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ARTICLE

Protocol for aerosol-free recombinant production and NMR analysis of prion proteins

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Abstract The central hallmark of prion diseases is the misfolding of cellular prion protein (PrP^C) into a disease-associated aggregated isoform known as scrapie prion protein (PrP^{Sc}). NMR spectroscopy has made many essential contributions to the characterization of recombinant PrP in its folded, unfolded and aggregated states. Recent studies reporting on de novo generation of prions from recombinant PrP and infection of animals using prion aerosols suggest that adjustment of current biosafety measures may be necessary, particularly given the relatively high protein concentrations required for NMR applications that favor aggregation. We here present a protocol for the production of recombinant PrP under biosafety level 2 conditions that avoids entirely exposure of the experimenter to aerosols that might contain harmful PrP aggregates. In addition, we introduce an NMR sample tube setup that allows for safe handling of PrP samples at the spectrometer that usually is not part of a dedicated biosafety level 2 laboratory.

Keywords Aerosol · Prion diseases · Recombinant prions · Biosafety level 2 · Prion protein oligomerization

Introduction

Prions are proteinaceous infectious particles that cause fatal neurodegenerative diseases in humans (Creutzfeldt Jakob disease, Gerstmann Sträussler Scheinker syndrome, and fatal familial insomnia) and animals (bovine spongiform encephalopathy, chronic wasting disease, and scrapie) (Prusiner 1998). During prion pathogenesis the normal host-encoded prion protein (PrP^C) converts into an aggregated isoform denoted as PrP^{Sc}. The conversion process is accompanied by a dramatic change in conformational characteristics. While native PrP^C is mainly an α-helical protein, PrP^{Sc} is largely enriched in β-sheet structure (Caughey et al. 1991; Pan et al. 1993). PrP^C and PrP^{Sc} differ significantly in their susceptibility to proteolytic digestion by proteinase K (PK). PrP^C is fully digested upon exposure to PK. However, PK digestion of PrP^{Sc} has revealed a protease-resistant core encompassing amino acid residues ~90–230 (Prusiner 1998).

PrP^{Sc} self-replicates in an autocatalytic manner (Prusiner 1998). PrP^{Sc} recruits PrP^C and templates its conversion into a PrP^{Sc} conformation. Importantly, it has been demonstrated that prions can be generated de novo from recombinant prion protein (Legname et al. 2004), leading to prion diseases even in wild-type animals (Makarava et al. 2010; Wang et al. 2010). While some of these studies require the use of cofactors such as ribonucleic acids (RNA) and lipids (Wang et al. 2010), others employ

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elevated temperatures for de novo prion generation (Makarava et al. 2010). These findings call for safety precautions not only when working with PrP^{Sc} from infected humans or animals but also when working with recombinant PrP under conditions favoring self-assembly into potentially harmful aggregates.

Incorporation of beef (Ward et al. 2006; Will et al. 1996), therapeutic human growth hormone, dura mater grafts, and neurosurgical instruments contaminated with prions (Brown et al. 2000) as well as transfusion of blood from affected individuals (Llewelyn et al. 2004; Peden et al. 2004) have been identified as possible routes for prion infection in humans. Infection by prion aerosols has not been documented thus far, and it is generally accepted that prions are not airborne. However, for a given experimental setting prion aerosols have been shown to infect mice (Denkers et al. 2010; Haybaeck et al. 2011) and deer (Denkers et al. 2013). Commonly, any work conducted with tissue from prion-infected humans or animals involving open vessels is performed in a class II biosafety cabinet to prevent exposure of the experimenter to potentially infectious prion aerosols. While this is standard practice when working with tissue from infected subjects, similar safety precautions for work with recombinant PrP have not been reported.

Recombinant PrP is usually expressed in *E. coli* and can be purified either from inclusion bodies (Makarava and Baskakov 2008; Mehlhorn et al. 1996; Rezaei et al. 2000; Zahn et al. 1997) or in a soluble form (Abskharon et al. 2012; Hornemann and Glockshuber 1996). Purification of protein solubilized from inclusion bodies generally involves a nickel-nitrilotriacetic acid (Ni–NTA) column and a subsequent size exclusion step. Refolding is accomplished either on-column or by dilution upon elution from the Ni–NTA column. During the purification procedure PrP can self-assemble into potentially harmful aggregates. First, on-column refolding leads to the formation of PrP precipitates (Zahn et al. 1997). Second, high PrP concentrations as required for NMR applications generally favor aggregation. Third, the same situation applies when working with PrP bearing single-point mutations that are associated with inherited human prion diseases and render the protein significantly more aggregation-prone. In the past, solution-state NMR spectroscopy has revealed only minor differences in the structural and dynamical characteristics for both the native (Lysek et al. 2005; Riek et al. 1996; Zahn et al. 2000) and unfolded states (Gerum et al. 2009, 2010) among different species. Solid-state NMR has given valuable insight into structural details of amyloid fibrils derived from full-length PrP (Tycko et al. 2010) and truncated versions thereof (Kumar et al. 2010; Walsh et al. 2009). However, only little is known about transiently formed PrP oligomers and about the dynamics of their interconversion with PrP monomers and fibrils. Recently, we have shown for the first time that real-time two-dimensional NMR can be used to monitor PrP oligomerization at atomic resolution revealing strikingly non-uniform effects across the polypeptide chain (Schlepckow and Schwalbe 2013).

The fact that prions can be generated de novo from recombinant PrP and generally bear the potential to be transmitted via aerosols has led us to reconsider standard laboratory practices for the generation of samples for biophysical characterization of PrP. Since PrP oligomers of the size of 14–28 mers have been reported to be the most infectious entities when isolated from diseased animals (Silveira et al. 2005), biophysical studies of PrP oligomerization should be given particular attention. Here, we report a protocol for aerosol-free production of recombinant PrP under biosafety level 2 conditions with an emphasis on producing isotopically labeled samples for NMR applications. Moreover, we introduce an NMR tube setup that enables safe handling of PrP samples at the NMR spectrometer.

Materials and methods

Bacterial strain and plasmid DNA

E. coli BL21(DE3) cells (Life Technologies, Darmstadt, Germany) were transformed with plasmid DNA based on the pRSET A expression vector coding for the C-terminal domain of murine PrP (residues 121–232, human numbering) including an N-terminal hexahistidine tag (Zahn et al. 1997).

Protein expression

As a general statement, all working steps involving open vessels were performed inside a class II laminar flow biosafety cabinet equipped with prefilters. The procedure for PrP expression and purification broadly follows the protocol reported by Zahn et al. (1997).

Expression of either wildtype or mutant murine PrP starts with the transformation of *E. coli* BL21(DE3) cells with the appropriate plasmid DNA. Agar plates with freshly transformed cells are tightly sealed with parafilm and incubated overnight at 37 °C outside the cabinet. On the next day, a single clone is picked and used for inoculation of a culture with minimal medium designed for production of isotopically labeled protein. For cell growth we use baffled plastic Erlenmeyer flasks made of polyethylene terephthalate glycol (PETG) (Thermo Fisher Scientific Inc.) that are break-resistant thereby excluding contamination of laboratory space with PrP-expressing cells outside the cabinet. Moreover, these flasks have screw

caps fitted with a filter membrane to allow for sterile aeration of the culture while shaking.

Cell growth is controlled by measurement of OD600. At regular time intervals, unbreakable plastic UV/Vis cuvettes for single use (Brand, Wertheim, Germany) are filled inside the biosafety cabinet. As these cuvettes are capped with a rubber plug, cell growth can conveniently be monitored using a spectrophotometer outside the cabinet. Protein expression is usually induced at an OD600 of 0.6–0.8 by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM final concentration) and conducted at 37 °C. PrP overexpression leads to formation of inclusion bodies. Cells are then harvested about 4 h after induction by centrifugation. Cell lysis is subsequently performed using BugBuster™ protein extraction reagent (Merck Millipore, Darmstadt, Germany). This procedure avoids formation of aerosols that are likely to form when using a homogenizer or microfluidizer for cell lysis. Inclusion bodies are collected by centrifugation at 16,000 *g* for 15 min and at 4 °C. Inclusion body resolubilization is done overnight in a sealed vessel in resolubilization buffer (6 M guanidinium hydrochloride (GdnHCl), 5 mM reduced glutathione, 5 mM imidazole, 100 mM sodium phosphate, 10 mM Tris pH 8.0 (Zahn et al. 1997)).

Protein purification

For PrP purification we use the Biologic LP FPLC system from Bio-Rad (München, Germany). Sample loading and collection are exclusively performed inside the biosafety cabinet. This is accomplished by pulling FPLC tubing into the cabinet by means of septa in the cabinet's side walls allowing connection of the space inside the cabinet with the outside.

Resolubilized inclusion bodies are usually loaded by means of tubing leading from the cabinet onto a 5 mL HisTrap column (GE Healthcare) with a flow rate of 0.5 mL/min. Depending on protein yield it may be necessary to dilute the solution with the resolubilization buffer as stated above before loading to prevent clogging of the column, which may severely compromise refolding and protein recovery. Upon completion of inclusion body loading, a previously reported protocol for PrP purification (Zahn et al. 1997) is followed. We have observed that rounds of PrP on-column refolding and elution can be repeated up to four times leading to substantial increases in protein yield. Samples are collected in the biosafety cabinet during elution and PrP-containing samples are pooled upon sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Upon overnight incubation with thrombin, cleaved PrP is concentrated using a table top centrifuge (4 °C, 8,000 *g*). As we have observed that concentrators can leak we only open the rotor inside the cabinet. The concentrated solution is subsequently rebuffered to 6 M GdnHCl, 50 mM Tris/HCl pH 7.2 to load the protein in a predominately monomeric form onto a size exclusion column (HiLoad 26/60, Superdex 75, GE Healthcare). The size exclusion run is performed at a flow rate of 1 mL/min using 1 M urea, 150 mM sodium chloride, 50 sodium acetate pH 5.5 essentially as described previously (Gerum et al. 2009). We usually observe only the peak for monomeric PrP. Only in case of high protein yield a minor peak is observed, which is well separated from the peak of monomeric PrP and corresponds to the dimer caused by formation of intermolecular disulfide bonds (data not shown). Protein yield after His tag cleavage is usually 6 mg/L in unlabelled medium and 3–4 mg/L in minimal medium. Sample purity is checked using SDS-PAGE and fractions containing pure monomeric PrP are pooled. The purified protein is either directly concentrated and transferred to denaturing conditions as employed for NMR studies of PrP oligomerization or rebuffered to 10 mM sodium acetate pH 4.0 for storage purposes (Cobb et al. 2008).

SDS-PAGE

SDS-PAGE was performed using precast 4–12 % Bis–Tris NuPAGE gels (Life Technologies, Darmstadt, Germany). 50 mM MES, 50 mM Tris, 0.1 % SDS, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.3 was employed as running buffer. Gels were typically run for about 45 min at 200 V. Gel staining was done using Simply Blue Safe Stain (Life Technologies, Darmstadt, Germany).

Protein determination

Protein content was typically calculated before tag cleavage and after size exclusion chromatography using an extinction coefficient of ε (280 nm) = 22,015 M^{-1} cm^{-1}.

Inactivation of material potentially contaminated with PrP

Liquid and solid wastes were inactivated in 1 M sodium hydroxide solution (final concentration) or by incineration, respectively, following biosafety guidelines that have been developed for prion-infected material (Schutzmaßnahmen bei Tätigkeiten mit Transmissibler Spongiformer Enzephalopathie (TSE) assoziierter Agenzien in TSE-Laboratorien. Beschluss 603 des Ausschusses für Biologische Arbeitsstoffe 2011; World Health Organization. WHO infection control guidelines for transmissible spongiform encephalopathies 1999).

In our biosafety cabinet, we collect all liquid waste in a 2 L glass bottle. Upon overnight inactivation with 1 M NaOH (final concentration), liquid waste is transferred to a container outside the cabinet by means of a pump

(EcoLine, IsmaTec, Wertheim, Germany). The glass bottle and the container are connected by tubing that passes through septa in the side walls of the cabinet. The container can be conveniently emptied at regular time intervals.

The interior of the biosafety cabinet including standard lab ware such as pipettes was decontaminated regularly.

NMR spectroscopy

Prion protein oligomerization in our setup is induced from the unfolded state in 8 M urea pH 2.0 (Gerum et al. 2009, 2010). Upon concentration of the sample, it is diluted to the appropriate oligomerization conditions (Schlepckow and Schwalbe 2013). Since a standard glass tube may accidentally break and lead to exposure of the experimenter to potentially harmful PrP aggregates or to contamination of the NMR probe we have developed an alternative NMR sample tube setup. The PrP sample is filled into a polytetrafluoroethylene (PTFE) liner (Deutero GmbH, Kastellaun, Germany) that is sealed with a PTFE plug. This tube cannot break thereby preventing exposure of experimenters to potentially harmful PrP aggregates. It is important to introduce the sample without the formation of air bubbles as we have observed that subsequent removal of air bubbles is difficult. For filling of the tube we use Pasteur glass pipettes that reach down to the bottom of the tube. As the sample tube is gradually filled, the pipette is slowly and gently moved upward. The outer diameter of the PTFE liner is designed such that the tube perfectly fits into a standard 5 mm NMR glass tube. The tube setup might require some manual shimming at the spectrometer, which should be considered when performing kinetic experiments as this can increase the experimental dead-time.

NMR spectra were recorded on Avance 700 and 800 MHz NMR spectrometers (Bruker) equipped with

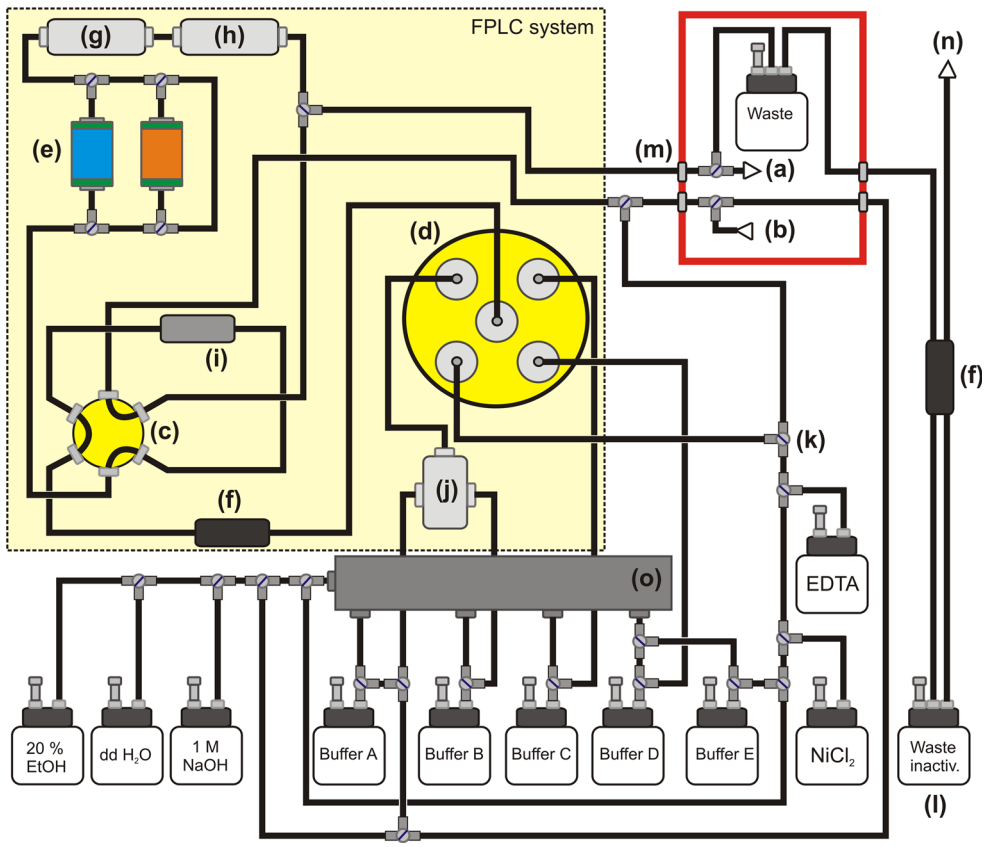

Fig. 1 Schematic showing the coupling of a FPLC purification system (*light yellow shaded area*) with a biosafety cabinet (*orange box*). PrP-containing samples are both loaded and collected from inside the cabinet. Vessels labeled buffer A, B, C, D, and E can be filled with solutions as required for chromatographic procedures. EDTA and $NiCl_2$ solutions allow for stripping and reloading of Ni–NTA columns. Tubings are connected with three way cocks. **a** Elution, **b** sample application, **c** manual sample inject valve, **d** buffer select valve, **e** Ni–NTA and size exclusion columns, **f** pump, **g** absorptivity detector, **h** conductivity detector, **i** sample loop for size exclusion chromatography, **j** mixer, **k** 3-way cock determining whether the left bottom port on the buffer select valve (**d**) is fed with either resolubilized inclusion bodies from inside the cabinet or buffers as required for chromatography from outside the cabinet, **l** container for collection of inactivated liquid waste from inside the cabinet, **m** septum, **n** drainage, **o** manifold that allows to rinse tubing emanating from buffers A, B, C, D, and E using 1 M NaOH/dd H_2O/20 % EtOH

cryogenically cooled HCN probes. ^{15}N, ^{1}H heteronuclear single quantum coherence (HSQC) spectra (Mori et al. 1995) as shown in the main text were acquired with 256 increments in the indirect dimension and 4 scans per increment. Protein concentrations of 470 and 160 μM were employed for samples in a standard 5 mm glass tube and in a PTFE liner, respectively. Proper chemical shift referencing was done using 3-(trimethylsilyl)propionate (TSP) − d_4. The pH dependence of TSP was taken into account (De Marco 1977).

Results and discussion

Aerosol-free production of isotope-labeled recombinant PrP

All fluids can act as sources of aerosols and since there is no safe dose of prions (Fryer and McLean 2011) we decided to develop a protocol for the expression and purification of recombinant PrP that prevents the exposure of the experimenter to any kind of aerosol.

During protein expression *E. coli* cultures are grown in plastic culture flasks. The two main advantages of using these flasks are (i) break-resistance preventing contamination of laboratory space outside the cabinet and (ii) screw caps with a fitted filter membrane that allow sterile aeration of the culture while shaking. For OD control during cell growth we employ plastic UV cuvettes with rubber plugs that allow for spectrophotometer usage outside the cabinet. Upon cell harvest cells are lysed inside the cabinet by applying BugBuster™ protein extraction reagent. This is a safe way of releasing PrP-containing inclusion bodies and preventing aerosol formation as might well be expected during cell lysis using homogenizers or microfluidizers. The key to subsequent PrP purification is the connection of the FPLC system outside the cabinet with the interior of the cabinet (Fig. 1). This is accomplished by pulling respective tubing through septa located in the side walls of the cabinet.

Crucially, with this setup it is possible both to load chromatography columns from the cabinet and collect eluting protein inside the cabinet. Exposure of laboratory workers to aerosols containing potentially harmful PrP aggregates is thus completely prevented.

Glass bottles with solutions needed for Ni–NTA affinity and size exclusion chromatography and for inactivation (1 M NaOH) and cleaning/storage purposes (water and 20 % ethanol) are located outside the cabinet and are supplied with filters allowing for sterile pressure compensation. The FPLC system can be opened once all the tubing has been thoroughly decontaminated with 1 M NaOH.

Since every round of PrP expression and purification leads to accumulation of several liters of potentially contaminated liquid inside the cabinet, it was important to devise a convenient way to dispose of the accumulated liquid. To this end, we collect all liquid waste in a 2 L glass bottle that is connected to a 10 L container outside the cabinet by means of tubing that passes through a septum in one of the side walls of the cabinet (Fig. 1). Upon inactivation in 1 M NaOH (final concentration) liquid waste is transferred to the outside container by means of a pump that is located outside the cabinet.

We typically obtain protein yields of 6 and 3–4 mg/L in LB and minimal media, respectively. These yields are somewhat lower as compared to the original protocol, which reported yields of 10–20 mg/L in either media (Zahn et al. 1997). This observation might be related to the fact that loading a prepacked Ni–NTA column as in our setup might not be as efficient as loading resin in a batch procedure.

NMR sample tube setup and NMR spectral quality

In addition to expressing and purifying recombinant PrP under completely aerosol-free conditions we here report a method for containing the NMR sample in an unbreakable tube to allow for safe handling of samples at the NMR spectrometer. The tube setup is given in Fig. 2.

The sample (typically 350 μL) is filled into a PTFE liner and sealed with a PTFE plug. The PTFE liner perfectly fits

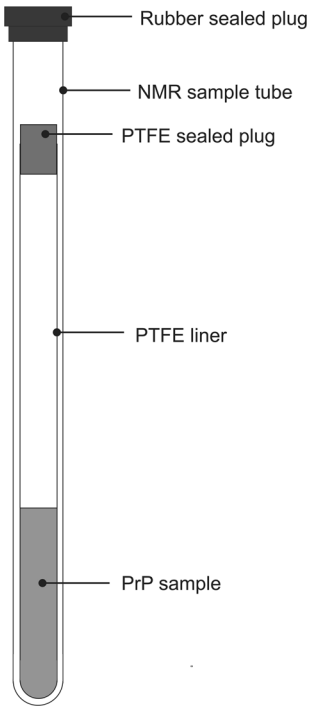

Fig. 2 NMR tube setup for safely handling PrP samples at the spectrometer. Schematic showing the sample inside a PTFE liner that is placed inside a standard 5 mm glass tube

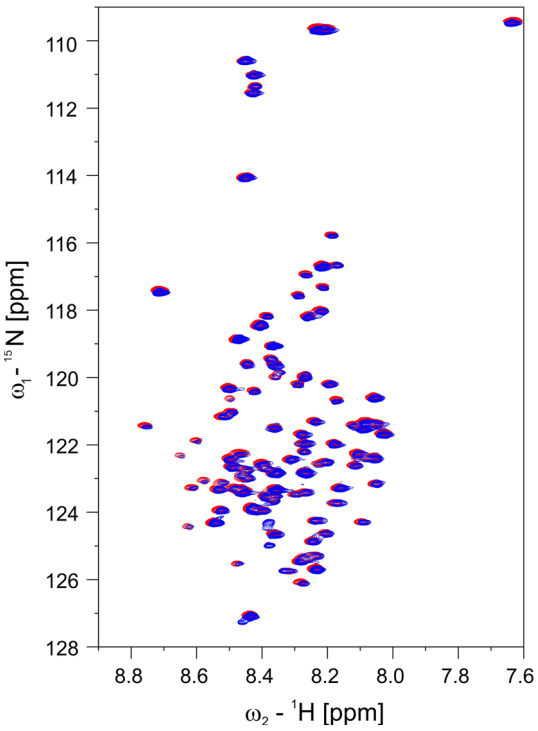

Fig. 3 NMR spectral quality for samples in a PTFE liner. Comparison of ^{15}N, ^{1}H HSQC spectra of wildtype mPrP(121-232) in 8 M urea at pH 2.0 and at 25 °C in a standard 5 mm glass tube (red spectrum) and in a PTFE liner (blue spectrum). Protein concentrations were 470 and 160 μM, respectively. Due to the higher sample volume, signal-to-noise for the sample in the glass tube is more favorable. Contour levels were adjusted such as to best illustrate the agreement between the two spectra. Resonance assignments have previously been reported (Gerum et al. 2010)

into a standard 5 mm NMR glass tube. Comparison of ^{15}N, ^{1}H HSQC spectra of wildtype mPrP(121–232) unfolded in 8 M urea pH 2.0 in a standard 5 mm glass tube and in our revised setup clearly shows that there are no artifacts introduced reflecting the inertness of the PTFE surface (Fig. 3).

Conclusions

Given recent studies that report on de novo generation of prions from recombinant PrP (Legname et al. 2004; Makarava et al. 2010; Wang et al. 2010) and on infection of mice (Denkers et al. 2010; Haybaeck et al. 2011) and deer (Denkers et al. 2013) using prion aerosols we have adjusted biosafety precautions that are normally taken when producing recombinant PrP for biophysical studies such as NMR investigations. We here report a revised collection of methods that allow for aerosol-free production of recombinant PrP in quantities sufficient for NMR applications. This procedure completely prevents exposure of laboratory workers to potentially infectious PrP aggregates and thus greatly improves the workers' safety. Moreover, we have introduced a PTFE liner setup for NMR samples enabling safe sample handling at the NMR spectrometer. Importantly, due to the inertness of the PTFE surface PrP structure is not perturbed as judged from analysis of chemical shifts in ^{15}N, ^{1}H HSQC spectra. We anticipate that the procedure described here to couple a biosafety cabinet with an external FPLC purification system will find applications in the purification of prions from diseased humans and animals.

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Conflict of interest The authors declare that they have no conflict of interest.

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