Table 3 Bioassay of test materials from Normal Immunoglobulin 2VI (Cohn) process

Sample	Parameter	Sample dilution							
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Control cryosupernatant	Mice infected/inoculated incubation period (days)[a]	0/8							
Control IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/9							
ME7 spiked cryosupernatant	Mice infected/inoculated incubation period (days)	5/5	5/5	5/5	4/5	1/5	1/5	1/5	0/4
		186 ± 6	235 ± 26	223 ± 8	279 ± 67	279	347	230	
ME7 IgG (Cohn)	Mice infected/inoculated incubation period (days)	0/20	0/5	0/5	0/5				

[a]Mean ± standard deviation.

Table 4 Calculation of Infectivity in spiked process starting materials and final materials of chromatography and Cohn process

Step/Fraction	Infectivity (log_{10} ID_{50}/ml)	Volume (log_{10})	Total infectivity (log_{10})	Reduction (log_{10})
Chromatography				
ME7 spiked SN1	5·4 (4·5–6·3)[a]	2·1	7·5	
ME7 albumin	≤ 0·7	1·2	≤ 1·9	≥ 5·6
ME7 IgG	≤ 0·7	1·4	≤ 2·1	≥ 5·4
Cohn				
ME7 spiked cyrosupernatant	5·4 (4·4–6·5)[a]	2·5	7·9	
ME7 IgG (Cohn)	≤ 0·7	1·6	≤ 2·3	≥ 5·6

[a]95% confidence interval of Spearman Kärber estimate of ID_{50}.

Discussion

The potential risk of vCJD transmission has led producers of plasma products to examine the prion clearance capacity of their fractionation processes. Whereas it is an accepted principle to add viral log reduction factors attained by mechanistically complementary steps [27], different approaches are needed to establish overall prion removal. The European Agency for the Evaluation of Medicinal Products (EMEA) guidance [28] advises that validation studies of removal/inactivation procedures for TSEs are difficult to interpret due to the necessity to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent.

This study programme looked initially at PrP^{sc} removal capacity of individual process steps then examined the potential of two or more combined process steps to remove prion infectivity. The study programme used scrapie 263K and ME7 as models for human prions, an approach that is supported by the finding that partitioning of human prions is similar to that observed in the hamster scrapie model [29]. The studies used different spiking materials (microsomal 263K and 10 000 g supernatant ME7) because the ME7 study sought to use an infectious spike which would consist not only of microsomal infectious units, but of smaller units of infectivity as soluble PrP^{sc} [17]. The study programme found substantial partitioning of prions away from the product streams of chromatographic albumin and immunoglobulin, and for immunoglobulin produced by Cohn fractionation. Importantly, this investigation shows that removal of infectivity from immunoglobulin preparations is similar whether chromatographic or Cohn purification processes are used.

The study with microsomal scrapie 263K showed substantial partitioning of PrP^{sc} away from the target proteins in all ion exchange steps examined. The log reductions across the anion exchange DEAE Sepharose and cation exchange CM Sepharose for the albumin process were ≥ 4·0 and ≥ 3·0, respectively. The log reductions across the DEAE Sepharose and anion exchange Macro-Prep for the immunoglobulin process were 3·3 and ≥ 4·1, respectively.

At the loading pH buffer ranges used for this experiment (pH 5·2 for DEAE Sepharose, pH 4·5 for CM Sepharose, and pH 6·2 for Macro-Prep), scrapie should be below its isoelectric point (pI) of pH 5·4–9·3 [30] on the DEAE and CM Sepharose columns, and hence would be positively charged. While pH 6·2 is within the pI range for scrapie, it is likely that scrapie is predominantly positively charged when loaded onto Macro-Prep. If scrapie bound to chromatography columns purely based on charge, it would be predicted that more binding should occur with the cation exchanger CM Sepharose, and less to the anion exchangers DEAE Sepharose and Macro-Prep. The substantial

removal of PrP^{sc} by the anion and the cation exchange gels, and lack of substantial amounts of PrP^{sc} in the wash fractions indicated that PrP^{sc} removal was more dependent on adsorption to the gel matrix than to the exchange group. There was a partitioning of 0·05% of loaded PrP^{sc} in the unbound IgG eluted from DEAE Sepharose. However, as the Macro-Prep column removed ≥ 4·1 logs, there is a level of confidence that this remaining PrP^{sc} would be removed from the product stream.

Similar results were reported using murine bioassay of BSE 301 V over Toyopearl DEAE-650 M [31], in which LRFs of 2·9 and 2·7 were found in eluted fibrinogen and factor VIII, leading to the conclusion that over 99% of BSE infectivity remained bound to the ion-exchange column. A 2 M NaCl wash removed 5·75% of this infectivity, and infectivity could not be detected in eluates following a 0·1 M NaOH wash.

In our study, new chromatography gels were used, as opposed to production gels that had been exposed to previous cycles. The possibility of infectivity binding to chromatography gels has led to further experimental work examining prion removal and or inactivation of infectivity from chromatography gels, in which it was ascertained that infectious prions did bind to DEAE Sepharose, and the cleaning cycle was able to remove or inactivate this infectivity [32].

A LRF of ≥ 5·6 across the DEAE and CM Sepharose ion exchange columns in the albumin process and a LRF of ≥ 5·4 across the DEAE Sepharose and Macro-Prep ion exchange columns in the immunoglobulin process was achieved. Both processes include a final concentration/diafiltration step using 30 kDa ultrafiltration, with the retentate containing either albumin or immunoglobulin. It is unlikely that substantial prion infectivity would be lost in the permeate stream, as infectious units are believed to have a minimum molecular weight of approximately 55 kDa [33]. Conversely, it is possible that some infectivity is adsorbed to the ultrafilter membrane surface; however, the membrane types used (polyethersulphone for albumin and regenerated cellulose for immunoglobulin) are both specified as low protein binding by the respective manufacturers.

If the starting titre for the ME7 bioassay study had been higher it may have been possible to show removal capacity equal to the addition of removals attained for each column in the Western blot study. Previous studies using scrapie 263K for validation of prion removal in bovine serum albumin production with sequential columns [34] have shown a 5·2 log removal of scrapie 263K over the first ion exchange column, and ≥ 6·2 when the second ion exchange column is included. This implies that the result is limited by the starting titre, and the question of additivity cannot be resolved without a higher infectivity spike. In our study programme, the spike material preparations and the method of detection (Western blot vs. bioassay) were different between the two studies. Higher titre spiking material would be needed to further elucidate the additive vs. non-additive nature of prion removal over sequential columns.

The LRF calculated for the Cohn immunoglobulin process was ≥ 5·6, which is similar to that shown previously, where a clearance of ≥ 4 logs of PrP^{sc} in fraction III effluent was detected by Western blot [7]. Removal over Fraction I + III precipitation with microfiber glass depth filtration and Seitz depth filtration has previously been reported [8] to give an overall removal of BSE 301 V infectivity of ≥ 2·9. The non-additive nature of ethanol precipitation and depth filtration was established by these authors, and further work [35] suggests that the likely cause of prion removal is the precipitation of PrP^{sc} by ethanol, and removal in centrifugation, followed by the 'polishing' effect of depth filtration.

Conclusion

Using both scrapie 263K and ME7 as spiking agents, these experiments have shown that the ion exchange chromatographic steps used in the production of albumin and immunoglobulin have the capacity to remove at least 5·6 and 5·4 logs, respectively, of infectious prions. Production of immunoglobulin by ethanol precipitation was shown to have the capacity to remove at least 5·6 logs of prion infectivity. Importantly, this investigation shows that removal of infectivity from immunoglobulin preparations is similar whether chromatographic or Cohn purification processes are used. In all the bioassay studies, the process steps removed scrapie ME7 to below the limit of detection, and further studies with a higher titred challenge, or more concentrated final product would be needed to finitely measure the removal capacity of individual process steps. The Western blot study indicated that prion removal by the columns was more dependent on adsorption to the gel matrix than the exchange group, and a ≥ 3·0 log removal of PrP^{sc} was achieved for all individually spiked columns.

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Prion removal by nanofiltration under different experimental conditions

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Abstract

Manufacturing processes used in the production of biopharmaceutical or biological products should be evaluated for their ability to remove potential contaminants, including TSE agents. In the present study, we have evaluated scrapie prion protein (PrP^{Sc}) removal in the presence of different starting materials, using virus removal filters of different pore sizes. Following 75 nm filtration, PrP^{Sc} was detected in the filtrate by Western blot (WB) analysis when a "super-sonicated" microsomal fraction derived from hamster adapted scrapie strain 263K (263K MF) was used as the spike material. In contrast, no PrP^{Sc} was detected when an untreated 263K MF was used. By using spike materials prepared in a manner designed to optimize the particle size distribution within the preparation, only 15 nm filtration was shown to remove PrP^{Sc} to below the limits of detection of the WB assays used under all the experimental conditions. However, infectious PrP^{Sc} was recovered following 15 nm filtration under one experimental condition. The results obtained suggest that the nature of the spike preparation is an important factor in evaluating the ability of filters to remove prions, and that procedures designed to minimize the particle size distribution of the prion spike, such as the "super-sonication" or detergent treatments described herein, should be used for the preparation of the spike materials.

Keywords: Prion; Removal; Filter; Clearance study; Spike material

1. Introduction

The transmission of variant Creutzfeldt–Jakob disease (vCJD) through blood transfusion has been of increasing concern, since a fourth possible transmission case was reported [1]. In addition, prions have been detected in the buffy coat separated from the blood of hamsters infected with scrapie, using a biochemical assay (protein misfolding cyclic amplification, or PMCA) [2]. Infectious prions are thought to be the causative agent of the transmissible spongiform encephalopathy (TSE) diseases, which include Creutzfeldt–Jakob disease (CJD), vCJD, and bovine spongiform encephalopathy (BSE). Therefore, to reduce the risk of transmission when raw materials for protein products (such as plasma) are contaminated with infectious prions, measures should be introduced to decrease the prion load, to evaluate the risk to the product, and to introduce prion removal/inactivation step(s) in the manufacturing process, if feasible [3–5]. Unlike viruses, the minimum infectious prion unit does not exist as a single particle. The infectious prion unit is believed to be composed of protein polymers/aggregates, rather than a prion particle. The unusual nature of the prion agent makes it particularly important to

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consider the effect of the prion spike material when evaluating process steps for prion clearance. A rationale for the choice of the spike preparation used for such evaluation studies should be provided [4].

Several prion strains have been used to evaluate manufacturing processes for their ability to remove TSE agents, including hamster scrapie prion protein (PrP^{Sc}, 263K or Sc237), and mouse PrP^{BSE} (301V). In a polyethylene glycol (PEG) fractionation process, hamster PrP^{Sc} and human PrP^{vCJD}, prepared using the same methodology, were reported to behave in a very similar manner [6]. Different prion spike preparations have been used to investigate prion removal, including crude brain homogenate (BH), microsomal fraction (MF), caveolae-like domains (CLDs), and purified PrP^{Sc}. Of these materials, purified PrP^{Sc} was reported to behave differently from the other preparations in an 8% ethanol fractionation step [7]. This result suggests that the methods used to prepare the prion spike material may be a critical factor in prion clearance studies. Furthermore, these reports are useful in providing a rationale for the choice of the prion source and spike preparation used for such evaluation studies [8].

Tateishi et al. reported that sarkosyl influenced the ability of BMM40 filters to remove prions, using BH derived from CJD-infected mice [9]. The presence of sarkosyl was also shown to significantly reduce the capacity of Planova (P)-35N to remove the scrapie agent ME7, while filtration with P-15N resulted in the complete removal of infectivity, to below the limit of detection of the bioassay used, in both the presence and absence of sarkosyl [10]. Van Holten et al. evaluated the capacity of Viresolve 180 membranes (designed for virus removal from proteins of <180 kDa) to remove prions by using BH which was lysolecithin-treated, sonicated, and subsequently passed through a 100 nm filter (SBH), and demonstrated removal of PrP^{Sc} down to the limit of detection of the Western blot assay used. They argued that by using a better defined spike material, where the size of the scrapie particles was limited, the results may be more relevant with respect to the removal of potential TSE infectivity in plasma than previous studies that used a less well-defined BH [11].

Aggregation of the prion protein is a critical parameter when evaluating nanofiltration steps. The actual form of the infectious agent present in plasma in natural infection is not known. In addition, nanofiltration is typically performed late in the downstream processing, after protein purification steps, which may result in removal of larger or aggregated prion forms. Therefore, use of a spike preparation containing large aggregates may result in an over-estimate of the prion removal capacity of a filter. Although the reports described above, and others, have shown excellent prion removal ability for a number of filters, most reports have not described the particle size distribution of the prion protein in the spike preparations used. Therefore, in this study we have investigated the prion removal capacity of P-35N, P-20N and P-15N filters under diverse conditions, considering the particle size distribution of the MF preparations used.

2. Materials and methods

2.1. Preparation of microsomal fraction (MF)

Brains removed from hamsters infected with scrapie strain 263K [12] (originally obtained from the Institute for Animal Health, Edinburgh, UK), were homogenized in phosphate buffered saline (PBS) until homogeneous, to a final concentration of 10% (w/v). The homogenate was clarified by low speed centrifugation, to remove larger cell debris and nuclei, and the supernatant material was then further clarified by centrifugation at 8,000 × *g* for 10 min at 4 °C, before being ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, to concentrate the scrapie fibrils, and small membrane vesicles and fragments. The pelleted material was resuspended in PBS, aliquoted, and stored at −80 °C. This material was designated 263K MF. Prior to use, stocks were thawed at 37 °C, and sonicated 2 × 4 min on ice water (Ultrawave ultrasonic bath model #U100, 130 W 30 kHz, Ultrawave Ltd., Cardiff, UK). Six independent batches of 263K MF were used in this study. These batches are designated 263K MF preparation lots A–F (Tables 1–3). Normal MF, derived from normal (i.e. uninfected) hamster brain material, was also prepared as described above.

Since we were unable to measure the particle size distribution of contaminated materials in our facility, we used normal MF, and investigated changes in the particle size distribution following strong sonication or treatment with detergent. Various concentrations of sarkosyl (*N*-lauroylsarcosine sodium salt, Nacalai Tesque, Inc., Kyoto, Japan), lysolecithin (L-α-lysophosphatidylcholine, Sigma-Aldrich Corp., St. Louis, USA), Triton X-100 (polyethylene glycol mono-*p*-isooctylphenyl ether, Nacalai Tesque, Inc.), TNBP (tri-n-butyl phosphate, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and/or 1% Tween 80 (Nacalai Tesque, Inc.) were added to normal MF. Changes in the particle size distribution were then monitored by dynamic light scattering method using volume-weighted gaussian analysis using a submicrometer particle sizer (NICOMP Type 370, Particle Sizing Systems, Inc., Santa Barbara, USA). To evaluate the effect of strong sonication, normal MF was sonicated using a closed system ultrasonic cell disruptor (Bioruptor UCD-200T, CosmoBio Co. Ltd., Tokyo, Japan) with a resonance chip set in the tube. Sonication was performed for 1 min at 20 kHz, 200 W in a cold water-bath. Ten cycles of sonication were performed, with a 1 min

Table 1
Scrapie infectivity in different 263K MF preparations[a]

	Log_{10} LD_{50}/ml	SE at 95% probability
Non-super-sonicated 263K MF lot C	5.7	0.44
Super-sonicated 263K MF lot C	6.0	0.53
Super-sonicated 263K MF lot D	5.3	0.69
SD-treated, ultracentrifuged, super-sonicated and 220 nm-filtered 263K MF lot C	6.9	0.69

[a] This bioassay study was performed in accordance with GLP regulations.

Table 2
Removal of PrP^{Sc} from PrP^{Sc}-inoculated PBS

	PVDF filter				Planova filter					
	220 nm		100 nm		P-75N (72 ± 2 nm)		P-35N (35 ± 2 nm)		P-15N (15 ± 2 nm)	
Super-sonicated	+	−	+	−	+	−	+	−	+	−
Before filtration	4.2/3.5[a]	3.5/4.2	4.2/3.5	3.5/4.2	4.2/4.2	3.5/4.2	4.2/4.2	3.5/4.2	4.2/4.2	3.5/4.2
Filtered	3.8/3.8	3.1/3.8	3.8/3.1	2.4/3.1	2.4/2.4	<1.0/<1.0	<1.0/<1.0	<1.0/<1.0	<1.0/<1.0	<1.0/<1.0
LRF[b]	0.4/−0.3	0.4/0.4	0.4/0.4	1.1/1.1	1.8/1.8	≥2.5/≥3.2	≥3.2/≥3.2	≥2.5/≥3.2	≥3.2/≥3.2	≥2.5/≥3.2

Data represents total PrP^{Sc} present in samples, expressed as $\log_{10}$ arbitrary units, following Western blot analysis as described for WB1. This study was performed in accordance with GLP regulations.

[a] Two independent batches of 263K MF were used: lot C (left) and lot D (right), respectively.

[b] LRF, log reduction factor = total PrP^{Sc} in input/total PrP^{Sc} in filtrate, expressed as a $\log_{10}$ value.

interval between each sonication treatment. During the treatment cycle, the particle size distribution was monitored. We named this treatment cycle "super-sonication".

Different preparations of 263K MF, treated with various combinations of detergent, ultracentrifugation and/or "super-sonication", were used as the spiking agent in the process evaluation studies, and are described in the relevant methods sections below.

2.2. Detection of PrP^{Sc} by Western blotting (WB)

To determine the relative levels of PrP^{Sc} present in different samples, WB assays were performed. Three slightly different WB methodologies were applied over the course of the studies, all of which are based on detection of the disease-associated, protease-resistant form of the prion protein (PrP^{Sc}), using the monoclonal antibody 3F4 (Signet Laboratories, Inc., Dedham, USA) [13]. WB methods 1 and 2 were developed independently, and use different approaches to calculate the titer of PrP^{Sc}. As these assays were performed as part of GLP studies intended for regulatory submission, the results are presented as reported in these studies.

2.2.1. Method 1 (WB1)

Samples and controls were either tested directly, or first ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, and the pelleted material then resuspended in PBS. Ultracentrifugation was performed to concentrate the PrP^{Sc} present in large volume samples, and to remove soluble proteins or buffer components that might interfere with the WB assay. Samples were digested with proteinase K (Roche Diagnostics, GmbH, Penzberg, Germany) for 60 min at 37 °C. The optimal concentration of proteinase K, to remove any background that could interfere with the detection of PrP^{Sc} and to allow effective recovery of the PrP^{Sc} protein, was previously established for each sample. Digested samples were mixed 1:1 with Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, and 0.01% (w/v) bromophenol blue, BioRad Laboratories Inc., Hercules, USA) containing 5% (v/v) β-mercaptoethanol. After boiling, serial 5-fold dilutions of

Table 3
Removal of PrP^{Sc} from PrP^{Sc}-inoculated plasma preparations[a]

Filter	P-35N (35 ± 2 nm)		P-20N (19 ± 2 nm)		P-15N (15 ± 2 nm)			
Preparation	IVIG	Haptoglobin	IVIG	Haptoglobin	Antithrombin			Thrombin
Spike material	263K sMF[c]	263K sMF[c]	263K sMF[d]	263K dsMF[e]	263K dMF[f]	263K sMF[c]		263K dsMF[e]
MF preparation lot.	C/D	B	E/F	E/F	A/A	B		C/D
Spike ratio	1/100	1/200	1/20	1/200	1/50	1/21		1/20
Detection method[b]	WB1	WB3	WB2	WB2	WB1	WB3	BA	WB1
Before filtration	3.2/2.5	2.4	6.8/6.8	6.7/6.1	3.1/3.1	3.6	+ve	3.7/3.7
Filtered	0.8/0.8	<1.0	4.8/4.3	4.8/4.7	0.0/0.0	<0.8	+ve	<0.2/<0.2
Log reduction factor	2.4/1.7	≥1.4	2.0/2.5	1.9/1.4	≥3.1/≥3.1	≥2.8	NA	≥3.5/≥3.5

Abbreviations used: 263K MF, microsomal fraction derived from hamster adapted scrapie strain 263K; IVIG, intravenous immunoglobulin; 263K sMF, "super-sonicated" 263K MF; WB, Western blotting; 263K dsMF, detergent treated and "super-sonicated" 263K MF; 263K dMF, detergent treated 263K MF; BA, bioassay; +ve, scrapie positive.

[a] Scaled down conditions were designed according to current guidelines. However, in a study using P-35N filter and haptoglobin, clogging of the filter occurred, and the filtration was subsequently terminated.

[b] WB1, WB2, and WB3 mean Western blotting methods 1, 2 and 3, respectively. The studies involving the use of WB1 and WB2 were performed in accordance with GLP regulations; the studies involving the use of WB3 and the qualitative BA shown in this table, were performed as non-GLP studies.

[c] 263K MF was "super-sonicated" then 220 nm-filtered prior to spiking.

[d] 263K MF was ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in buffer equivalent to the starting material without protein, "super-sonicated", and 220 nm-filtered prior to spiking.

[e] 263K MF was "SD-treated", ultracentrifuged at 141,000 × *g* for 60 min at 4 °C, resuspended in the starting material (thrombin) or saline (haptoglobin), and "super-sonicated". These materials were 220 nm-filtered prior to spiking.

[f] 263K MF was treated with 0.1% sarkosyl for 30 min at room temperature.

the sample were then prepared and subjected to electrophoresis using 12% (w/v) SDS-polyacrylamide gels. Proteins were transferred from the gels to 0.45 μm PVDF membranes (Immobilon-P, Millipore Corp., Billerica, USA), and non-specific binding sites on the membranes were then blocked by overnight incubation in buffer containing dried milk and Tween 20. The blocked membranes were incubated with monoclonal antibody 3F4, washed extensively, and then incubated with a secondary horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Sigma-Aldrich Corp.). After further extensive washing, bound antibody was detected using an ECL-Plus detection system (GE Healthcare UK Ltd, Buckinghamshire, UK) and exposure to blue-light sensitive film.

The level of PrP^{Sc} present in each sample was calculated based on the end-point dilution after analysis by WB. The end-point dilution for each titration was taken as the first dilution at which the 28 kDa PrP^{Sc} protein could not be detected. The reciprocal of this dilution was then taken as the titer of the agent, and expressed in arbitrary units/ml.

2.2.2. Method 2 (WB2)

WB was performed essentially as described by Lee et al. [14]. Briefly, samples were digested with proteinase K at approximately 6 U/ml for 60 min at 37 °C and centrifuged at approximately 20,000 × *g* for 60 min at 4 °C. The pellet was then resuspended and denatured in a 1:1 mix of supernatant and sample buffer (62.5 mM Tris—HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 0.0025% (w/v) bromophenol blue, Invitrogen Corp. Carlsbad, USA), by heating at approximately 100 °C. Serial 3.2-fold (0.5 $\log_{10}$) dilutions of the sample were prepared, and loaded onto 12% (w/v) SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Invitrogen Corp.), and the membranes blocked using buffer containing dried milk and Tween 20 for 1—2 h at room temperature. The blocked membranes were then incubated with monoclonal antibody 3F4, washed extensively, and incubated with a secondary alkaline phosphatase (AP)-conjugated anti-mouse antibody (Cambridge Biosciences Ltd., Cambridge, UK). After further extensive washing, bound antibody was detected using a CDP Star/Nitroblock II detection system (Applied Biosciences, Bedford, USA) and exposure to blue-light sensitive film.

The titer of PrP^{Sc} present in each sample was calculated slightly differently from WB1 and WB3. The end-point dilution for each titration was taken as the last dilution at which the 28 kDa PrP^{Sc} protein could be detected. The reciprocal of this dilution was then taken as the amount of agent in the sample volume tested, and was adjusted for the volume tested and any concentration factors, to give a titer/ml for the original process sample.

2.2.3. Method 3 (WB3)

Samples were ultracentrifuged twice at 150,000 × *g* for 1 h. The samples in the precipitates were then resuspended in PBS at 1/1 or 1/10th volume of the original. Resuspended samples were treated with proteinase K at a final concentration of 10—100 μg/ml. After incubation at 37 °C for 60 min, samples were treated with 10 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride (AEBSF) at room temperature for 10 min, then mixed with 5× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (300 mM Tris—HCl, 12% (w/v) SDS, 25% (v/v) glycerol, and 0.025% (w/v) bromophenol blue, pH 6.8, with 25% (v/v) β-mercaptoethanol) and heated at 100 °C for 5 min. Samples were serially 5-fold diluted with 1× PAGE dilution buffer (60 mM Tris—HCl, 2.4% (w/v) SDS, 5% (v/v) glycerol, and 0.005% (w/v) bromophenol blue, pH 6.8). SDS-PAGE was performed at 30 mA per gel for approximately 42 min. The proteins in the gel were transferred to 0.45 μm PVDF membranes. After treating with blocking buffer (5.0% (w/v) skimmed milk in PBS, 0.05% (v/v) Tween 20), the membrane was incubated with monoclonal antibody 3F4 at 4 °C overnight, then incubated with HRP-conjugated sheep anti-mouse IgG (Sigma-Aldrich Corp.). Bound antibody was visualized by chemiluminescence (ECL-Plus) on X-ray film. The titer of PrP^{Sc} present in the samples was calculated as described for method 1 in Section 2.2.1.

2.3. Evaluation of PrP^{Sc} removal by filtration

A 10% (v/v) concentration of "super-sonicated" 263K MF was prepared in PBS, and 10 ml aliquots were then filtered through a 220 nm or a 100 nm 4 cm^2 PVDF filter (Millex-GV or -VV, Millipore Corp.). In addition, 25 ml aliquots of "super-sonicated" 263K MF in PBS were filtered through a 0.01 m^2 P-75N (72 ± 2 nm), P-35N (35 ± 2 nm), or P-15N (15 ± 2 nm) filter (Asahi Kasei Medical Co., Ltd. Tokyo, Japan). Two independent batches of 263K MF were used. WB1 analysis of samples before and after filtration was performed to determine the removal of PrP^{Sc} under the different conditions. Non-sonicated 263K MF (from the same batch of 263K MF) was also filtered as a control.

2.4. Hamster bioassay to determine the infectious titer of 263K scrapie stocks

Three- to four-week-old female specific pathogen-free (SPF) Syrian hamsters were used in these experiments. Serial 10-fold dilutions of each sample or positive control were prepared in PBS. Six hamsters per sample dilution were inoculated intra-cerebrally with 0.02 ml per animal. The inoculated animals were monitored daily for general health, and weekly for clinical evidence of scrapie. Animals were euthanized once advanced signs of scrapie were evident, or at the end of the assay period (200 days). The brain was removed from each hamster following euthanasia: one half was fixed for histopathology and the other half was stored frozen at −70 °C for further analysis if required. For histopathological analysis, sections taken at four standard coronal levels, to cover the nine areas of the brain which are recognized to be mostly infected by the scrapie agent, were stained with hematoxylin and eosin, and scored for the presence or absence of scrapie lesions [15]. Histopathological analysis was performed on samples from around the clinical end-point of the titration assays, to confirm the clinical results. Hamsters that died during the