Harnessing Prions as Test Agents for the Development of Broad-Range Disinfectants

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Abbreviations and acronyms:
Aβ, amyloid-β; AD, Alzheimer’s disease; APP, amyloid precursor protein; CJD, Creutzfeldt-Jakob disease; PMCA, protein misfolding cyclic amplification; PrP, prion protein; PrP\textsubscript{C}, cellular isoform of the prion protein; PrPres, protease-resistant form of misfolded prion protein; PrP\textsuperscript{TSE}, pathological isoform of the prion protein; qPMCA, quantitative protein misfolding cyclic amplification; sCJD, sporadic Creutzfeldt-Jakob disease; TSE, transmissible spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease; 263K, hamster-adapted scrapie agent
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Abstract

The development of disinfectants with broad-range efficacy against bacteria, viruses, fungi, protozoa and prions constitutes an ongoing challenge. Prions, the causative agents of transmissible spongiform encephalopathies (TSEs) such as Creutzfeldt-Jakob disease (CJD) or its variant (vCJD) rank among the pathogens with the highest resistance to disinfection. Pilot studies have shown that procedures devised for prion disinfection were also highly effective against microbial pathogens. This fueled the idea to systematically exploit prions as test pathogens for the identification of new potential broad-range disinfectants. Prions essentially consist of misfolded, aggregated prion protein (PrP) and putatively replicate by nucleation-dependent, or seeded PrP polymerization. Recently, we have been able to establish PrP seeding activity as a quantitative in vitro indicator for the disinfection of 263K scrapie prions on steel wires used as surrogates for medical instruments. The seeding activity on wires re-processed in different disinfectants could be i) biochemically determined by quantitative protein misfolding cyclic amplification (qPMCA), ii) biologically detected after qPMCA in a cell assay, and iii) correctly translated into residual titres of scrapie infectivity. Our approach will substantially facilitate the identification of disinfectants with efficacy against prions as promising candidates for a further microbiological validation of broad-range activity.
Introduction

The need for pathogen-free surgical instruments and medical devices

Modern medicine depends on clean, pathogen-free materials and instruments. Therefore, safe disinfection is of utmost importance in the maintenance of re-usable surgical instruments and requires ongoing improvement in order to keep up with the challenge of newly emerging pathogens and increasingly complex medical devices. An ideal disinfectant would be effective against all classes of pathogens, suited for routine application, material-friendly, compatible with heat-sensitive devices, and free of effects that fix proteins or other organic material. However, some microbial pathogens (e.g. bacterial spores, protozoal oocysts, mycobacteria, non-enveloped viruses or fungal spores) can be highly tolerant to disinfection. The same holds true for prions, the causative agents of transmissible spongiform encephalopathies such as ovine scrapie, bovine spongiform encephalopathy (BSE), or Creutzfeldt-Jakob disease and its variant in humans.

Prions represent a biological principle of infection that substantially differs from that of bacteria, viruses, fungi or protozoa. Prions are essentially composed of a pathologically misfolded and aggregated isoform of the host-encoded prion protein referred to as PrP\textsuperscript{Sc} or PrP\textsubscript{TSE}.\textsuperscript{4} The replication of prions is thought to occur via a mechanism of nucleation-dependant, or seeded PrP polymerization. In this process PrP\textsubscript{TSE} oligomers or -polymers act as templates that recruit cellular prion protein (PrP\textsuperscript{C}) and integrate it into their aggregate structure. When PrP\textsubscript{TSE} particles break up into smaller units PrP nuclei with proteinaceous seeding activity are multiplied which causes further autocatalytic replication of the pathological protein state. Being devoid of coding nucleic acids and characterized by an amyloid-like molecular structure prions rank amongst the most resistant pathogens in hierarchical scales of resistance to disinfection.\textsuperscript{1}

Epidemiological evidence suggests that transmission of human TSEs through contaminated medical instruments or devices can occur in invasive diagnostic or surgical procedures, and that such risk, while so far being only “hypothetical” for vCJD, is “real”
(i.e. effectively relevant) with respect to sporadic CJD (sCJD).\textsuperscript{7-14} Hence, safe re-processing of medical instruments and devices is mandatory not only for the prevention of nosocomial infections caused by microbial pathogens but also for the prevention of secondary transmissions of human prion diseases in hospitals and other medical settings.

**Towards integrated research on prion and microbial disinfection**

The development of disinfectants with simultaneous efficacy against bacteria and fungi (including spores), viruses, protozoa and prions constitutes a challenging task. This task has been additionally complicated by the fact that experimental research on classical microbial and prion disinfection was not tightly integrated for many years. So far, there is only a relatively small number of studies that have attempted to bridge this gap. In two reports, published in 2009\textsuperscript{15} and 2010\textsuperscript{16} different formulations that had been originally established for prion disinfection were shown, partly after targeted optimization, to be highly effective against bacteria, viruses and fungi as well. These findings demonstrated that in principle the disinfection of microbial pathogens and prions can be smoothly combined. They also provided a proof-of-concept that prions are not only a challenge but potentially also an informative paradigm for the broad-range disinfection of surgical instruments and medical devices. This fueled the idea to systematically exploit prions in future studies as model pathogens for the development of novel broad-range disinfectants. It has to be noted, however, that efficacy against prions does not necessarily imply that a disinfectant is also effective against other pathogens.\textsuperscript{1} Therefore, any claim for broad-range efficacy of formulations or processes with prioncidal activity needs to be carefully validated in appropriate microbiological test systems.

Bioassays in animals provide the gold standard for the detection of prions and their inactivation.\textsuperscript{17,18} However, since they are time consuming, expensive, tightly restricted in throughput and potentially critical in both ethical and regulatory respect they do not provide a real option for the extensive screening of large numbers of disinfection samples. Biochemical testing for the removal, destabilization or degradation of pathological prion protein PrP$^{\text{TSE}}$, the
molecular surrogate marker for prion infectivity, has been performed as an alternative in disinfection studies using steel wires as surrogates for medical instruments and devices, but this detection method is less reliable and sensitive than animal bioassays. Only during the past few years the situation has profoundly changed. The infectivity of certain prion strains can now be titrated by quantitative cell-based assays in solution or on solid test surfaces, and PrP^TSE-associated seeding activity converting normal protease-sensitive PrP^C into pathological and usually Proteinase K-resistant prion protein (PrPres) has become amenable to in vitro monitoring by protein misfolding cyclic amplification (PMCA). Recent technical advancements of the PMCA technology, called quantitative PMCA (qPMCA) and real-time quaking induced conversion assay (RT-QuIC), showed that the measurement of prion titres and PrP^TSE seeding activity, respectively, are biochemically feasible in vitro with high sensitivity and accuracy.

Since 2006 PMCA has been increasingly used for probing prion disinfection in vitro, initially in a non-quantitative manner and by now also in quantitative approaches. Prion replication by nucleation-dependent PrP polymerization implicates the seeding activity of PrP^TSE as a key biochemical counterpart of biological prion infectivity. Based on this concept we examined in the recent study by Pritzkow et al. whether prion disinfection could be assessed without animal bioassays by biochemical and biological in vitro monitoring of PrP seeding activity. For this purpose we pursued a three-stage approach: The residual seeding activities on prion-contaminated steel wires that had been exposed to different disinfectants were at first biochemically determined by quantitative PMCA and translated into estimates of scrapie infectivity, and afterwards biologically detected in a non-quantitative cell assay by inoculation of glial cultures with qPMCA products derived from the test wires. Finally, the scrapie titre estimates from the qPMCA assay and the findings from the cell assay were compared to and validated by actual infectivity data from animal bioassays. The design, results and implications of this study which aimed at establishing a sensitive and practical screening test for prion disinfection are reviewed in the following sections of this article.
**Probing prion disinfection in vitro**

**PMCA-based seeding activity assay**

As a prerequisite for our study we first established a protocol for serial PMCA\(^{29,35,36}\) that allowed the quantitative detection of the seeding activity of 263K scrapie-associated Pr\(^{P}\)TSE in solution and on steel wires. In accordance with findings independently reported by others\(^{37}\) we observed that the presence of glass beads in reaction batches substantially increased the sensitivity and robustness of PMCA. We also noticed that comprehensive precautionary measures aiming at the prevention of cross-contamination were required to warrant PMCA specificity in our hands. These safeguards included sealing and stringent decontamination of reaction tubes, collection of PMCA samples by vial puncture, and addition of the chaotropic prion-inactivating reagent guanidine thiocyanate to the water bath above the ultrasonic transducer.

Next, we applied our protocol for quantitative PMCA to steel wires in a carrier assay for prion disinfection. Residual seeding activities on test steel wires that had been contaminated with 10\(^{-1}\)-diluted 263K scrapie hamster brain homogenate (SBH) and subsequently exposed to different disinfectants were quantitatively determined by comparing the PrPres amplification seeded by test steel wires to the PrPres amplification seeded by reference steel wires after 1, 2, 3 and 4 rounds of qPMCA. Thus, our seeding activity assay was based on an internal calibration. The reference wires were contaminated with 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\), 10\(^{-5}\), 10\(^{-6}\), 10\(^{-7}\) or 10\(^{-8}\)-diluted SBH and carried known amounts of scrapie infectivity which had been determined in a previous study by endpoint titration in hamsters.\(^{38}\) We then used these infectivity titres of the reference steel wires as conversion factors in order to translate the seeding activities detected on test steel wires into estimates of 263K scrapie infectivity.

**Cell-based seeding activity assay**

Prion bioassays in animals essentially rely on the transmission of a TSE infection that eventually becomes evident by the onset of TSE-typical symptoms, cerebral...
neuropathological changes (e. g. vacuolation) and/or accumulation of misfolded prion protein
in the central nervous system. Thus, as to the formation, deposition and accumulation of
PrP\textsuperscript{TSE}, animal bioassays for prions basically detect PrP seeding as a biologically
transmissible principle. In our disinfection study biologically transmissible PrP seeding
activity that remained present on test steel wires after exposure to different disinfectants was
indirectly detected by a surrogate cell assay for 263K scrapie prions. We found that primary
cultures of hamster glial cells displayed both an accumulation of PrPres and amplification of
PrP seeding activity upon exposure to 263K scrapie brain tissue or to PMCA products that
were derived from steel wires contaminated with this prion strain. On this basis we subjected
re-processed test steel wires to qPMCA for the biochemical detection and quantification of
their residual seeding activity, and subsequently inoculated glial cultures with aliquots from
such qPMCA reactions. Our non-quantitative glial assay confirmed the biological
transmissibility of all PrP seeding activities that had been amplified from incompletely
decontaminated wires.

**In vivo validation of seeding activity testing for prion disinfection**

Our *in vitro* assessment of prion disinfection was verified by comparing the estimated
infectivity levels on the steel wires and the findings from the cell assay to actual scrapie titres
that had been determined previously\textsuperscript{16,38} or in our present study in hamster bioassays for
identical, similarly disinfected test carriers. We tested 15 different formulations or procedures
for prion disinfection. While the test limit of hamster bioassays for the reduction of scrapie
titres on test steel wires was found to be about 5.5 log\textsubscript{10} units (logs),\textsuperscript{38} at least 7 logs of
seeding activity reduction could be monitored by qPMCA testing of steel wires. We found that
the residual levels and reduction factors of scrapie infectivity indicated by the detected
seeding activities were always consistent with, and within the detection limit of titrations in
animals virtually identical to the findings from hamster bioassays. Taken together, the
seeding activity of PrP\textsuperscript{TSE} on test steel wires for prion disinfection could be biochemically
titrated by qPMCA, biologically demonstrated by inoculation of glial cultures with qPMCA
products, and correctly translated into titres of prion infectivity.

**Conclusion and outlook**

**Using prions as test pathogens in search of novel broad-range disinfectants**

The validation by cell assay and titration of qPMCA-detected seeding activity were found to indirectly reproduce two key features of animal bioassays in our study, i.e. a qualitative biological detection and quantitative determination of scrapie agent. The findings provided a proof-of-principle that cell assay-coupled qPMCA can substantially reduce, or possibly even replace, animal bioassays in screening studies of prion disinfection. This opens an avenue for the more extensive use of prions as potentially highly informative test pathogens when searching for novel broad-range disinfectants suitable for the routine maintenance of surgical instruments and medical devices.

The decontamination of surgical instruments and medical devices usually comprises cleaning, disinfection and sterilisation. While cleaning refers to the reduction or removal of the mass of contaminating material from an instrument, disinfection and sterilisation reduce the biological infectivity of any pathogens remaining on an instrument after cleaning. We suggest a simple test schedule for the rapid *in vitro* screening of the efficacy of candidate formulations against prion contaminations on instrument surrogates: Firstly, in order to specify the cleaning efficacy with respect to the prion protein, the reduction of the total load of PrP (i.e PrP<sup>C</sup> and PrP<sup>TSE</sup>) on test wires will be tested by SDS-PAGE and Western blotting as described previously.<sup>20</sup> Then, cell assay-coupled qPMCA will be used for gauging disinfection by an indirect quantitative *in vitro* assessment and biological detection of the prion infectivity that remains present on test carriers after re-processing. When the cleaning/disinfection solutions in which test wires had been incubated for decontamination are included in these analyses this will further elucidate the relative contributions of cleaning and inactivation to the reduction of the load of prion contamination. Formulations found to be
sufficiently effective against prions in the screening procedures for cleaning and disinfection will be subsequently subjected to microbiological *in vitro* assays that test the efficacy against bacteria, viruses, fungi,\textsuperscript{15,16} and ideally also against protozoa. It has to be pointed out that our *in vitro* method for the screening of prion disinfectants intrinsically cannot reproduce all features of prion bioassays in animals. Therefore, formulations which show efficacy against prions and microbial pathogens in the preceding *in vitro* tests need to be finally validated for the reduction of prion infectivity by bioassays in animals.

Several different studies have shown that the susceptibility to individual disinfection methods may vary between different prion strains.\textsuperscript{39-42} In the light of these findings it has been recommended that any prion inactivation procedures should be validated by bioassay against the prion strain for which they are intended to be used.\textsuperscript{41} This would require comprehensive experiments in animals, but there are no animal models commonly available that would allow the sensitive titration of the agents of vCJD and the different forms of sCJD over a broad range of infectivity. In order to further promote the development of novel highly-effective disinfectants it might therefore be helpful to adapt PMCA- and cell culture protocols to the propagation and detection of PrP seeding activities associated with sCJD or vCJD. Furthermore, a pragmatic approach may focus on the most relevant human TSEs such as sCJD/subtypes MM1 and VV2\textsuperscript{43} or vCJD when validating candidate formulations for broad-range disinfection for their efficacy against human prions.

**Transmissible protein misfolding in prion diseases and beyond**

The proteinaceous seeding activity of PrP\textsuperscript{TSE} transmits protein misfolding to cellular PrP in a cell-free manner in PMCA. This seeding activity seems to be also the driving force underlying the transmission of PrP misfolding at different levels of biological host organisms, i. e. from molecule-to-molecule, between cells, and in tissues.\textsuperscript{44} Because of their ability to transmit disease-causing PrP misfolding even between individuals, prions are genuinely infectious agents. According to a wealth of data nucleation-dependent, or seeded polymerization governs the misfolding and aggregation of proteins also in other amyloidoses
such as Alzheimer’s Disease (AD), type 2 diabetes or amyloid A amyloidosis. Yet, apart from TSEs, amyloid diseases are generally not considered as being infectious.

The infectiousness of a human disease can be experimentally assessed in animal species that are closely related to humans. When inocula from more than 100 cases of AD were tested at the National Institutes of Health (USA) in primates for their ability to transmit disease this produced negative results with animals surviving an average of about 9 years (range: 1-24 years) after inoculation. However, other researchers claimed to have found evidence for an induction of amyloid-β- (Aβ-) amyloidosis in primates by intracerebral injection of AD brain homogenate and concluded that “beta (A4)-amyloidosis is a transmissible process comparable to the transmissibility of spongiform encephalopathy”. In fact, inducible protein misfolding diseases such as amyloid A amyloidosis or apolipoprotein A II amyloidosis show remarkable similarities to transmissible prion diseases, and an increasing number of publications recently reported the “molecular transmissibility” or “inducibility” of amyloidosis by intracerebral inoculation of proteins such as Aβ into susceptible laboratory animals. Notably, also the induction of cerebral Aβ-amyloidosis through Aβ-contaminated steel wires or peripherally applied Aβ-rich extracts could be demonstrated in transgenic mice that expressed a mutant form of the human amyloid precursor protein (APP). These findings have been interpreted by some experts as indications for an infectious transmissibility of Alzheimer disease (AD), but they may rather reflect an acceleration of disease in genetically-altered predisposed hosts than a genuine infection. The caveats to AD “transmission” were expressed also at various prion conferences, for example by TSE expert Paul W. Brown who has repeatedly pointed to the uniformly negative results for AD transmission in the comprehensive National Institutes of Health primate study.

Most recently Morales et al. described the induction by intracerebral injection of AD brain extracts of Aβ-pathology in transgenic mice expressing human wild-type APP. Since such mice do not naturally develop amyloid deposits during their lifespan the reported findings of this study seem to suggest that Aβ-deposition in the mouse brains had been caused by a
prion-like transmission and propagation of protein misfolding. At the end of their report the authors concluded: “It remains to be studied whether at least a proportion of AD cases could be initiated through a transmissible prion-like mechanism under natural conditions in humans”. Such possibility is still not proven. However, if proteinaceous seeding activity actually emerged as a naturally transmissible principle of non-PrP amyloids this would have far-reaching fundamental and practical implications not least in the field of disinfection. Again, much could possibly be learned from prions.

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