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Journal of Chromatography A, 1183 (2008) 135–142
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Hollow-fiber flow field-flow fractionation of whole blood serum

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Received 5 October 2007; received in revised form 8 January 2008; accepted 10 January 2008
Available online 16 January 2008

Abstract

Hollow-fiber flow field-flow fractionation is here applied to untreated, whole human blood serum. Matrix-assisted, laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS) of serum fractions shows mass signals in the <30 000 M_r range where low-abundance, serum protein components are known to be present, though a membrane of nominal 30 000 Da cutoff was employed for the fractionation device. Using diluted sera spiked with low amounts (0.06–0.1%, w/w) of an artificial mixture constituted the human adrenocorticotropic hormone fragments 18–39 (M_r = 2465.7) and 7–38 (M_r = 3659.2), and of bovine insulin (M_r = 5734), horse cytochrome *c* (M_r = 12 384) and chicken lysozyme (M_r = 14 388), a hybrid fractionation/microfiltration mechanism shows to govern the separation of the low-M_r components.

Keywords: Field-flow fractionation (FFF); Hollow-fiber flow field-flow fractionation (HF FlFFF); MALDI-TOF-MS; Blood serum profiling; High-abundance proteins (HAP); Low-abundance proteins (LAP)

1. Introduction

Flow field-flow fractionation (FlFFF) is well suited to separate intact proteins and protein complexes [1]. The FlFFF separation mechanism is structured by the action of a hydrodynamic field across an empty, capillary channel [2]. This makes retention, in principle, proportional to the protein diffusion coefficient [3,4]. Since the FlFFF channel is empty, there is very little (if any) mechanical or shear stress on the protein molecule. This allows for the separation of high-M_r proteins, protein complexes or aggregates without sample entanglement/adsorption on the stationary phase, which can degrade these analytes. In case of high-M_r proteins and protein complexes, moreover, FlFFF is particularly selective since mass-based selectivity in FlFFF increases with increasing M_r. Almost any solution can be used as mobile phase. This is the key point to avoid possible protein degradation due to the mobile phase. Because of all the above features, FlFFF shows very effective for non-degrading separation and further characterization of proteins and protein oligomers [5].

Hollow-fiber (HF) FlFFF is a micro-volume (<100 μL) version of FlFFF. Possible use of HF channels for FFF was first reported in 1979 [6], but fundamentals and first examples of HF FlFFF were described somewhat later [7–10]. A wide selection of currently available HFs, either of polymeric or ceramic composition, makes this technique enable to fractionate samples of very different origin, shape and size [11–16]. Methods for HF FlFFF of proteins were recently improved [17,18], also using microbore HF channels [19]. Still at a prototype stage, HF FlFFF has shown to be an effective technology which is able to give reproducible results, which may become a valid alternative to standard, flat-channel FlFFF [20].

Mass spectrometry (MS) with soft-impact ion sources and time-of-flight (TOF) analyzers is the most applied method today for the analysis of proteins. Separation methods such as LC and/or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) are coupled to reduce sample complexity

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doi:10.1016/j.chroma.2008.01.022

and desalt the sample. We have successfully coupled HF FlFFF with matrix-assisted, laser desorption/ionization (MALDI)-TOF-MS, and with electrospray ionization (ESI)-TOF-MS for desalting, separation and MS-based characterization of protein samples [21–23]. HF FlFFF in combination with MALDI-TOF-MS and a chemiluminescence enzyme assay has been used to characterize a recombinant protein drug [24]. Microbore HF FlFFF has been hyphenated with capillary isoelectric focusing (cIEF) as a non-gel-based 2D separation technique for intact urine protein separation before shot-gun proteomics by nanoflow LC–MS/MS [25]. HF FlFFF shows unique features for the separation of proteins before MS analysis. Low channel volume (in the order of 100 μL, or less in the case of microbore HF FlFFF [19]) reduces sample dilution. This is key point not to affect MS detection sensitivity. A mobile phase which is not able to affect ionization can be used, and sample desalting is also obtained because very low-M_r components such as salts are filtered through the pores of the HF [22]. Finally, possible disposable usage of the HF channels eliminates the risk of run-to-run sample carry-over. This is the crucial aspect for accurate and reproducible protein MS profiling of complex protein samples.

Human blood serum is a very complex protein sample since it contains the majority of proteins circulating in the body. Moreover, while high-abundance protein (HAP) components such as albumin (human serum albumin, HSA), immunoglobulins (IgA, IgG), transferrin, haptoglobin, and antitrypsin are known to be the major constituents of human serum (>85% in weight of total proteins), the low-abundance protein (LAP) components, which are present in less than 15% in weight of the total protein content, may be in the order of millions in number, and span 10 orders of magnitude in relative concentration. This makes whole blood serum a very challenging sample for protein profiling methods. Microbore HF FlFFF has shown able to fractionate HAP/LAP components of whole blood serum [19], though no MS-based characterization of the obtained fractions has been as yet performed.

In this work we show HF FlFFF is able to fractionate whole human serum. The fractionation effectiveness of HF FlFFF is first evaluated by SDS-PAGE on serum fractions collected at different elution times, corresponding to different ranges in diffusion coefficient and, thus, to different M_r values. The HAP/LAP ratio in each fraction is shown to be modified after HF FlFFF. Using a compatible mobile phase, the obtained serum fractions are then characterized by MALDI-TOF-MS. We have demonstrated that the use of HF FlFFF increases the ability of MALDI-TOF-MS to recover signals of proteins with M_r values below the nominal pore cut-off value of the HF membrane. This may be explained by the possible presence of lower-M_r protein components in the form of complex with higher-M_r protein components. Possible onset of a hybrid fractionation/filtration mechanism is proposed to explain the partial retention along the HF channel, and the partial filtration across the HF wall, of low-M_r protein components. Membrane-selective, flat-channel FlFFF was first described by other authors to increase the detection of lipoproteins and albumin aggregates [26,27]. Proteins of M_r higher than the pore cut-off value of the HF membrane (e.g. HSA) were shown to be possibly filtered through the pores, though analysis of the filtered fractions was not therein described. Results of HF FlFFF/microfiltration are here investigated by spiking, with peptides and small proteins, human sera at different dilution, and a diluted bovine serum albumin (BSA) sample. The actual presence of the added species in the retained fractions and in the filtered fluid is then evaluated by MALDI-TOF-MS.

2. Experimental

2.1. Samples

Serum samples were obtained from healthy donors who gave informed consent. Standard peptides were the adrenocorticotropic hormone (ACTH) fragments 18–39 (M_r = 2465.7) and 7–38 (M_r = 3659.2) from AnaSpec (San Jose, CA, USA). The standard proteins insulin from bovine pancreas (M_r = 5733.49), horse cytochrome *c* from equine heart (M_r = 12 384), lysozyme from chicken egg white (M_r = 14 300), and BSA (M_r = 66 430) were from Sigma–Aldrich, St. Louis, MO, USA.

2.2. HF FlFFF

The HF FlFFF system was a prototype equipment, whose scheme, set-up, and run operations were described in previous papers [21–24]. The HF FlFFF channels were homemade of a piece of polysulfone HF membrane (from SKU, Seoul, South Korea) sheathed by two pieces of 1/8 in. O.D. Teflon tube. A tee connection was positioned between the two tubes to make the radial flow outlet. Hand-tight male fittings were positioned at the channel inlet and outlet. The HF membrane had a nominal 30 000 M_r cut-off, with nominal inner radius of 0.040 cm (referred to dried conditions), and a length of 24 cm. The HF FlFFF system employed two pumps, a HPLC pump Model LC-2000Plus (Jasco, Tokyo, Japan), and a syringe pump Model Pump 11 (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 required sample injection/focusing/relaxation process was set for 5 min, with the focusing point determined as described in the literature [11]. The radial flowrate (V_{rad}) was set at 0.4 mL/min, and the longitudinal, outlet flowrate (V_{out}) at 0.3 mL/min. Spectrophotometric UV/vis detection was made by an UV6000LP spectrophotometer (Thermo Finnigan, Austin, TX, USA) operating at 280 nm.

The mobile phase was a 5-mM solution of ammonium acetate (NH_4Ac) (Sigma–Aldrich) in Milli-Q water (Millipore, Bedford, MA, USA) at pH 7.0. This solution is commonly employed as a non-degrading protein solvent for MS operations. We already proved that this mobile phase neither degrades proteins nor suppresses their ionization in ESI-TOF-MS or MALDI-TOF-MS after HF FlFFF [22–24]. Serum samples were diluted in the mobile phase in a dilution range of 1:5–1:10 (v/v).

2.3. MALDI-TOF-MS

MALDI-TOF-MS was performed using a Voyager DE Pro (Applied Biosystems, Foster City, CA, USA) equipped with a

pulsed-N_2 laser operating at 337 nm. Positive ion spectra were acquired in linear mode with *m/z* ranging from 2000 to 200 000 using a 20 000 V accelerating voltage, an 18 000–19 000 V grid voltage, and a delay extraction time of 250–550 ns. The spectrum for each spot was obtained by averaging the result of 150 laser shots, and five spots were considered for each analysis. External mass calibration was performed using the Calibration Mixture 2 (Applied Biosystems) in the 2000–10 000 *m/z* range, the Calibration Mixture 3 (Applied Biosystems) in the 5000–20 000 *m/z* range, or the single- and double-charged ions of BSA (Sigma–Aldrich) in the 20 000–70 000 *m/z* range. The analysis was performed by spotting on the target plate 1 μL of sample, mixed with an equal volume of the matrix solution. A 3% (w/v) solution of sinapinic acid in 50% acetonitrile (ACN)/50% H_2O/0.1% trifluoroacetic acid (TFA) was used as matrix.

Sinapinic acid, ACN, and TFA were purchased from Sigma–Aldrich. Water was obtained from a Milli-Q Plus system (Millipore).

2.4. SDS-PAGE

Serum fractions were vacuum-dried, and suspended in 20 μL of H_2O. The obtained samples were analyzed by 12% (w/v) PAGE in sodium dodecylsulfate (SDS) buffer (25 mM Tris pH 6.8, 5%, w/v, glycerol, 0.2%, w/v, SDS, 1%, w/v, β-mercaptoethanol). After electrophoresis, the gel slabs were stained using Coomassie Blue (Biorad, Hercules, CA, USA).

2.5. SPE

Solid-phase extraction (SPE) prior to MALDI-TOF-MS analysis was always performed on the fluid filtered from the radial outlet to prevent information loss due to possible excessive dilution. Isolute C18 cartridges (500 mg, International Sorbent Technology, Hengoed, UK) were employed. Before sample loading the SPE cartridges were conditioned with 5 mL of ACN and 5 mL of water. The sample was loaded on the conditioned cartridge, washed with 10 mL of water, and finally eluted with 5 mL of ACN. The eluate was dried under vacuum, and then solubilized with 20 μL of water.

3. Results and discussion

Fig. 1a reports an example of fractogram obtained for an untreated serum sample 1:5 (v/v) diluted in the mobile phase (NH_4Ac 5 mM). According to the HF FlFFF retention theory [8,11], and to a hard-sphere model for protein conformation, proteins of larger M_r are eluted later than proteins of smaller M_r. As a consequence, higher abundances of higher-M_r components should be increasingly found in the fractions collected at longer retention times. Fractions were collected at 1-min intervals, and eight of them were run on SDS-PAGE. Fig. 1b shows different protein patterns in the different fractions. It is known that HSA is the lowest-M_r (M_r = 66 453) and, by far, the most abundant HAP. The highest, relative amount of HSA was however found in fractions 3–5, although fraction 2 was collected in correspondence of the fractogram maximum in Fig. 1a. This

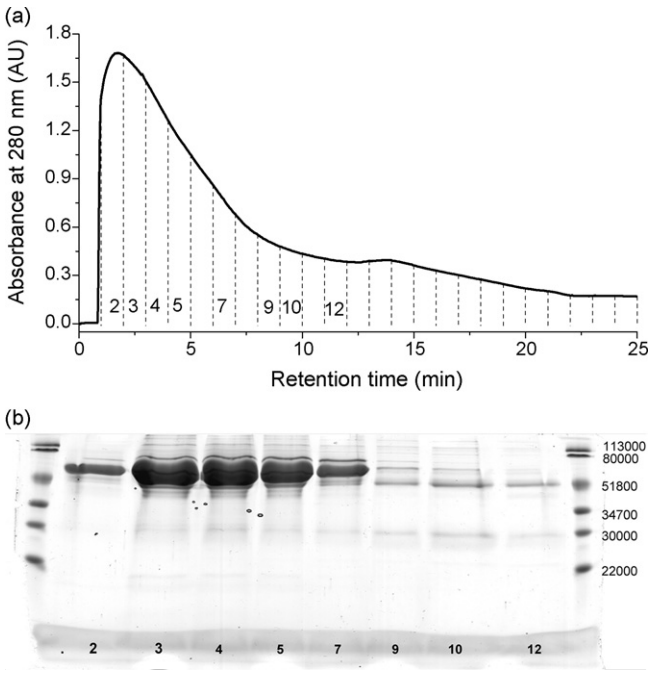

Fig. 1. Human blood whole serum, 1:5 (v/v) diluted in NH_4Ac 5 mM. (a) Fractogram and fractions collected for SDS-PAGE. (b) SDS-PAGE of the collected fractions.

is not surprising, since we have shown that commutation of flow patterns from focusing to elution mode generates pressure pulses and variations of the mobile phase flowrate that are responsible for an intense void peak observed at the beginning of the fractograms [14,21]. For this reason, even if in fraction 2 the UV signal is more intense than in fractions 3–5, the proteins present in fraction 2 are more diluted (less intense SDS-PAGE bands) than in the more retained fractions. The HSA content in the fractions collected after the retention time of the fractogram maximum (i.e. in fractions 7–12) then progressively decreases with respect to the content of less abundant components of relatively high M_r, as expected from retention theory.

3.1. HF FlFFF and MALDI-TOF-MS of whole serum

To further characterize the fractionation extent, MALDI-TOF-MS was performed on the fractions. In the early fractions, a significant number of *m/z* signals, probably corresponding to the lowest-M_r LAP components, was observed. This is particularly interesting because low-M_r LAPs are acknowledged to include peptides and proteins of diagnostic relevance (i.e. actual or potential biomarkers of diseases). The spectra obtained from the fractions 2 and 3 in Fig. 1a are reported in Fig. 2a–c, respectively. In fraction 2 (Fig. 2a), a significant amount of signals in the $m/z < 20\,000$ range are present. However, in the $m/z > 20\,000$ range, the signal due to HSA was also found (data not shown). This finding agrees with the presence of HSA in the SDS-PAGE lane of fraction 2, although the bands corresponding to the low-M_r components had not been therein found (Fig. 1b). In fact, the relatively poor resolution of one-dimensional SDS-PAGE, and the relatively high detection limit for such diluted bands

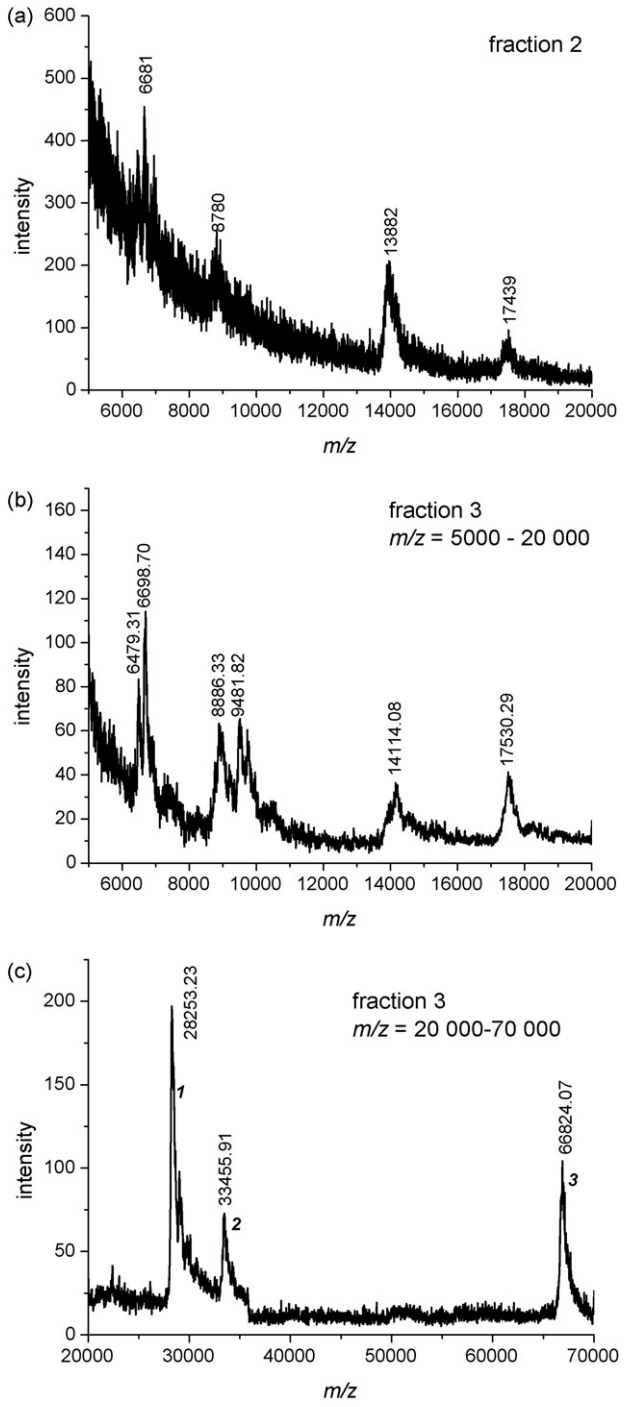

Fig. 2. MALDI-TOF-MS of the fractionated serum sample of Fig. 1. (a) Fraction 2; (b) fraction 3, *m/z* range: 5000–20 000; (c) fraction 3, *m/z* range: 20 000–70 000; signal 1: IgG light chain; 2: HSA($2H^+$); 3: HSA(H^+).

when using Coomassie Blue could have made the LAP bands invisible. Otherwise, some low-M_r LAPs associated to high-M_r HAPs could indeed dissociate when they are run by SDS-PAGE. In fraction 3, signals at higher *m/z* values are also found, among which the ones corresponding to the IgG light chain, and to the HSA($2H^+$) and HSA(H^+) species (Fig. 2c, signal 1, 2, and 3, respectively).

In the late fractions, the signals corresponding to high-M_r components like, for instance, the HSA dimer, and the IgG complex were found, as shown for fraction 15, 30 in Supplementary Fig. S1. This confirms that, because of the gentle mechanism, HF FlFFF does not alter the non-covalent chemistry of protein complexes. HF FlFFF then shows promising to fractionate also highest-M_r serum protein complexes of diagnostic relevance (e.g. lipid–proteins, immunocomplexes) for their further characterization. Although it lies beyond the aims of the present work, this is object of current studies.

High signal/noise levels were generally observed in the spectra of all the fractions. This can be ascribed not only to the effect of fractionation but also to sample desalting during fractionation. In previous work describing on-line HF FlFFF-ESI-TOF-MS we have shown that the signals associated with protein/Na^+ adducts were totally suppressed [22]. This is because, due to the radial flow field, the ionic species present in the protein sample are actually filtered through the HF membrane pores. As a consequence, in the fractions that undergo to MALDI-TOF-MS they are present at very low, if not negligible, amount. Although the HF FlFFF process reduces protein concentration because of sample dilution, it must be however noted that sample desalting during fractionation avoids the presence of signals due to the formation of protein adducts in the ion source [23]. This should eventually enhance recovery of LAP signals in the fractionated sample. Moreover, if fraction concentration, obtained by freeze-drying or by other procedures, were necessary to increase MALDI-TOF-MS detection of LAP components, sample desalting through HF FlFFF would become the key point to avoid salt concentration increase in the concentrated sample.

It may be surprising that a significant number of relatively low *m/z* signals (i.e. of $M_r < 30\,000$) was found in the early fractions: the nominal cut-off value of the employed HF membrane indeed was 30 000 Da. Protein components with M_r lower than the HF pore cut-off had been identified also in the spectra obtained by a shot-gun approach applied to urine proteome samples processed through HF FlFFF, though no explanation of this fact was given [25]. Formation of aggregates can make low-M_r components be in fact retained. For the same reason, possible complexation can also make lower-M_r components be carried by proteins of higher M_r and, then, be co-eluted with them. This has been reported in previous work on HF FlFFF and MALDI-TOF-MS of protein impurities present in uricase samples [24]. If complex formation were the reason for which species with $M_r < 30\,000$ were retained, their mass signals should have been found in the fractions collected at times corresponding to the retention times of higher-M_r species. The spectrum of fraction 2 (Fig. 2a) actually shows signals the *m/z* values of which are lower than what expected from the fraction collection time (in Fig. 1a). This is also observed in the spectra from fraction 3 (Fig. 2b and c), which was collected at the retention time corresponding to the M_r of HSA: *m/z* signals of even one order of magnitude lower than the *m/z* value of HSA are in fact present. Although at low retention the M_r-based selectivity of HF FlFFF is relatively low [8,11], co-elution with HSA of much lower-M_r components could be hardly explained if formation of complexes with HSA did not actually occur. This hypothesis is also supported by the presence

in the SDS-PAGE lanes of fractions 9 and 10 of distinct bands with $M_r < 30\,000$ (Fig. 1b). The retention times at which fractions 9 and 10 were collected indeed corresponded to $M_r \gg 30\,000$. This confirms not only that components of $M_r < 30\,000$ present in fractions 9 and 10 were actually retained rather than filtered through the pores, but also that they were retained more than expected from their M_r values. It must be noted that in fractions 9 and 10, HSA ($M_r = 66\,453$) was barely present, being mostly retained at shorter retention time as expected from the M_r value of the HSA monomer.

All the above findings support the fact that during HF FlFFF the low-M_r LAPs possibly associated with high-M_r HAPs are maintained in the associated form. The presence of SDS-PAGE lanes in the low-M_r region could be ascribed to dissociation during electrophoresis. On the other hand, it must be noted that the actual pore cut-off value of the HF membranes can be lower than the nominal value, particularly when sample tends to be adsorbed on the membrane. A decrease in sample recovery after multiple runs of standard protein samples was observed in previous work [17]. Actual adsorption of proteins on the inner surface of the HF membrane has been determined by surface, elemental analysis [28]. Adsorption of serum proteins, particularly HAP components such as HSA, on the HF inner wall should however make the low-M_r components that tend to associate to HAPs be co-adsorbed on the membrane wall. We have in fact observed that low-M_r components were either filtered through the pores as single species or retained likely as complexes.

3.2. HF FlFFF/microfiltration of low-M_r LAPs

The actual occurrence of HF FlFFF/microfiltration of complex protein samples deserved further investigation. MALDI-TOF-MS analysis of different fractions was performed to give indications of the possible association between LAP/HAP components. Since identification of native serum low-M_r LAPs present either in the fractions or in the filtered fluid was not the actual object of an approach based on MALDI-TOF-MS, serum samples were spiked with a known mixture of two peptides and of three low-M_r proteins in the 2000–14 000 M_r range. The mixture composition is reported in Table 1.

The fractogram in Fig. 3a was obtained. In Fig. 3b, the spectrum from the earliest fraction (fraction 1) reveals the presence of signals of the lowest-M_r added components (1: ACTH (18–39), $M_r = 2466$; 2: ACTH (7–38), $M_r = 3660$; 3: bovine

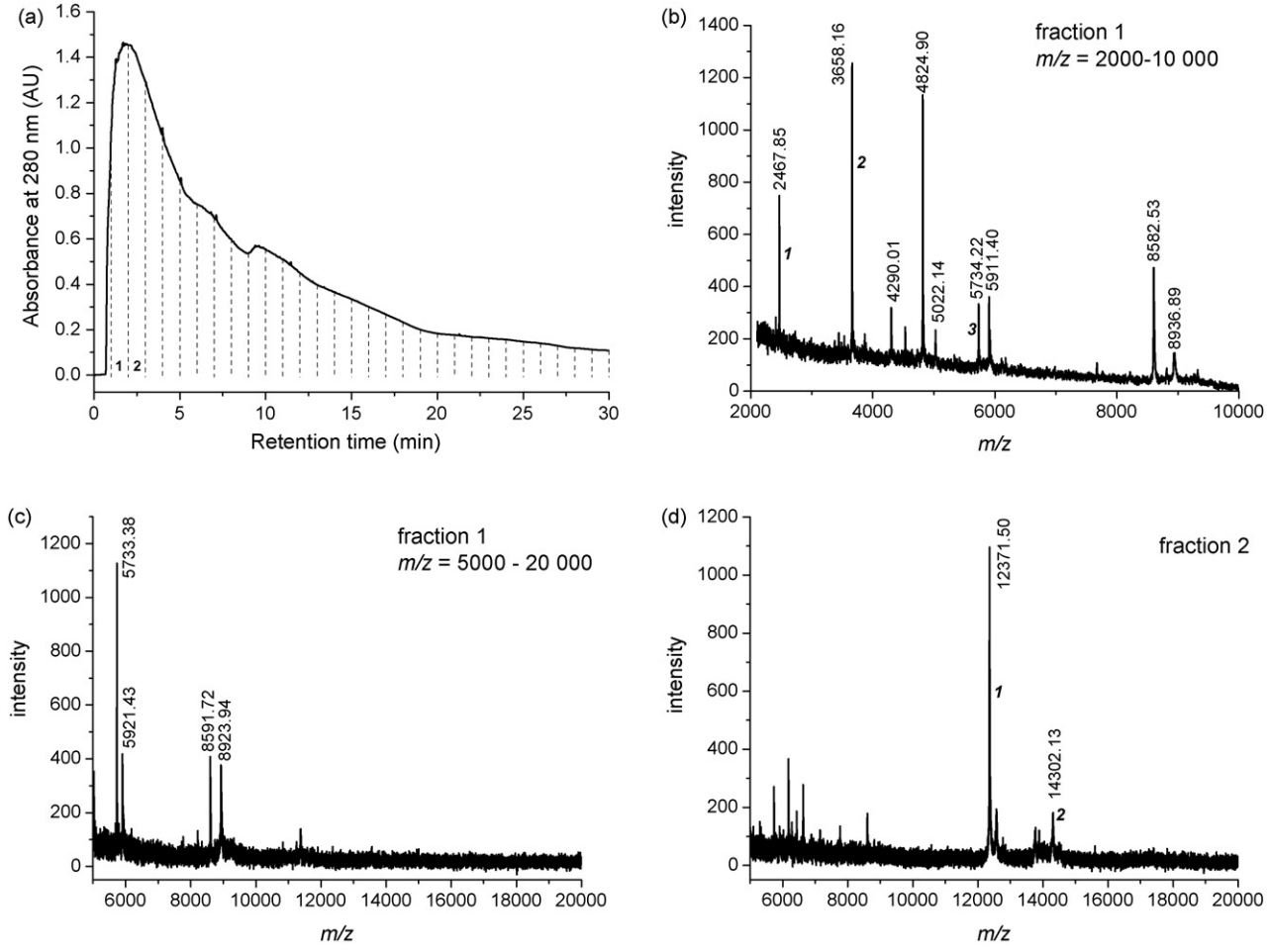

Fig. 3. Human blood whole serum, 1:5 diluted in NH_4Ac 5 mM, and spiked with the mixture in Table 1. (a) Fractogram and fractions collected for MALDI-TOF-MS; (b) fraction 1, *m/z* range: 2000–10 000; signal 1: ACTH (18–39), $M_r = 2466$; 2: ACTH (7–38), $M_r = 3660$; 3: bovine insulin, $M_r = 5734$; (c) fraction 1, *m/z* range: 5000–20 000; (d) fraction 2; signal 1: cytochrome *c*, $M_r = 12\,384$; 2: lysozyme, $M_r = 14\,388$.

Table 1
Mixture of low-M_r peptides and proteins added to human serum samples

	M_r	Concentration % (w/v)
ACTH (18–39)	2 466	0.06
ACTH (7–38)	3 660	0.2
Bovine insulin	5 734	0.35
Cytochrome *c*	12 384	0.1
Lysozyme	14 388	0.1

insulin, $M_r = 5734$), while the highest-M_r added components were not found (Fig. 3c). It must be noted that, when the serum sample spiked with this mixture underwent MALDI-TOF-MS analysis without previous fractionation, the signals of these lowest M_r peptides had not been found, probably because of competitive ionization by HAPs, and because of adduct formation. Only the signals of the relatively highest-M_r components (cytochrome *c*, $M_r = 12\,384$; lysozyme, $M_r = 14\,388$) had in fact been found (see Supplementary Fig. S2). In Fig. 3d, the spectrum from the later fraction (fraction 2) shows the presence of signals of the highest-M_r added components (1: cytochrome *c*; 2: lysozyme). No signals of the lowest-M_r added components found in fraction 1 were found in fraction 2. This would indicate that the added components were selectively and increasingly retained according to their increase in M_r. This finding agrees with retention theory. However, it must be also noted that the added components were eluted together with HSA, the M_r of which (66 453) indeed is far higher than the M_r values of the added components. This bears further evidence on the possible formation of complexes between the added components and HSA. Possible HF membrane wall polarization caused by HSA adsorption would reduce the actual pore size value. In fact, it would also induce co-adsorption of the added components, leading to the absence of relevant signals in the eluted fractions.

If main cause of co-elution with HSA were the formation of complexes with the added components, increasing dilution of serum samples before the addition of the added components would affect complexation equilibria. Otherwise, it must be also noted that increasing dilution of the injected serum sample reduces the sample band thickness during sample focusing, which should increase diffusion kinetics. Sample dilution would also reduce possible protein adhesion to the membrane. For these reasons, sample dilution would favor filtration rather than retention of the added components. Fig. 4a shows the fractogram obtained for the serum sample diluted 1:10 (v/v), and spiked with the same mixture reported in Table 1. That made concentration ratio between HSA and the added components twofold reduced. Fig. 4b shows the spectrum from the earlier fraction (fraction 1). The lowest-M_r components (1: ACTH (18–39), $M_r = 2466$; 2: ACTH (7–38), $M_r = 3660$; 3: bovine insulin, $M_r = 5734$; Fig. 4b) are present, which mean that they were still retained. However, Fig. 4c shows that, once trapped and extracted using SPE, such added components were found also in the filtered fluid, that is in the fluid collected from the cross-flow outlet. These findings indicate that these lowest-M_r components, when added to an 1:10 (v/v)-diluted serum sample, actually underwent a hybrid HF FlFFF/microfiltration mechanism, as they were partly frac-

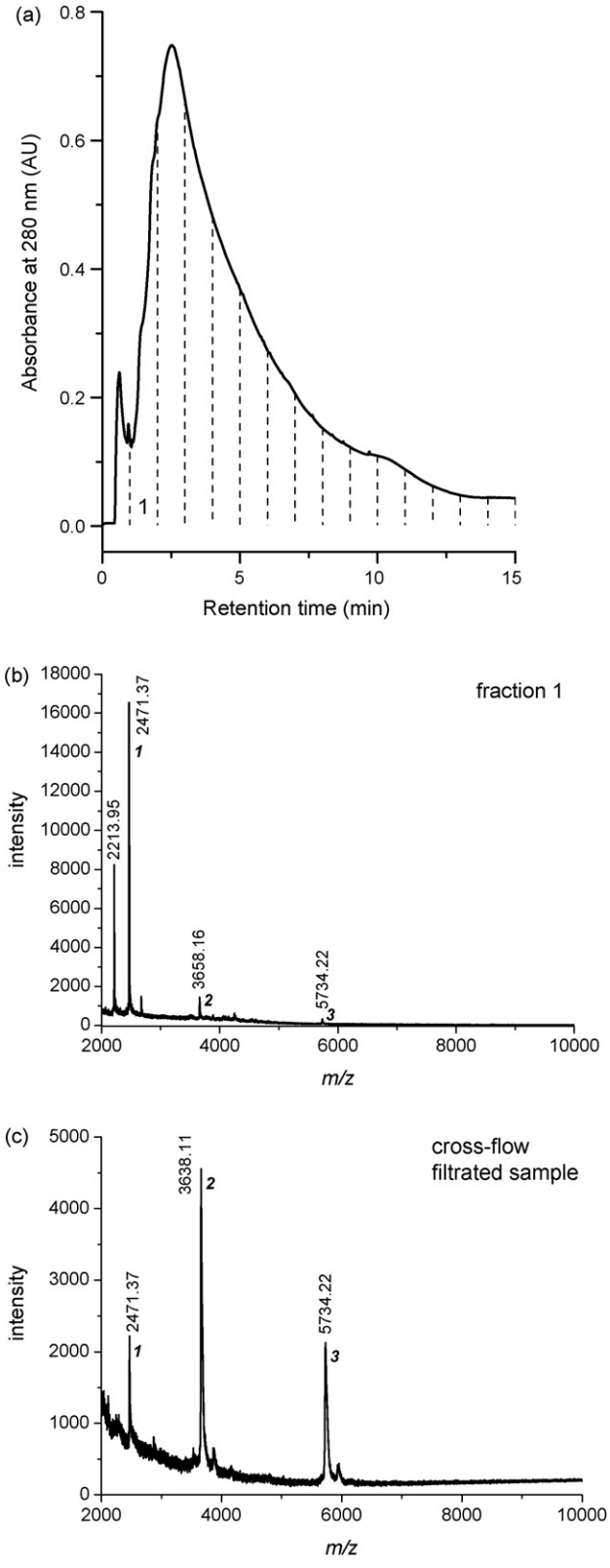

Fig. 4. Human blood whole serum, 1:10 diluted in NH_4Ac 5 mM, and spiked with the mixture in Table 1. (a) Fractogram and fraction collected for MALDI-TOF-MS; (b) fraction 1; signal 1: ACTH (18–39), $M_r = 2466$; 2: ACTH (7–38), $M_r = 3660$; 3: bovine insulin, $M_r = 5734$; (c) MALDI-TOF-MS of the cross-flow filtered sample undergone to SPE.

tionated along the HF channel and partly filtered through the HF pores. A scheme of such a split along two different flow streams is reported in Supplementary Fig. S3. Since this dual behavior was not in fact observed in the case of a more concentrated serum sample (see Fig. 3), reducing HAP (and, particularly, HSA) concentration via serum dilution shows to reduce the possibility to fractionate the lowest-M_r components in the form of complexes with HAPs. This was further investigated by the following study using a reference protein sample.

Albumin is the most abundant protein among HAPs. Because of its known ability to form complexes with LAPs, a case study was performed by spiking a diluted bovine serum albumin (BSA, 0.1%, w/v) sample with the same amounts of bovine insulin, and of the peptides of human origin ACTH (18–39) and ACTH (7–38) that were used to spike the human serum samples of Figs. 3 and 4. The occurance of BSA/bovine insulin complexation would have made bovine insulin signals to be found in the retained fractions as observed for the serum sample. In fact, the added components were all found only in the filtered sample, as shown in Supplementary Fig. S4. A low albumin concentration therefore seems to favor filtration of the low-M_r components, as dissociated species, rather than their retention as albumin-associated species. It must be noted that this experiment with BSA was performed with a HF FlFFF channel already employed for previous, multiple runs of whole serum samples. This implies that the low-M_r components added to the diluted BSA sample were filtered through the pores even though serum protein adsorption on the HF wall had previously occurred. In our opinion this bears further evidence on the fact that retention of low-M_r LAP components may not depend on possible membrane polarization due to protein adsorption, but actually to the possibility to form complexes with HAP, which depends on the HAP concentration. Further investigations on the fractionation/filtration equilibrium conditions are however necessary, which are beyond the aims of this first paper on HF FlFFF for MS-based serum analysis. For instance, chaotropic reagents to control dissociation of possible LAP/HAP complexes under weak degrading conditions may be used, as well as HF membranes with different pore cut-off values under different sample dilutions.

4. Conclusions

HF FlFFF shows effective to fractionate untreated, whole blood serum for its further MALDI-TOF-MS-based characterization. After HF FlFFF, a significant number of signals at relatively low *m/z* values is always present in the earliest (pre-HSA) fractions of the serum samples. These signals can be likely ascribed to low-M_r LAP components, which are known to be of relevant diagnostic interest. After HF FlFFF, signals due to some selected, low-M_r LAP components added to the serum sample, and which had M_r values lower than the nominal pore cut-off value of the HF membrane, were found both in the fractionated and in the sample filtered through the HF pores. This can be ascribed to a hybrid HF FlFFF/microfiltration mechanism. The balance between retention and filtration appeared, from these first experiments, to depend on possible HAP/LAP complexation, which depends on sample concentration.

Since HF FlFFF does not alter protein complexes, in perspectives we could distinguish by means of HF FlFFF/microfiltration the low-M_r LAPs that may (in the fractions) or may not (in the filtered sample) form complexes with HAPs. Hybrid HF FlFFF/microfiltration could be then applied to MS-based methods for proteomics (e.g. LC-Q-TOF-MS/MS) to distinguish and identify biomarkers that are or are not carried by HAPs. To further investigate these aspects, work is on progress in our laboratories. Preliminary interactomic maps obtained by Nanochip-ESI-Ion Trap-MS/MS on fractionated serum samples seem to support the presence of native, low-M_r LAP/HSA complexes in the most retained fractions.

HF FlFFF therefore shows interesting for MS-based methods for serum proteomics, since current methods used to remove HAPs could also remove the LAPs associated to the removed HAPs. Being a prototype technique, we must however note that HF FlFFF still requires some significant technical developments to possibly find a routine-scale application to proteomics. When a rugged channel design, and HF membranes specifically made for HF FlFFF usage become available, reproducibility studies can be performed for such applications. Channel cartridge engineering and system operation optimization shall be effective also to increase the relatively poor separation efficiency currently observed in HF FlFFF. We wish that such a technological gap could be filled up in a relatively short time to finally make HF FlFFF evolving from a promising, prototype technique to a widely applied technology.

Acknowledgments

Authors are grateful to L. Barbieri, University of Bologna, for his assistance in the experimental work.

The work was partially supported by the University of Bologna (funds for selected research topics), and by the Italian Ministry of the University and Research (MiUR, PRIN 2006, 2006033944). P.R. and M.-H.M. duly acknowledge the Significant Bilateral Project “Novel Methods for Top-down Analysis of Macromolecular and Nanosized Samples of Biotechnological and Environmental Interest” within the VIII Executive Programme of Scientific and Technological Co-operation between Italy and Korea for the years 2007–2009.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.chroma.2008.01.022.

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