]. The re-injection mode was introduced to improve FFF resolution in the analysis of polydisperse colloidal samples. This mode allowed discovering artifacts due to concentration effects or sample interactions [4,5]. Up to now reinjection in FFF has been always off-line performed. However, this is a time-consuming and labor-intensive procedure since it requires manual collection and processing of the fractions to be reinjected. Moreover, the analyte dilution occurring during the 1st run often requires a reconcentration step of the collected fractions before the 2nd (reinjection) run. In asymmetrical flow FFF (AF4) [6], reconcentration of high-volume sample fractions can be effectively obtained in-channel by means of the relaxation/focusing procedure performed before sample elution. Relaxation/focusing concentrates the sample in a confined space, and allows the introduction/injection of high sample volumes (even higher than the channel volume) with no loss in fractionation efficiency [7].

Hollow-fiber (HF) flow FFF (HF5) is a micro-volume (<100 μL) version of flow FFF (F4) [8]. The use of HF channels for FFF was reported for the first time in 1979 [9]. Fundamentals and first examples of HF5 were described somewhat later [10–13], and HF5 system advances and applications to a broad range of analytes have been reported in relatively recent years [14–23]. Still at a prototype stage, today's HF5 is becoming a valid alternative to standard, flat-channel F4 [24].

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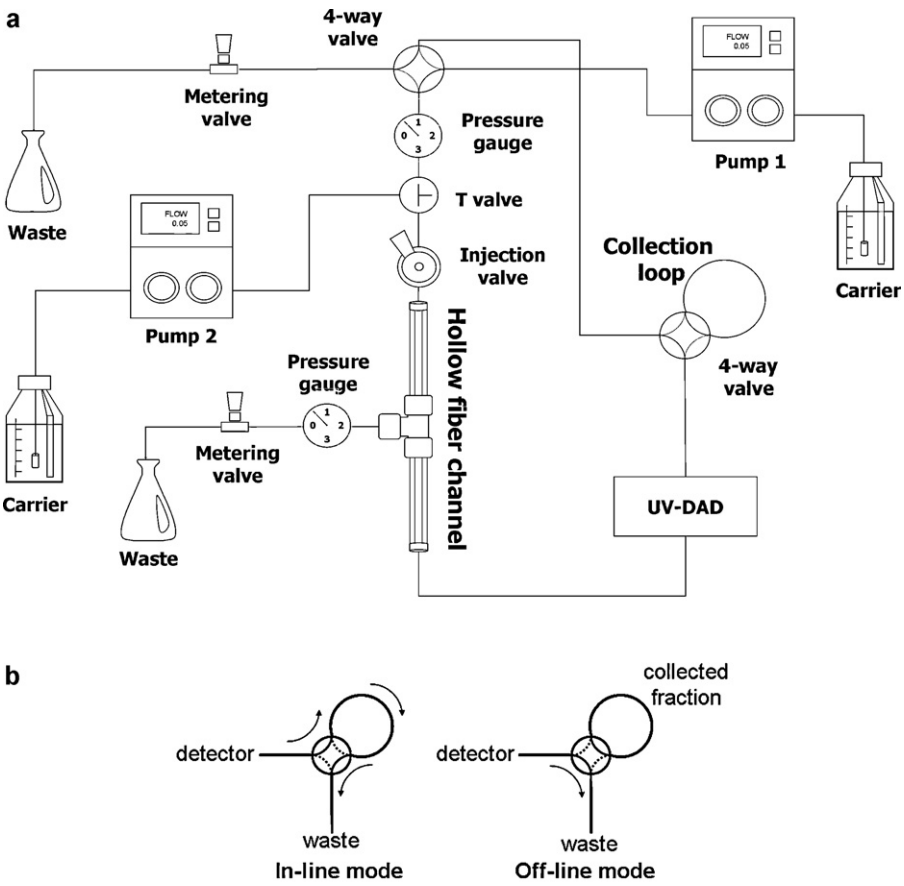

Fig. 1. (a) Schematic of the prototype system for HF5/HF5. Details of the system operation in HF5 mode were provided in Ref. [27]; (b) close-up of the collection device, and collection valve operations.

HF5 is particularly suited for either on-line or off-line coupling with other separation methods [25,26]. Low HF5 channel volume and low flow rate operations allow reducing the dilution factor of the eluted fractions. As with AF4, in HF5 a relaxation/focusing procedure is performed before sample elution, which allows reducing the sample dilution effect due to multiple separations. Reinjection in HF5 can take advantage of these features because sample components are eluted from HF5 in a relatively low volume and, therefore, at higher concentration than from flat-channel AF4. Consequently, a sample trap device of relatively low volume, which can be automated, can be placed at the HF5 longitudinal outlet for collection of the sample fraction that shall be reinjected. Moreover, with respect to AF4 a relatively low volume of the reinjected fraction potentially allows reducing refocusing time, thus reducing the risk of sample loss due to interactions with the channel wall.

In this work we present a novel method for in-line, reinjection-mode HF5, which we call "tandem HF5" (HF5/HF5). The term tandem not only expresses that the method is based on the reinjection of a selected fraction of eluted sample but also that the sample trapped at the end of the first fractionation is reinjected in a continuous, in-line mode.

For HF5/HF5 operations, the instrumental setup was modified with respect to what we described in the literature [27] by including a four-port, two-way valve positioned downstream the UV detector, and connected to a loop of proper volume. In this HF5/HF5 system, the 4-way valve with the loop constitutes the "sample trap" device. The eluted fraction to be reinjected is trapped in the downstream sample loop and, then, in-line fed back to the HF channel inlet by a reversed, focusing flow stream. This makes the trapped sample be reinjected and, subsequently, refocused at the same longitudinal position at which it was focused for the 1st run. The refocusing step reduces the sample plug volume to be reinjected, and it brings the analytes present in the trapped sample fraction back to their original concentration. The 2nd run is performed under same or different flow rate conditions with respect to the 1st run.

HF5/HF5 is here applied for the fractionation of stable oligomers of some model protein samples such as bovine serum albumin (BSA) and ferritin from horse spleen. The sample components eluted in correspondence of a selected, eluted band are trapped and refractionated. The HF5/HF5 performance is evaluated for the 1st and 2nd run in terms of retention reproducibility and efficiency (i.e. number of theoretical plates). Absolute sample recovery in the 1st and 2nd run, and the relative sample recovery between the two runs also are evaluated.

The HF5/HF5 mode shows particularly promising in the case of fractionation of complex protein samples such as blood serum. It is known that in whole serum a very high percentage of the total protein content is constituted of few proteins, while low abundant proteins are perhaps millions though they represent a small percentage of the total content. The possibility to analyze low abundance proteins is therefore dependent on the availability of proper methods to deplete high abundance proteins and increase the relative amount of low abundance components. HF5 has been applied to fractionate whole blood serum [28]. In particular, it has been used to separate serum lipoproteins (LPs) either on analytical [20,29] or semi-preparative scale [23]. HF5/HF5 is here shown able to improve fractionation of a selected LP fraction containing high-density LP (HDL) components.

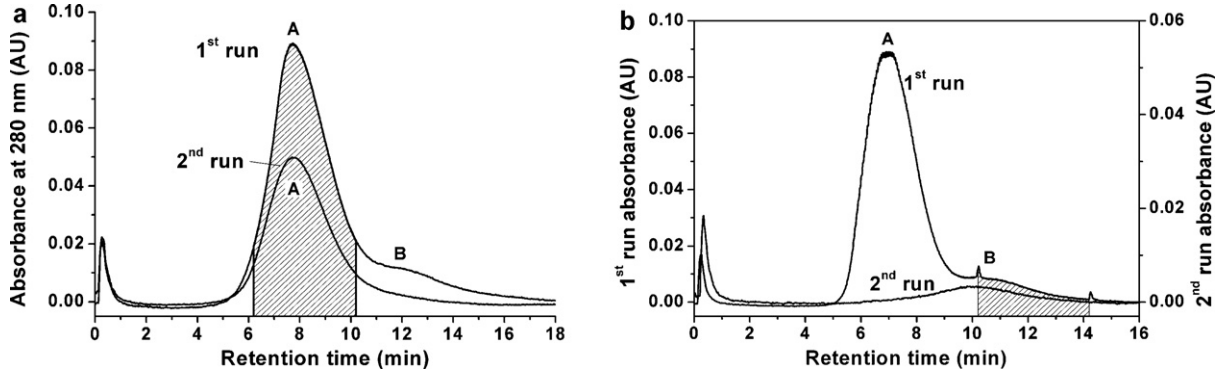

Fig. 2. HF5/HF5 of BSA. Experimental conditions: sample concentration: 0.7 mg/mL; injected volume: 20 μL; focusing time (1st run): 5 min; refocusing time (2nd run): 16 min; elution flow rates (both 1st and 2nd run): inlet flow rate (V_{in}) = 0.7 mL/min; radial flow rate (V_r) = 0.45 mL/min; collection loop volume: 1 mL. Shaded area: collected fraction. Collection and refractionation of the (a) monomer peak and (b) dimer peak.

2. Experimental

2.1. Samples

BSA and ferritin from horse spleen were obtained from Sigma–Aldrich, St. Louis, MO, USA. Samples were diluted in 5 mM ammonium acetate (NH_4Ac, Sigma–Aldrich) at concentrations ranging from 0.07 to 0.1 mg/mL. Serum samples were obtained from healthy donors who gave informed consent, and stored at −20 °C for few days before the analysis. Lipoproteins contained in serum samples were stained by mixing 200 μL of serum with 7 μL of 1% (w/v) Sudan Black B (SBB) in DMSO. Serum samples were diluted 20:80% in 5 mM NH_4Ac before the injection.

2.2. HF5/HF5 mode

2.2.1. HF5 system

The instrumental system for the HF5/HF5 mode (Fig. 1a) was derived from a HF5 prototype system whose scheme, set-up, and run operations were described in our previous papers [27–32]. The HF5 channel was home-made with a piece of polysulfone HF membrane produced for protein filtration in hemodialysis applications (SK Chemical, Seoul, Korea). The HF membrane had a nominal cut-off of 30,000 M_r, and nominal inner radius of 0.040 cm (referred to dried conditions). A HF piece of 24.0 cm in length was sheathed by two pieces of 1/8 in. O.D., 2 mm I.D. Teflon tube, and docked at the inlet and outlet extremities of the module by compression of the Teflon tube [30]. A tee connection was positioned between the two Teflon tubes to make the radial flow outlet. Hand-tight male fittings were positioned at the channel inlet and outlet. The actual channel thickness was calculated from the retention time of a protein (BSA) of known diffusion coefficient, based on the HF5 retention theory [14].

Two pumps were used to generate the flows and to inject/focus the samples: a Model LC-2000Plus HPLC pump (Jasco, Tokyo, Japan), and a Model 11 syringe pump (Harvard Bioscience, Holliston, MA, USA). Sample injection was made via a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) equipped with a 20 μL external PEEK loop. The focusing point position was determined as described in the literature [14]. The radial flow rate (V_{rad}) was set at 0.4 mL/min, and the longitudinal, outlet flow rate (V_{out}) at 0.3 mL/min.

A 4-way diagonal valve (Upchurch Scientific) equipped with a 1 mL PEEK loop was placed downstream the UV/vis detector to trap a selected fraction of the eluate, as described in Fig. 1b.

UV/vis detection was made by a photodiode array UV6000LP spectrophotometer (Thermo Finnigan, Austin, TX, USA), equipped with a 5-cm pathlength, light pipe cell. The use of this detector showed able to increase sensitivity and decrease detection limits of HF5 methods [17]. The detector operated at a wavelength (λ) of 280 nm for protein detection or at both λ = 280 and λ = 600 nm for the selective detection of protein and lipid components in serum.

The mobile phase was a 5 mM solution of NH_4Ac in Milli-Q water (Millipore, Bedford, MA, USA) at pH 7.0. This solution is commonly employed as a non-degrading solvent for intact proteins and protein complexes.

2.2.2. HF5/HF5 operations

A HF5/HF5 run is made of the following steps:

a. The system is set to injection/focusing/relaxation with the trapping loop in line. The sample is injected through the injection valve.
b. After the relaxation time the flow pattern is switched to elution, and the analytes are eluted and detected.
c. When the selected fraction of the eluate flows through the trapping loop, the 4-way valve is switched to off-line position to isolate the trapped eluate while the mobile phase flow is not stopped.
d. The 1st run is completed when all the analytes are eluted.
e. For the 2nd run, the system is newly set to injection/focusing/relaxation, and the 4-way valve is switched to in-line connect the trapping loop to the channel inlet. By this way the fraction trapped in the loop is flushed back to the channel inlet, and subjected to a second relaxation/focusing step (refocusing) for a proper time.
f. The system is set to elution mode to perform the second fractionation.

3. Results and discussion

3.1. Fractionation of protein oligomers

Representative fractograms obtained from HF5/HF5 of BSA samples are reported in Fig. 2. In Fig. 2a the 1st-run fractogram of BSA shows one main retained peak (A) that can be assigned to the protein monomer, and a second band at higher retention time (B), which is partially resolved from the monomer peak, and which can be ascribed to the BSA dimer. The sample fraction eluted in correspondence to the first peak (shaded area) was collected and fed back to the HF5 channel for the 2nd run. The fractogram obtained from the 2nd run (Fig. 2a) shows only one retained peak (A) at the same retention time of the monomer peak in the 1st run. In the 1st and 2nd run the half-height peak widths of the monomer band are comparable. These findings prove effectiveness of the HF5/HF5 procedure: the coincidence of retention time and peak width indicates that the relaxation/focusing conditions achieved during the

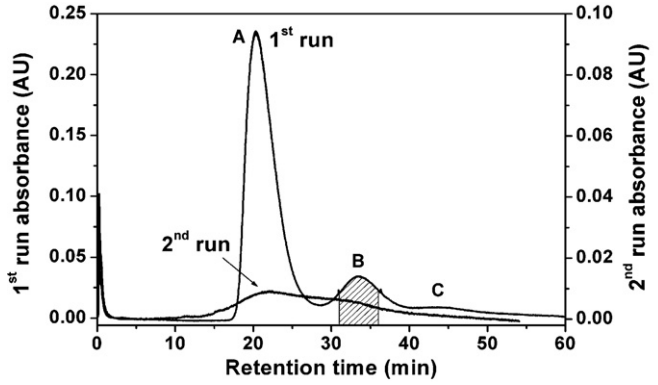

Fig. 3. HF5/HF5 of ferritin (horse spleen). Experimental conditions: sample concentration: 1.0 mg/mL; injected volume: 20 μL; focusing time (1st run): 5 min; refocusing time (2nd run): 16 min; elution flow rates (both 1st and 2nd run): V_{in} = 0.7 mL/min; V_r = 0.5 mL/min; collection loop volume: 1 mL. Collection and refractionation of the dimer peak.

1st run were effectively reestablished during refocusing. Moreover, the absence of a dimer peak (B) in the 2nd-run fractogram indicates that only the separated BSA monomer had been actually trapped after the 1st run, and that during the 2nd run the monomer did not undergo aggregation. Fig. 2b shows a different HF5/HF5 run of BSA in which the band ascribed to the protein dimer (shaded area) is collected and re-run. The spikes that are visible in the 1st-run fractogram in correspondence to both ends of the collected fraction are the consequence of pressure pulses due to manual switching of the valve in the trapping device. As in the case of Fig. 2a, the 2nd-run fractogram shows only one retained peak (B), the retention time of which is close to the 1st-run retention time of the band that was collected. No peak is visible at the retention time corresponding to the 1st-run retention time of the monomer peak (A). These findings prove that the protein dimer that was trapped after the 1st run maintained its oligomeric form, and that during the 2nd run it did not significantly dissociate into the monomeric form.

The above results suggest that HF5/HF5 may possibly be able to provide information on the equilibria between protein oligomeric forms under the concentration conditions typical of the HF5 process. This is particularly appealing if HF5 is to be applied for the characterization of self-assembling protein products. Fig. 3 shows HF5/HF5 fractograms of a ferritin (horse spleen) sample. The 1st-run fractogram shows three retained peaks with decreasing intensities as a function of increasing retention time, which can be respectively ascribed to the protein monomer (A), dimer (B), and trimer (C). The collected fraction (Fig. 3, shaded area) corresponds to the dimer peak. The 2nd-run fractogram shows not only a band at a retention time corresponding to the 1st-run retention time of the dimer (B), but also a band at the expected retention for the protein monomer (A), the intensity of which is higher than the intensity of the dimer band. Blank runs could have been due to sample components that had not been completely eluted from the channel in the 1st run (data not shown). In comparison to the behavior observed in the case of HF5/HF5 of BSA, the ferritin (horse spleen) dimer thus appears to undergo a significant dissociation process after the 1st run, which partially reestablished the original equilibrium between the monomeric and dimeric forms.

3.2. Evaluation of fractionation performance

The efficiency of the relaxation/focusing process during the refocusing step may critically affect the HF5/HF5 performance. For instance, incomplete relaxation of the sample fraction that is fed back to the channel may lead to a loss in sample recovery after the 2nd run. Moreover, poor focusing of the sample plug before the 2nd-run elution may reduce fractionation efficiency, and possible shift of the focusing position with respect to the 1st run may result in a shift in retention times. The effects of refocusing on the HF5/HF5 performance were evaluated by varying refocusing time of the BSA monomer fraction. Table 1 reports the values for retention time, number of theoretical plates (N) for the monomer peak, and absolute % sample recovery for the 1st and 2nd run as a function of refocusing time. Retention time shift between the runs ($\Delta t_r = t_r$(2nd run) − t_r(1st run)), and the relative % sample recovery (2nd-run to 1st-run recovery) are also reported. Absolute recovery values for the 1st and 2nd run were determined by comparing the area of the eluted peaks with the area of the peak obtained by directly injecting the sample into the detector cell. The relative % recovery values were calculated as the ratio between the area of the 2nd-run peak and the area of the band collected in the 1st run (see shaded area in Fig. 1a). The N values were calculated from peak width values measured at half peak height to prevent possible inaccuracy due to the presence in the fractogram of a partially unresolved dimer peak. The results show that, with increasing refocusing time from 4 to 16 min, (a) the time shift values, which are negative for low refocusing times, approach zero; (b) the relative sample recovery increases from 40% to about 80%; (c) the N values for the 2nd run increase from 13 to 45, that is the efficiency increases from 20% to 90% of the average, 1st-run efficiency ($N = 50 \pm 2$, $n = 18$). The obtained N values are somewhat lower than the values expected for flat-channel F4. However, they are comparable to the N values previously reported in HF5 literature [24], and we expect they can significantly increase in future evolutions of the HF5 systems [33].

All the above findings indicate that the application of a long refocusing time (in this case, a minimum value of ca. 16 min) is necessary to obtain (1) refocusing at the same channel position as in the 1st run, which is a critical point to have reproducible retention times between the runs; (2) complete relaxation of the refocused sample, which is critical to (a) minimize the loss in efficiency between the 1st and 2nd run due to the elution of sample components under non-equilibrium conditions, and to (b) achieve comparable sample recovery levels in the 1st and 2nd run.

Table 1
HF5/HF5 of the BSA monomer. Retention time, number of theoretical plates (N), and % recovery the BSA monomer in the 1st and 2nd run are reported as a function of refocusing time. Experimental conditions: sample concentration: 0.7 mg/mL; injected volume: 20 μL; focusing time (1st run): 5 min; elution flow rates (for both 1st and 2nd run): V_{in} = 0.7 mL/min, V_c = 0.45 mL/min. Uncertainty is reported as standard deviation (n = 3).

Refocusing time (min)	1st run			2nd run			Δt_r ((2) − (1)) (min)	Relative %recovery ((3)/(4))
	t_r (min) (1)	N	% recovery (3)	t_r (min) (2)	N	% recovery (4)		
6.0	7.8 ± 0.2	48 ± 3	85 ± 1	5.8 ± 0.2	13 ± 2	29 ± 2	−2.0	34
7.0	8.0 ± 0.2	46 ± 2	88 ± 3	6.5 ± 0.3	15 ± 3	58 ± 3	−1.5	65
8.0	7.7 ± 0.1	47 ± 3	84 ± 2	7.0 ± 0.2	16 ± 3	65 ± 3	−0.7	77
10.0	7.6 ± 0.3	53 ± 4	87 ± 1	7.2 ± 0.1	32 ± 1	62 ± 2	−0.4	71
12.0	7.6 ± 0.2	51 ± 1	85 ± 3	7.1 ± 0.2	31 ± 3	66 ± 1	−0.5	78
14.0	7.9 ± 0.2	49 ± 2	90 ± 2	7.8 ± 0.1	39 ± 1	70 ± 3	−0.1	78
16.0	7.6 ± 0.1	50 ± 2	86 ± 1	7.6 ± 0.1	45 ± 2	67 ± 3	0.0	78

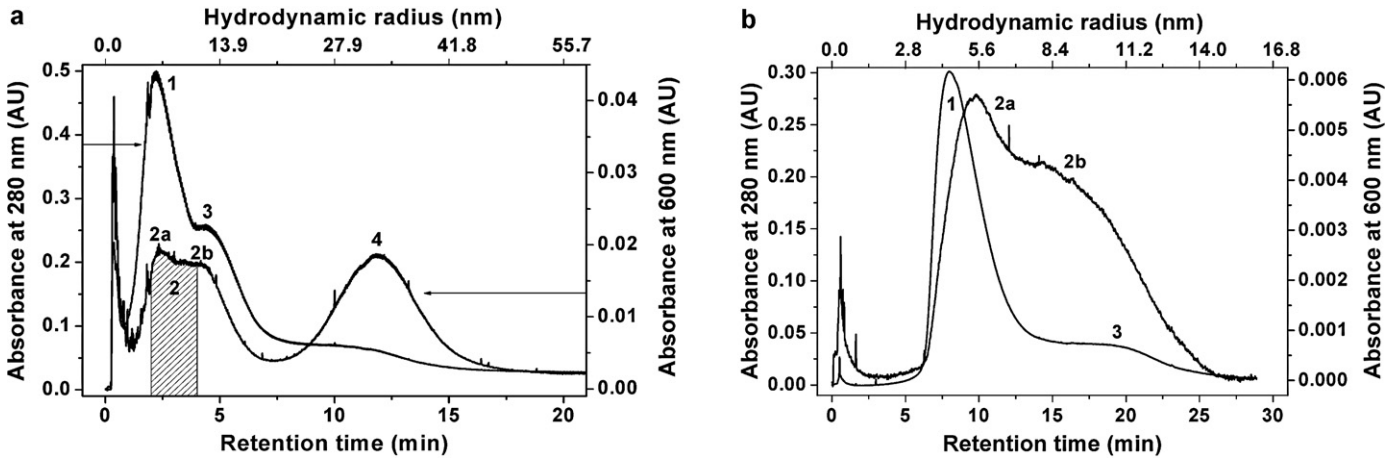

Fig. 4. HF5/HF5 of SBB-stained human serum. Serum samples (200 μL) were stained with 1% (w/v) SBB in DMSO (7 μL), and then 20:80% diluted in 5 mM NH_4Ac before injection. Injected volume: 20 μL; UV/vis detection at λ = 280 nm and λ = 600 nm. (a) 1st run. Focusing time: 5 min; elution flow rates: V_{in} = 0.7 mL/min, V_r = 0.2 mL/min; collection loop volume: 1 mL. Shaded area: collected fraction. (b) 2nd run. Refocusing time: 16 min; elution flow rates: V_{in} = 0.7 mL/min, V_r = 0.5 mL/min.

3.3. Serum lipoprotein fractionation

Fig. 4 shows representative HF5/HF5 fractograms of a SBB-stained, blood serum sample. SBB selective staining of serum lipid components allows for selective spectrophotometric detection of LP populations at λ = 600 nm [20]. Fig. 4a shows the representative, 1st-run profiles of the serum sample recorded at λ = 280 nm and λ = 600 nm. Baseline separation of the HDL (band 2) and LDL (band 4) components confirms the results reported in the literature [29]. Band 2 however appears to be a convolution of at least two bands (2a and 2b). This would agree with the expected presence in the HDL fraction of differently sized subpopulations [34]. However, under the applied separation conditions, the HDL component co-elutes with the high-abundance proteins (HAPs): comparison of the λ = 600 nm trace with the λ = 280 nm trace shows that two intense bands (band 1 and 3) due to HAPs are eluted at the retention times of bands 2a and 2b, respectively. High concentration of the co-eluted proteins would hinder further characterization of the HDL fraction. Moreover, the hypothesis that the HDL band profile could be due to the unspecific staining of co-eluted HAPs cannot be excluded, because it is known that human serum albumin can form complexes with lipid components. The HDL band (shaded area in Fig. 4a) was thus collected and reinjected in HF5/HF5 mode. Fig. 4b shows the 2nd-run profiles obtained under higher radial flow rate conditions with respect to the 1st run, and recorded at λ = 280 nm and λ = 600 nm. Due to the increase in the radial flow rate, with respect to the 1st run the collected fraction components are eluted at higher retention times. The trace at λ = 280 nm shows two bands (1 and 3) that can be associated to the bands 1 and 3 in Fig. 4a by a comparison of the hydrodynamic radius values calculated from the HF5 retention theory (upper *x*-axes in Fig. 4a and b) [11]. The profile at λ = 600 nm in Fig. 4b indicates the presence of two subpopulations (bands 2a and 2b) as in the case of band 2 in Fig. 4a. By comparing the profiles at λ = 600 nm and λ = 280 nm the retention time values of bands 1 and 3 are clearly different from the retention time values of bands 2a and 2b. Bands 2a and 2b cannot thus be ascribed to an artifact originated from co-elution with HAP components, and they could be then due to separation of two different HDL subpopulations. It is worth noting that such a resolution between HDL and HAP components would have been hardly achieved if the whole serum sample had undergone single-run HF5 under the conditions applied for the 2nd HF5/HF5 run. This is because whole serum injection would have likely generated overloading effects due to co-elution of the HAP components, which were in fact depleted after the 1st run.

4. Conclusions and perspectives

A simple instrumental modification of a HF5 prototype system was developed to allow for tandem HF5 operation. In the case of protein samples containing different oligomers, HF5/HF5 allowed separating, trapping and reinjecting each fractionated oligomer. Comparison between 1st and 2nd-run elution profiles provided information on the stability of protein aggregates, and possible occurrence of reversible association. This information could be particularly valuable for the characterization of protein drugs (e.g. antibodies) that are known to undergo concentration-dependent association phenomena, and for which the evaluation of the aggregation state is a fundamental requirement in quality control. Work is on progress on this subject.

HF5/HF5 operations should be particularly interesting when applied to complex protein samples, since preliminary results here presented show that HF5/HF5 can improve resolution and detection of low abundance components present in the fraction trapped after the 1st run and then fed back to the channel for the 2nd run. Moreover, by reinjecting a selected sample fraction the overloading effects due to high-abundance components that during the 1st run possibly co-eluted with the selected components can be reduced in the 2nd-run fractionation of the selected, trapped components. Increase of the analyte concentration at the channel outlet might also be obtained in the 2nd run by eluting the collected fraction at a lower retention level. This can improve detection if further characterization by off-line methods such as mass spectrometry wants to be performed on selected fractions of biological fluids of particular interest. However, it must be noted that, from first principles of separation science, concentration cannot be obtained without potential losses in separation.

The HF5/HF5 method here presented matched the performance (in terms of separation efficiency and recovery) of previous, single-run HF5 methods. In very recent work we have introduced instrumental and operational improvements that make HF5 able to reach the separation performance of commercial AF4 [33]. In the near future we then expect to enhance performance of the HF5/HF5 method, and make it ready to be more extensively applied to complex samples for which highly efficient separations of a selected sample component want to be obtained.

Competing interests

The mission of byFlow S.r.l. includes know-how transfer, development, and application of novel flow-assisted technologies and

methodologies for the analysis and characterization of samples of nano-biotechnological interest.

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