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Methodological Aspects on Microdialysis Protein Sampling and Quantification in Biological Fluids: An In Vitro Study on Human Ventricular CSF

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There is growing interest in sampling of protein biomarkers from the interstitial compartment of the brain and other organs using high molecular cutoff membrane microdialysis (MD) catheters. However, recent data suggest that protein sampling across such MD membranes is a highly complex process that needs to be further studied. Here, we report three major improvements for microdialysis sampling of proteins in complex biological matrixes. The improvements in this in vitro study using human ventricular cerebrospinal fluid as the sample matrix include increased fluid recovery control, decreased protein adsorption on the microdialysis membrane and materials, and novel quantitative mass spectrometry analysis. Dextrans in different concentrations and sizes were added to the perfusion fluid. It was found that dextrans with molecular mass 250 and 500 kDa provided a fluid recovery close to 100%. An improved fluid recovery precision could be obtained by self-assembly triblock polymer surface modification of the MD catheters. The modified catheters also delivered a significantly increased extraction efficiency for some of the investigated proteins. The final improvement was to analyze the dialysates with isobaric tagged (iTRAQ) proteomics, followed by tandem mass spectrometric analysis. By using this technique, 48 proteins could be quantified and analyzed with respect to their extraction efficiencies. The novel aspects of microdialysis protein sampling, detection, and quantification in biological fluids presented in this study should be considered as a first step toward better understanding and handling of the challenges associated with microdialysis sampling of proteins. The next step is to optimize the developed methodology in vivo.

In recent years, the hunt for potential protein biomarkers for neurodegenerative diseases has intensified. The main focus has been to investigate the protein content in cerebrospinal fluid (CSF) due to its direct contact and neurochemical exchange with the brain. CSF, which is a colorless body fluid, contains a vast amount of proteins that range over at least 10 orders of magnitude in concentration.[1] This large concentration range requires proper and reliable sampling, sample treatment, and detection techniques.

Microdialysis (MD) is a well-established sampling technique that has been used routinely for more than 20 years.[2,3] MD is today a clinical sampling tool used for, for example, neurochemical brain monitoring in neurointensive care patients.[4−8] MD is a diffusion-based sampling technique in which a semipermeable, hollow fiber membrane is inserted into the sampling area of interest. Inside the tubular membrane, a continuous flow of perfusate transports the collected molecules into a fraction collector or online instrumentation for further analysis. The most important parameter in MD is the extraction efficiency, also known as the relative recovery.[3] The extraction efficiency is defined as the concentration of an analyte in the dialysate divided by the concentration of the analyte in the bulk sample. It is an important indicator that describes the overall efficiency of the sampling.[2] To enable correct biological interpretation of the MD data, it is of major importance that the extraction efficiency of the MD membrane remains stable during the entire sampling period.

The main application for MD has been to sample small hydrophilic molecules (e.g., glucose, lactate, pyruvate, glycerol, glutamate and urea) from different biological matrixes.[2,4] With the introduction of MD membranes with a molecular weight cutoff (MWCO) of 100 kDa or higher, the focus has been to develop MD methods for sampling larger biomolecules, such as cytokines[9−13] and other proteins.[14−19] The reported extraction ef-

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ficiencies for protein biomarkers range between 1% and 5% at perfusion flow rates of 0.5−1.0 μL/min, when using 100 kDa MWCO membranes and sampling larger proteins.[17] However, more importantly, the extraction efficiency for individual macromolecules has been shown to vary extensively, despite their similarities in molecular mass. The reproducibility, that is, the precision between different studies, as well as the repeatability, precision within the same study, have also been reported to be poor,[11,19,20] indicating that the methods used are not robust. Li et al.[21] listed five challenges that must be considered when sampling proteins with MD. First of all, proteins are often present in low concentrations in the sample of interest; for example, in CSF. Second, the diffusion velocity of proteins is low due to the size of the proteins. Third, there is a possibility of perfusion fluid losses when large-pore-sized membrane catheters are used. Fourth, proteins adsorb onto the membrane and tubing of the catheter, leading to catheter fouling. Finally, MD produces submicroliter sample volumes containing low concentrations of proteins, which is highly challenging to analyze properly.

To ease the transfer of a newly developed MD method from in vitro to in vivo, it is important to have maximum control over all five challenges. It is essential to mimic the conditions present in the cerebrospinal fluid in vivo by using a representative CSF sample and perform the sampling at 37 °C. By mimicking the sample properties, the concentration and diffusion properties of the proteins remain similar to in vivo conditions.

It is also of great importance to control the fluid balance. Ideally, the recovery of the perfusion fluid should be 100%; that is, the volume that is delivered into the MD catheter should also exit to secure a diffusion-driven dialysis sampling. When MD membranes have a high MWCO, their fluid characteristics are transferred from the traditional dialysis region into that of ultra filtration, where fluid flow has to be considered and diffusive transport may not always dominate the molecular flux. At lower pressure, the permeate flux will transport fluid to the perfusate and the recovery will be above 100%, and at higher pressure, the perfusate will be transported into the body and the recovery will be less than 100%. Hence, being far away from 100% may challenge the biological relevance of the sample. One way to manipulate fluidic flow and, hence, the permeate flux is to increase the osmotic pressure of the perfusate. This is often done by adding albumin as a colloid to the perfusate.[13−15] However, adding albumin effectively rules out consecutive proteomic applications using liquid chromatography (LC) in combination with mass spectrometric (MS)-based detection. The added albumin would completely dominate the sample loading in both the LC and the MS signal, especially when the proteins usually are enzymatically digested to peptides at an initial stage to comply with the LC−MS approach. Thus, the addition of any stabilizing proteins should be avoided if MS is used for detection. Dextrans, with a molecular mass of 60−70 kDa, are other additives that have been used successfully to increase the osmotic pressure.[9,18,19,22−24] Dextran is the collective name for neutral polysaccharides with large dimensions that are used extensively in many biological applications. Dextrans do not affect the MS signal, since they can easily be removed prior to separation and detection.

When foreign material, for example, a MD membrane, is implanted into biological material, a series of processes occurs. The immediate response is formation of a protein layer that spontaneously adsorbs onto the membrane.[25] This highly complex process affects the membrane by decreasing the pore size and altering the MWCO.[18,19,26] Protein adsorption is the initial step required for subsequent interaction with host cells, which often is referred to as biofouling.[25] Biofouling further leads to formation of a tissue scar, that is, encapsulation.[27] This is a severe problem in microdialysis, since it initiates a cascade of response actions that may complicate further result interpretation.[28] Protein−membrane interaction on microdialysis catheters has been studied previously,[29−31] and biofouling and encapsulation have been demonstrated by microscopic methods.[12,13,18,27] Protein adsorption occurs within seconds to minutes;[26] encapsulation evolves over days or weeks.[27]

Since the proteins adsorbed to the membrane act as initiators for further bio processes, it is of great interest to decrease the adsorption rate and delay this process. One way to reduce protein adsorption is to modify the membrane surface. Torto et al.[32] adsorption-coated a microdialysis membrane with high-molecular-weight poly(ethylene imine) with the aim of enhancing the interaction of the membrane with enzymes. Kazuhiko et al.[33] coated MD membranes using a polymer with phospholipid polar groups. The modified catheter was implanted subcutaneously for

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an extended time period without significant inflammatory response, which was regarded as a sign of decreased initial protein adsorption and delayed encapsulation.

An alternative and simple way to modify MD catheters is to cover the surfaces of the membranes and tubings with triblock copolymers. One widely used triblock polymer family is the polaxamers, better known as Pluronics Poloxamers, the listed name in the US and European Pharmacopoeia, are used widely in many areas, such as cosmetics, medical applications, tissue engineering, and food additives.[35,36] The molecule has a triblock structure consisting of one hydrophobic polypropylene oxide (PPO) group flanked by two hydrophilic polyethylene oxide (PEO) units. The structure is $PEO_n-PPO_m-PEO_n$, where n and m vary, depending on which one is chosen of the more than 50 different poloxamers available. The PPO chain, which is hydrophobic, is attached to the hydrophobic catheter material and the hydrophilic PEO chains self-assemble into a cilia-like surface on the hydrophobic polymer material that effectively repels proteins.[35] The degree of protein repellency is dependent on the length of the PEO chains and the surface coverage.[37−39]

MD, as a sampling technique, generates small sample volumes containing low concentrations of proteins, which requires subsequent separation and detection methods to be selective and sensitive. The combination of liquid separation techniques with mass spectrometry is a good choice due to universality and high sensitivity. This methodology is also easy to automate and enables simultaneous protein and peptide identification, characterization, and quantification.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become an important analytical tool in the field of proteomics[40] and can readily be combined with nanoflow LC separations for peptide and protein identification and quantification. In recent years, strategies for quantitative MS proteomics have become increasingly important because only identification of proteins in samples is often insufficient.

Quantitative MS techniques have been developed to specifically quantify peptides/proteins in complex biological samples[41] and to compare the relative abundance of proteins for multiple samples.[42] One of the most popular approaches for relative quantification is the isobaric tag for relative and absolute quantification (iTRAQ), which enables multiplexed quantitative analysis of up to eight samples simultaneously.[43] The major benefits of using a multiplexed quantitative strategy, such as iTRAQ, are the possibility to perform differential quantification of complex samples under identical conditions and the increased sample throughput in the analysis. Consequently, iTRAQ labeling can be successfully applied to compare the extraction efficiencies in different MD setups with high sensitivity and the possibility to simultaneously identify multiple proteins in the dialysate.

In this study, a novel MD method for sampling proteins has been developed. The study was performed in vitro using human ventricular CSF as the sample matrix. Different concentrations and molecule sizes of dextran added to the perfusion fluid were evaluated for their effect on perfusion fluid recovery and protein extraction efficiency. Perfusion fluid recovery and protein extraction efficiency were compared between untreated MD catheters and PEO−PPO−PEO surface-pretreated catheters. Finally, the extraction efficiencies of proteins in CSF obtained from the different MD setups were quantified using iTRAQ tagging followed by nanoflow LC/MALDI-TOF/TOF-MS detection.

EXPERIMENTAL SECTION

Chemicals and Reagents. Acetonitrile (ACN), acetic acid (HAc), and ammonium dihydrogen phosphate ($NH_4H_2PO_4$) were of proanalysis grade (>99.5% pure) and obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) (p.a.) and Pluronic F-127 were purchased from Sigma Aldrich (St. Louis, MO). Dextran 60 Ph.Eur (molecular mass 60 kDa, batch no. HF 1021), Dextran T250 (molecular mass 250 kDa, batch no. HE104), and Dextran T500 (molecular mass 500 kDa, batch no. HE518) were purchased and kindly provided by Pharmacosmos (Holbaek, Denmark). Perfusion fluid (CNS), composed of aqueous solution of 147 mM NaCl, 2.7 mM KCL, 1.2 mM $CaCl_2$, and 0.85 mM $MgCl_2$, was obtained from CMA Microdialysis (Solna, Sweden). Deionized water was produced by a Milli-Q^+ system, Millipore Corp. (Marlborough, MA).

Cerebrospinal Fluid Collection. CSF was collected by ventricular drainage from neurointensive care patients with invasive intracranial pressure monitoring owing to severe spontaneous subarachnoid hemorrhage. The total protein amount in the pooled ventricular CSF (vCSF) was measured to 303 ± 10 mg/L ($n = 9$) using the Bradford Coomassie Brilliant Blue G-250 protein assay (595 nm, bovine serum albumin as standard, Bio-Rad, Hercules, CA). The vCSF was centrifuged before being stored in a −70 °C freezer. In total, 400 mL vCSF was collected; pooled; and finally, divided in 20 aliquots, each containing 20 mL vCSF. The aliquots were stored at −70 °C until further use.

Surface Modification of Membranes and Vials. To modify the surface of the inner tubing and the membrane, MD catheters were soaked in and continuously perfused with 5% w/v Pluronic F-127 solution at a flow rate of 0.5 μL/min for 24 h at ambient temperature. After the surface modification, the catheters were washed with water for 12 h. The 300-μL collection vials (CMA Microdialysis) were treated in a similar way: first, the vials were tagged for identification purposes and then washed with Milli-Q water. Second, the vials were filled with 5% w/v Pluronic F-127 solution for 24 h before they were washed with Milli-Q water, dried, and weighed.

Microdialysis Setup. Four catheters, two surface-modified and two untreated, were used simultaneously to increase the accuracy when comparing different perfusion flow compositions and surface treatments. A new micro vial stand was constructed to facilitate the collection of the dialysate at the same height as

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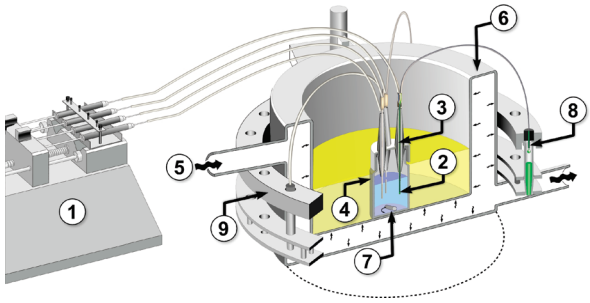

Figure 1. In vitro MD setup. The majority of the parts are cross-cut for illustration purposes. (1) Syringe pump, (2) MDs catheters, (3) in-house-constructed catheter holder, (4) CSF sample beaker 20 mL, (5) 37 °C heated water inlet, (6) double-jacketed glass beaker, (7) magnetic stirrer, (8) dialysate collection and (9) in-house-constructed microvial stand. The figure is not drawn to scale.

the membranes on the MD catheter, thus minimizing possible siphoning effects, which in turn could otherwise affect the overall flux across the membrane. The design of the stand made it possible to change vials without changing the position of the outlet of the MD catheter. It also facilitated encapsulation of the vials during sample collection, minimizing dialysate evaporation during sampling.

The MD setup is shown in Figure 1. A syringe pump, PHD 2000 Programmable, (Harvard Apparatus, Holliston, MA) was used to deliver a constant flow of 0.3 μL/min of perfusate to the four CMA 71 MD catheters (10 mm membrane length, 100 000 Da MWCO, outlet tube dimensions: 290 mm length and inner diameter 150 μm; CMA Microdialysis AB, Solna, Sweden). The catheters were placed in an in-house-constructed holder that was mounted on the lid of a 20 mL beaker. This allowed the membranes to be positioned exactly at 8 mm from each other and 17 mm from the bottom of the sample container. Two of the catheters were treated with Pluronic F-127 according to the described procedure, and the other two catheters were untreated. The holder with the four catheters was put in 19 mL of CSF sample. The sample was held at 37 °C throughout all experiments with the help of a Lauda B thermostat, which provided a heated flow of water through a double-jacketed glass beaker. The vCSF sample was stirred with a magnetic stirrer. The dialysates were collected in 300 μL collection vials, which were put in the in-house-constructed microvial stand mounted at the same height as the catheter membrane. Before the start of each 24-h experiment, the MD catheters were treated as recommended by the manufacturer (CMA Microdialysis).

Microdialysis Sampling Procedure. The rationale of the study was to develop the MD method for in vivo applications with the aim of sampling protein biomarkers. Therefore, we tried to simulate in vivo conditions as much as possible. To mimic the natural vCSF turnover and to minimize the effect of protein breakdown and bacterial growth, the vCSF sample was exchanged with a fresh CSF aliquot sample after 24 h. No protein standards were added to the CSF sample, and the experiments were performed at 37 °C. The vCSF sample was stirred to minimize concentration polarization effects.

A 20 mL aliquot of the vCSF sample was first thawed at room temperature and then transferred into a 20-mL disposable MD sampling beaker. The MD beaker was heated to 37 °C under continuous stirring. After 30 min, a 1 mL sample was taken from the vCSF sample for iTRAQ label 113 and put into the −70 °C freezer. The four MD catheters were transferred from a water container to the vCSF sample. The catheters were allowed to equilibrate in the vCSF sample with a flow rate of 0.3 μL/min for 20 min before starting the MD fraction collection. Each MD sample collection was performed over a 24 h period. After collection, the sample vials were weighed and stored at −70 °C. In total, four fractions, each corresponding to 6 h of sampling from each catheter, were collected each 24-h period. After 24 h of sample collection, a 1-mL sample was taken from the vCSF solution to be used for iTRAQ 118 labeling. The catheters were then rinsed with Milli-Q water for 24 h at a flow rate of 0.5 μL/min. This sampling procedure was repeated three times with varying perfusion fluid composition, as described in Table 1.

Table 1. Composition of the Perfusion Fluid in the Different Experiments and MD Catheters (Catheters 1−4)[a]

	catheter 1 modified	catheter 2 modified	catheter 3 untreated	catheter 4 unterated
Day 1	Dex^{60} 3%	CNS	Dex^{60} 3%	CNS
Day 2	Dex^{60} 2%	Dex^{60} 4%	Dex^{60} 2%	Dex^{60} 4%
Day 3	Dex^{250} 3%	Dex^{500} 3%	Dex^{250} 3%	Dex^{500} 3%
	iTRAQtag 114	iTRAQ tag 115	iTRAQ tag 116	iTRAQ tag 117

[a] Catheters 1 and 2 were surface-modified; catheters 3 and 4 were used without prior surface treatment. Days 1−3 represent three consecutive 24-h sampling periods. Dextran is abbreviated as Dex. The isobaric tags used for the quantification purposes are given in the last row of the table.

iTRAQ Labeling. Since the iTRAQ labels are designed to label between 5 and 100 μg of total proteins, the amount of total protein in each sample should not exceed 100 μg. The iTRAQ (Applied Biosystems, Foster City, CA) tagging was done accordingly: A 260 μL volume was taken from the start vCSF sample and tagged with isobaric tag 113. Then 65 μL was taken from each of the four collected MD fractions for each catheter and pooled into a total volume of 260 μL. These four samples were tagged according to Table 1: that is, catheter 1, isobaric tag 114; catheter 2, isobaric tag 115; catheter 3, isobaric tag 116; and catheter 4, isobaric tag 117. Finally, 260 μL was taken from the vCSF sample after the 24 h experiment and tagged with the isobaric tag 118. The remaining two tags in the 8-plex iTRAQ were not used.

Normalization of the samples was based on equal weighted volume. The samples were tryptically digested in parallel and then subsequently labeled with one of the eight iTRAQ reagents following a slightly modified protocol. Each sample was first dried using a Speedvac system ISS110 (Savant Holbrook, NY) and then redissolved in 20 μL of dissolution buffer and 1 μL of 1 M urea solution (instead of SDS, as suggested in the standard protocol) for protein denaturation. Afterward, the samples were reduced by adding 2 μL of the delivered reduction solution and incubated at 37 °C for 60 min, followed by free cysteine blocking for 10 min with 1 μL of the corresponding alkylation solution provided with the kit. Four trypsin vials provided by the kit where redissolved in 25 μL of Milli-Q water. The trypsin solution was then combined into one 100 μL aliquot, and 10 μL of this solution was added to each sample. The samples were tryptically digested at 37 °C overnight. The iTRAQ labels were adjusted to room temperature, and 50 μL of isopropyl alcohol (provided by the kit) was added to

every vial containing the iTRAQ label. Each sample was then labeled for two hours at ambient temperature with the corresponding iTRAQ label. After iTRAQ labeling, equal volumes (60 μL) of the six samples were combined, and the sample mixture was dried under vacuum.

Sample Desalting. The sample was redissolved in 1 mL of 2.5% acetic acid and desalted on an Isolute C18(EC) (1 mL, 50 mg capacity, Biotage, Uppsala, Sweden) SPE column using the following schedule: The column was first wetted in 1 mL of 100% acetonitrile and equilibrated with 5 × 1 mL of 1% acetic acid. The tryptic peptides were adsorbed to the media using five repeated cycles of sample loading by collecting the 1 mL of sample eluent and reapplying it on the top of the SPE column. The column was washed using 5 × 1 mL of 1% acetic acid, and finally, the peptides were eluted in 250 μL of 50% acetonitrile and 1% acetic acid. After desalting, the eluate was vacuum-centrifuged to dryness. Prior to nanoflow, LC/MALDI-TOF/TOF-MS analysis, the peptides were redissolved in 20 μL of 0.1% TFA.

Nanoflow Liquid Chromatography. The reversed-phase liquid chromatography separation was performed with an 1100 Nanoflow LC system (Agilent Technologies, Waldbronn, Germany), equipped with a fraction collector for direct fractionation onto a MALDI target plate. Digestion products were injected into a 10-μL sample loop. For separation of the peptides, a 15 cm × 180 μm C_{18} column (Thermo Electron, Waltham, MA) with 5-μm particle size and a H_2O/ACN/TFA solvent system (H_2O, 0.1% TFA mobile phase [A]; ACN, 0.1% TFA mobile phase [B]) was used. A flow rate of 2 μL/min was applied, starting with isocratic elution at 2% B over 20 min; followed by gradient elution from 2% to 8% B over 5 min; then from 8% to 32% B within 86 min; then from 32% to 40% B over 5 min; and finally, from 40% to 80% B over 1 min. The peptide elution was followed by online fractionation onto a MALDI target with a collection rate of four fractions per minute for 96 min within the elution period from 20 min (2% B) and 116 min (40% B), resulting in 384 fractions. For optimal MS results, disposable prespotted anchorchip targets (PAC-targets, Bruker Daltonics, Bremen, Germany) were chosen. The targets were washed with 10 mM $NH_4H_2PO_4$/0.1% TFA prior to MALDI-TOF/TOF-MS analysis.

MALDI-TOF/TOF-MS Analysis. Data were acquired with an Ultraflex II MALDI-TOF/TOF-MS (Bruker Daltonics) in reflector positive mode. A mass-over-charge (m/z) range of 700−4000 Da was analyzed with a sum of 300 shots/spot and 50 shots/position, respectively, in a hexagonal pattern. The laser frequency was set to 100 Hz. MALDI-TOF/TOF tandem MS analysis was performed in LIFT mode with 30% increased laser energy to get the fragmentation spectra. Post-LIFT mother ion suppression was applied to deflect the precursor and elevate fragment ion intensity. Peptide monoisotopic signals were analyzed using the SNAP algorithm implemented in the FlexAnalysis software (Bruker Daltonics). The spectra were calibrated externally using the prespotted calibrants adjacent to the sample spots. WarpLC Software (Bruker Daltonics) was used to acquire real data acquisition for automatic TOF-MS spectra. WarpLC Software was also utilized for background signal filtering; grouping of signals into a peptide profile (with respect to their distribution and intensity); and finally, as selection for optimized precursor ions for subsequent MS/MS experiments.

For final protein identification, all collected MS/MS data were run in a comprehensive MS/MS ion search using the Mascot search engine, version 2.2.2 (Matrix Science, London, U.K.). Acquired MS/MS spectra were evaluated with the Matrix Science MASCOT database SwissProt, version 51.6. The search parameters were set to taxonomy, *Homo sapiens*; enzyme, trypsin; fixed modifications, methylthio (C); variable modifications, oxidation (M); peptide mass tolerance, ± 50 ppm; fragment mass tolerance, ± 0.8 Da; maximum 1 missed cleavage site; and quantitation, iTRAQ 8-plex. Proteins were considered to be positively matched if at least one MS/MS spectrum fulfilled an individual MASCOT MS/MS ionscore > 27 (significance threshold set to 95% ($p \leq 0.05$)), MudPIT scoring, no normalization, and minimal precursor charge =1. Protein quantification was achieved by normalizing all reporter iTRAQ intensities to the corresponding control reporter ion (iTRAQ 113).

Ethical Considerations. Permission to use ventricular CSF has been granted by the Regional Ethics Committee (Dnr UPS 01−367).

Safety Considerations. Precautions should be taken to avoid exposure to acetonitrile and all acids. Acetonitrile is combustible, volatile, and toxic if exposed to lungs or skin. The vCSF samples are biologically active and should be handled with great precaution using protective gloves. Used MD catheters and biological sample material should be destroyed using appropriate destruction methods.

RESULTS AND DISCUSSION

Perfusion Fluid Recovery. Dextrans can be obtained in a large variety of sizes; they are often abbreviated by "Dextran" followed by the size of the molecule in kDa. For example a dextran with molecular weight of 60 kDa will be named Dextran 60. When using membranes with MWCO 100 kDa or larger, the first and most pertinent step is to consider the possibility of fluid loss when sampling dialysates. Adding dextran to the perfusion fluid alters the osmotic pressure and makes it possible to adjust the permeate flux across the membrane. In this way, a fluid recovery of close to 100% can be obtained, and a diffusion-driven dialysis sampling was achieved. The osmotic pressure is dependent on the molar concentration of the added dextran. This concentration can be adjusted by either changing the amount of dextran added to the perfusion fluid or by using a dextran with a dissimilar molecular weight.

An addition of 3% w/v Dextran 60 to the CNS perfusion fluid was used as a starting point of the study since this is the dextran size and amount recommended by the MD catheter manufacturer when 100 kDa MWCO membranes are used in vivo. As seen in Figure 2, the addition of 3% Dextran 60 produced a fluid recovery of 146% and 110% for untreated and surface-modified MD catheters respectively, resulting in a convective flux from the CSF sample to the perfusion fluid. A fluid recovery exceeding 100% was also obtained when 2% and 4% Dextran 60 were used in the perfusion fluid. When no dextrans were added to the CNS perfusion fluid, a fluid recovery of 68% was obtained when a surface-modified MD catheter was used. This combination produced a convective flux of perfusion fluid to the surrounding CSF sample. When the combination of untreated MD catheter and CNS fluid was used, no dialysate fluid could be recovered. These results

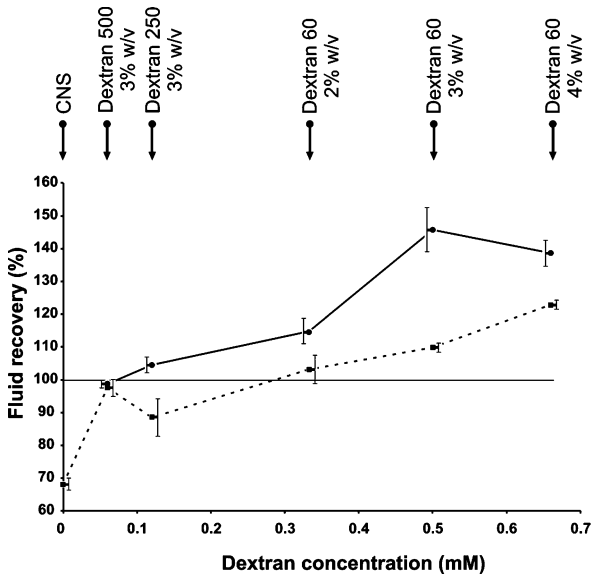

Figure 2. The fluid recovery in percentage (%) as a function of Dextran concentration in the perfusion fluid presented in moles per liter. The solid line represents the untreated catheters, whereas the dotted line represents the surface-modified catheters. At the top of the figure, the exact perfusion fluid composition for each point is presented. Error bars represent ±1 SD, where $n = 4$ in all cases except for Dextran 500 and Dextran 250, where $n = 3$. The line at 100% represents the ideal perfusion fluid recovery where the MD is governed by diffusion only. No dialysate fluid could be recovered from the untreated catheter when no dextran was added to the perfusion fluid. Two tailed, paired *t* tests confirmed a significant difference ($p <$ 0.05 for 2−4% w/v Dextran 60 and 3% w/v Dextran 250) between surface-modified and untreated catheters.

are similar to previous findings by Helmy et al.[13] using MD perfusate with or without human serum albumin as colloid.

It is important to preserve the microenvironment around the catheter, that is, the interstitial compartment of an organ, for in vivo applications of MD. In addition to a fluid recovery of 100%, one must also consider the fact that diffusion entails fluid and molecular exchange in both directions across the MD membrane. This makes it important to not only control what has diffused into the catheter but also what may have diffused out from the catheter. Since leakage of Dextran 60 could theoretically perturb the interstitial microenvironment, dextrans with molecular sizes of 250 or 500 kDa were used as alternative additives to the perfusion fluid.

As seen in Figure 2, an addition of 3% Dextran 250 to the perfusion fluid produced a fluid recovery of 105% for the untreated catheter; the surface-treated catheter produced a fluid recovery of 89%. The same tendency was observed when 3% Dextran 500 was added; however, the difference between the untreated MD catheter (99%) and surface-modified (98%) was smaller. These results suggest that large dextran additives should be used in the perfusion fluid because this results in fluid recovery close to 100% and, theoretically, a reduced risk of dextran leakage into the sampling area. The overall effect on the MD sampling microenvironment is thus decreased, since effects generated by the MD perfusion fluid are diminished. It was observed that a higher concentration of large dextrans (>8% w/v, Dextran 500, data not shown) increased the viscosity of the perfusion fluid. This should be avoided, since it leads to other complications, such as clogging of the MD catheter.

There is some concern about possible adverse tissue reactions based on anaphylactic reactions following intravenous administration of Dextran. Even though, to our knowledge, there are no reports of such reactions during cerebral MD, the use of large dextran molecules that do not leak out into the interstitial compartment in vivo appears advantageous also in this perspective. Since the nominal molecular cutoff of the MD membrane is not a precise limit[44] and since the molecular weight of the dextran molecules within a batch is variable, to back up our reasoning, we are in the process of trying to rule out any leakage of Dextran 250 or Dextran 500 with the 100 kD MD catheter in vitro.

Surface Modifications. There are many ways to modify membrane surfaces for improved antifouling properties. The field has just recently been reviewed by Chen and co-workers.[45] It should be possible to carry out modification of MD membranes under gentle aqueous conditions with minimal effect on the membrane architecture and on membranes already fitted to the MD catheter.[32] It is also important that the modification be nontoxic and that it can be sterilized.

Pluronic F-127 was chosen as a surface modifier in this study, since it has large hydrophobic and long hydrophilic units, $PEO_{98}-PPO_{67}-PEO_{98}$. The best protein resistance is obtained when PEO with the longest chain length is used, provided that an optimum surface density has been chosen.[39] The surface density may be controlled by the size of the PPO unit;[46] however, it is important to form a strong hydrophobic attachment to the membrane surface to minimize desorption. Pluronic F-127 has an average molecular mass of 12 600 Da[34] and forms a loose monolayer with a thickness of 2−9 nm, depending on the substratum surface hydrophobicity.[46] Two other reasons for choosing Pluronic F-127 are that solutions of Pluronic F-127 can be sterilized by autoclaving (120 °C, 15 min, 1 bar) and that the Food and Drug administration (FDA) guide has presented Pluronic F-127 as an inactive ingredient for different types of preparations, such as oral solutions, inhalations, suspensions, and ophthalmic or topical formulations,[34] making the prospect of transition from in vitro to in vivo studies promising.

As illustrated in Figure 2, surface-modified MD catheters produced a lower transmembrane flux into the perfusion fluid as compared with untreated catheters. A two-tailed, paired *t*-test confirmed a significant difference ($p < 0.05$ for 2−4% w/v Dextran 60 and 3% w/v Dextran 250) between surface-modified and untreated catheters. However, no significant difference between the surface-modified and untreated catheters was found when 3% w/v Dextran 500 was used as the colloid in the perfusion fluid.

Decreased protein adsorption on the surface-modified catheters is presented in Figure 3, where the perfusion fluid recovery is plotted as a function of time for each collected 6-h fraction. Figure 3 compares surface-modified and untreated catheters during the first 24-h period of sample collection with a perfusion fluid of 3%

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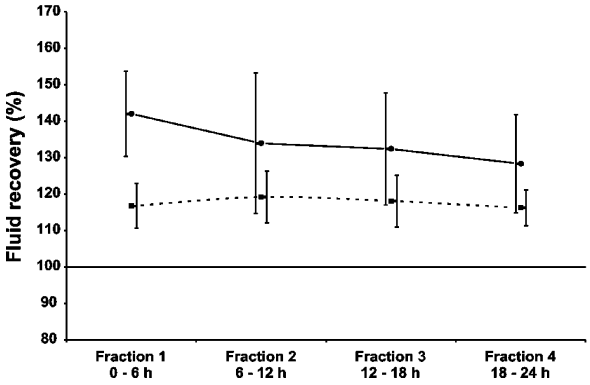

Figure 3. Fluid recovery as a function of fraction order. Perfusion fluid composition, 3% w/v Dextran 60. Solid line symbolizes untreated MD catheters and dotted line symbolizes surface-modified MD catheters. Error bars are based on ±1 SD of four surface-modified and untreated catheters, respectively. For statistical analysis, see the Results and Discussion.

w/v Dextran 60. The surface-modified catheters gave, on average, a 16.6% decrease in fluid recovery, as compared with the untreated catheters, which is in agreement with the data in Figure 2. More importantly, the precision of the surface-modified catheters was increased as compared with the precision for the untreated catheters with respect to the fluid loss among samples. A one-tailed *F*-test, which considers the ratio of the two sample variances, gave a probability value of $p = 0.086$, suggesting that the surface-modified catheters do have a better precision regarding fluid recovery with 91.4% certainty.

Protein Selection. In total, 48 proteins were identified and quantified using isobaric tagging and mass spectrometry. In a previous study from our group,[47] 70 proteins were quantified in vCSF using the same methodology. The number of proteins may seem low, but it should be noted that to receive a relative quantity using ITRAQ, the protein must be present in all samples that are compared. This means that the lowest concentrated sample (in this case, the microdialysates) determines the total number of proteins quantified. It should also be noted that the proteins were analyzed using stringent search criteria with settings preventing a tryptic peptide to be matched to multiple proteins.

The false discovery rates for the peptides were less than 2.7% in all experiments. For comparison, the study by Mauer et al.[44] identified 27 proteins in MD samples from stroke patients using MS and 20 kD MWCO catheters. Out of the 48 proteins, 16 were retrieved in all the individual tests. Among these 16 proteins, six were selected for further analysis. The selection was based on the five most abundant proteins with respect to their MASCOT score: albumin, transferrin, clusterin, complement C3, and hemopexin. In addition, hemoglobin-beta was chosen due to its molecular weight and importance as a marker in subarachnoidal CSF.

All proteins are presented in Table 2. The molecular mass and the isoelectric point are based on data from the Uniprot protein database[48] in which the signaling peptide of the protein is excluded. Table 2 also gives the average number of detected tryptic peptides and the average MASCOT score. According to Roche et al,[1] the relative protein abundance in CSF is as follows: albumin 60% IgG 7%, transthyretin 5%, transferrin 4%, α1-antitrypsin 2%, apolipoproteins 2%, etc. All these proteins are identified and quantified, except for transthyretin. All of the other proteins in the list are expected in CSF, although at very different concentrations. For instance, complement factor B and protein 14-3-3 are expected to be present in very low concentrations. These proteins are biomarkers of immune responses and neuronal injury, respectively. Since the vCSF was obtained from patients with acute brain injury, it is not surprising to find these proteins in our sample material.

As previously observed,[16] we also detected proteins with significantly larger molecular masses than the designated 100-kDa MWCO of the membrane. For example, complement C3 and complement C4A and B have molecular masses larger than 180 kDa. This could be explained by the fact that these proteins are built up by chains that may be split up into smaller subunits. By comparing the detected tryptic peptides with the sequence of complement C3, 9 of 10 chains were found. No tryptic peptide overlapped two chains, suggesting that the complement C3 has split into subunits before MD sampling. It should also be noted that the MWCO of a membrane is defined as the molecular mass at which 80% of the molecules are prevented from flux through the dialysis membrane,[44] meaning that the MWCO is not an absolute measure of the membrane pore size. Therefore, even proteins that do not split up into smaller subunits but are larger than the designated MWCO may diffuse across the MD membrane.

Protein Extraction Efficiencies. The proteins that we chose to investigate further differ widely regarding their physical and chemical properties and also the concentration in vCSF. As presented in Table 2, the molecular mass ranges from ~15 kDa up to 185 kDa, and the isoelectric point ranges from 5.67 for albumin up to 7.07 for transferrin. In Figure 4, the proteins are plotted as a function of moles per liter of dextran used in the perfusion fluid. Paired *t*-tests (one-tail) were used to compare the statistically significant differences between untreated and surface-modified catheters.

Albumin shows no significant difference in extraction efficiency between modified and untreated catheter. The change in dextran concentration does not affect the extraction efficiency of albumin significantly. The extraction efficiency for albumin is, on average, 17.5 ± 2.4% and 17.3 ± 2.4% for surface-treated and untreated catheters, respectively, on the basis of all collected samples. Clusterin and complement C3 show a little but not statistically significant difference between surface-treated and untreated catheters ($p = 0.069$ and $p = 0.061$). Transferrin ($p = 0.003$), hemopexin ($p = 0.05$), and hemoglobin-β ($p = 0.01$) significantly increase in extraction efficiencies when comparing surface-modified to untreated catheters. Clusterin and hemopexin have very similar molecular masses. The extraction efficiency for clusterin and hemopexin is also similar when comparing the untreated catheters. However, surface-modified catheters increase the extraction efficiency for hemopexin by, on average, 5.6%. The largest extraction efficiency for hemopexin is 44% and is obtained when 3% w/v Dextran 250 is added to the perfusion fluid of the surface-modified catheter. Transferrin is the protein mostly affected by surface treatment.

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Table 2. All Proteins That Were Quantified in the Study[a]

	Uniprot	protein	MW	pI	av amt of tryptic peptides	av MASCOT score
1	P02768	serum albumin[c]	66 472	5.67	76.0	1813
2	P02787	transferrin[c]	74 597	7.07	28.0	208
3	P10909	clusterin[c]	50 063	5.89	9.3	192
4	P01024	complement C3[c]	184 951	6.00	13.7	150
5	P02790	hemopexin[c]	49 295	6.43	10.0	140
6	P0C0L4	complement C4-A and B[b]	190 519	6.60	12.7	122
7	P01034	cystatin-C[b]	13 347	8.75	3.3	113
8	P02671	fibrinogen-α	91 359	5.79	7.5	112
9	P68871	hemoglobin-β[c]	15 867	6.81	3.0	98
10	P01009	α-1-antitrypsin[b]	44 325	5.37	7.7	97
11	P62942	peptidylprolyl *cis*−*trans* isomerase[b]	9 893	7.03	1.0	79
12	P04217	α-1B-glycoprotein[b]	51 941	5.65	3.3	77
13	P36222	chitinase-3-like protein[b]	40 489	8.65	4.3	75
14	P01834	Ig κ chain C region	10 821	5.58	1.3	73
15	P10451	osteopontin[b]	33 714	4.35	1.7	60
16	Q92876	kallikrein-6	24 500	6.91	1.0	59
17	P41222	prostaglandin-H2 D-isomerase	18 698	8.37	6.0	56
18	P00450	ceruloplasmin[b]	120 085	5.41	3.3	55
19	P01876	Ig α-1 chain C region[b]	37 654	6.08	6.3	52
20	P36955	pigment epithelium-derived factor (PEDF)	44 418	5.90	4.0	50
21	P69905	hemoglobin-α	15 126	8.73	3.0	50
22	P01860	Ig γ-3 chain C region	41 287	8.23	3.0	50
23	Q9Y287	integral membrane protein 2B	30 338	5.00	2.0	48
24	Q14624	interalpha-trypsin inhibitor heavy chain H4	100 300	6.11	3.0	47
25	P07477	trypsin-1 precursor	24 114	7.64	3.7	46
26	P08571	monocyte differentiation antigen	37 215	5.58	1.5	45
27	P02749	β-2-glycoprotein	36 255	8.37	1.0	44
28	P10599	thioredoxin	11 606	4.28	1.0	44
29	P01859	Ig γ-2 chain C region[b]	35 900	7.66	4.0	40
30	P14174	macrophage migration inhibitor	12 345	8.24	1.0	40
31	P02675	fibrinogen-β	50 763	7.95	6.0	38
32	P01861	Ig γ-4 chain C region	35 354	6.73	2.0	38
33	Q86XX4	extracellular matrix protein FRAS1	440 216	5.33	2.3	38
34	P30086	phosphatidylethanolamine-binding protein	20 926	7.43	1.0	37
35	P31946	14-3-3 protein β/α	28 082	4.67	2.0	37
36	P02763	α-1-acid glycoprotein	21 560	5.00	3.0	36
37	P01857	Ig γ-1 chain C region	36 105	8.46	4.0	35
38	Q96HE9	proline-rich protein 11	40 085	10.13	3.0	35
39	P01011	α-1-antichymotrypsin	45 266	5.32	3.0	34
40	P02647	apolipoprotein A-I	28 079	5.27	4.5	33
41	Q9UBP4	Dickkopf-related protein 3	36 185	4.52	1.0	32
42	P02649	apolipoprotein E	34 237	5.52	8.0	32
43	P22413	ectonucleotide pyrophosphate	104 924	6.76	4.0	32
44	P00738	haptoglobin	43 349	6.13	3.5	30
45	Q03164	zinc finger protein HRX	431 764	9.22	2.0	30
46	O75607	nucleoplasmin 3	19 212	4.55	1.0	29
47	P00751	complement factor b	83 001	6.66	1.0	28
48	P07196	neurofilament triplet L	61 385	4.64	1.0	28

[a] The proteins are sorted after the average MASCOT score obtained in the three 24-h experiments. The proteins' theoretical molecular masses (MW) and isoelectric points (pI) are presented without the proteins' signalling peptide and were obtained from the Uniprot database.[48] Columns 6 and 7 represent the average amount of tryptic peptides found for each protein and their MASCOT score, respectively. [b] Proteins that were found in all three experiments. [c] Six proteins that were studied in more detail.

The average extraction efficiencies for surface-treated catheters are 32.1% compared with 23.9% for untreated catheters; that is, a difference of 8.2%. The lowest extraction efficiencies for all proteins except transferrin were obtained when CNS fluid without added dextran was used as the perfusion fluid. The reason for this may be an outflow of perfusion fluid to the sample area that counteracts the diffusion mass transfer. It is also apparent that a fluid recovery over 100% that gives rise to an increased inflow of sample due to convection is not favorable for increased extraction efficiencies of proteins.

All untreated catheters gave a higher fluid recovery and in general lower protein extraction efficiency as compared to surface-modified catheters. As earlier discussed, this is most likely due to the surface treatment that decreases the effective pore size of the membrane, that is, decreases the MWCO. One may therefore expect an increased dilution effect due to increased influx of water and salt in the nonmodified catheters.

The extraction efficiencies obtained were normalized according to the total amount of collected sample for each MD experiment, removing the possible effect of water and salt dilution of the sample.[49] Two of the analyzed proteins, transferrin and hemoglobin-β, still showed a significant ($p < 0.05$) increase in the absolute amount of collected proteins when comparing surface-modified and nontreated catheters. For surface-modified catheters, the amount of transferrin and hemoglobin-β was 59.7 v % and 61.5 v %, respectively. For the nontreated catheter, the amount of

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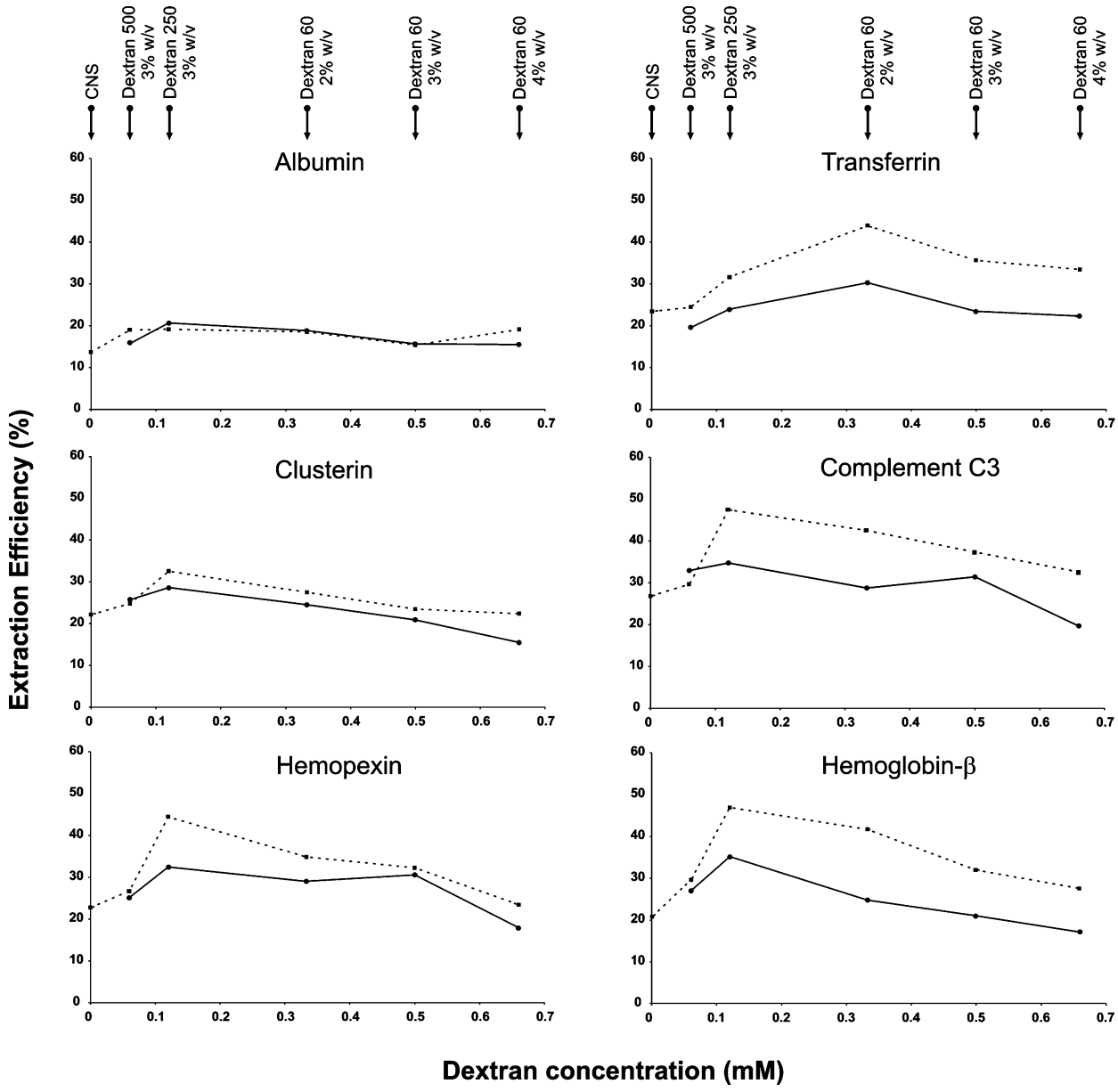

Figure 4. The extraction efficiencies for the six proteins studied in more detail as a function of dextran concentration in the perfusion fluid, presented in moles per liter. At the top of the figure, the exact perfusion composition for each point is presented. The dotted lines represent surface-treated MD catheters, and the solid lines represent untreated MD catheters. The *p* values, which are derived from a two-tailed *t*-test (paired) comparing surface-treated and untreated catheters for albumin ($p = 0.41$), transferrin ($p = 0.003$), clusterin ($p = 0.069$), complement C3 ($p = 0.061$), hemopexin ($p = 0.05$), and hemoglobin-β ($p = 0.01$).

transferrin was 48.4 v %, and for hemoglobin-β, 49.1 v %. Hemopexin, which differed significantly when the extraction efficiencies were compared, did not differ significantly ($p = 0.74$) when the normalized data were compared. These results illuminate the complexity of sampling amphilic macromolecules such as proteins in complex biological samples with MD.

Limitations of the Study. MD sampling from human CSF mimics only some aspects of the complex brain/catheter interface in vivo. Important features, such as tortuosity, change in the interstitial volume fraction, and cellular interactions with the MD membrane are not modeled. Thus, extraction efficiency determinations in vitro are just a rough estimate of the in vivo extraction efficiency.[4] The rationale for this study was that improving our understanding of the mechanisms determining the extraction efficiency of the MD membrane in vitro may lead to improvements of in vivo MD protein sampling performance. Such improvements need to be confirmed by in vivo experiments. Furthermore, the use of the iTRAQ method demanded a large volume of sample, resulting in a decreased time resolution, since the method used follows a standard protocol that demands large amounts of proteins. The aim of this study was to improve the microdialysis sampling performance and analyze a spectrum of proteins. A study focused on specific individual proteins, however, would demand smaller sample volumes and, hence, result in a higher time resolution. In addition, we are currently developing more sensitive mass spectrometric detection methods.

CONCLUSIONS

A novel microdialysis method for sampling proteins was developed. The study presents three major improvements. First,

the use of dextrans with considerably larger molecular mass (250 and 500 kDa) as compared with the MWCO of the MD membrane (100 kDa) were used in the perfusion fluid. Dextran 250 and 500 may be used to adjust the fluid recovery close to 100%, thus achieving a diffusion-driven dialysis sampling. Dextran 250 and 500 would also, theoretically, be hindered to a larger extent to diffuse from the perfusion fluid to the surrounding sample compared to smaller osmotic agents, such as Dextran 60 and albumin, due to the 100 kDa MWCO of the membrane. This may be advantageous for minimizing the degree of microenvironmental perturbation of the MD sampling in vivo. Second, by surface modification of the MD catheters a significantly increased precision of the fluid recovery was obtained, probably reflecting decreased protein adsorption on the surface. A decreased risk of protein fouling would diminish the risk of cell growth on the membrane. The result is not only a prolonged lifetime of the MD catheter but also a catheter that generates more accurate results over time.

Surface-treated catheters also provided significantly increased extraction efficiencies for some proteins. The third improvement is that we have developed and implemented an isobaric tagging protocol (iTRAQ) for quantitative analysis of proteins in dialysates using nanoflow LC coupled to mass spectrometry. By using iTRAQ in combination with nanoflow LC/MS, 48 different proteins sampled from the vCSF samples could be identified and quantified in the dialysates.

The novel aspects on microdialysis protein sampling, detection, and quantification in biological fluids presented in this study should be considered as a first step toward better understanding and handling of the challenges associated with microdialysis sampling of proteins. The next step is to optimize each improvement further and apply the developed methodology in vivo.

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