Review

A Broad Scope of Prion Biochemical Assays and its Reproducibility, Utility and Applications

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Abstract

An increasing number of neurological diseases have been linked to prion-like processes. Prions are made of a normal cellular protein that has undergone a pathological alteration that allows it to replicate through a template-assisted mechanism. The prion protein, or PrP, provided the first example of this process in which the normally alpha helical PrP undergoes a conformational change becoming beta sheeted that promotes its aggregation. The fact that the converted protein can serve as a template allows it to function as an infectious agent. Aggregation and transmission between cells has also been observed with other neurodegeneration associated proteins such as alpha synuclein in Parkinson’s disease and Aß in Alzheimer disease. The basis for new and sensitive tests for prion diseases is the ability of abnormal TSE-associated forms of prion protein to seed the formation of amyloid fibrils from recombinant PrP(Sc). In this review we will summarize the improvements in prion biochemical assays including Standard Quaking-Induced Conversion (S-QuIC), Enhanced QuIC (eQuIC), Real-Time QuIC (RT-QuIC), rPrP-PMCA (rPMCA) and the Amyloid Seeding Assay (ASA). We also contrast the molecular pathology of prion diseases with other neurodegenerative diseases. Finally, we will discuss the possibility of implementing some of these tests for other neurodegenerative diseases, such as Alzheimer disease, and how these tests might be adapted for the diagnosis of Alzheimer disease.

Introduction

A number of human neurodegenerative diseases are associated with the misfolding and aggregation of specific proteins. The archetypal example is provided by prion diseases, also known as transmissible spongiform encephalopathies (TSEs). Prion diseases are unique in that they occur in transmissible, sporadic and inherited forms. In the infectious disease, the misfolded proteins (“prions”) are believed to be the infectious agent having the ability to replicate by a template-assisted mechanism converting the normal host-encoded prion protein to the pathogenic conformation.

The first prion disease described was scrapie in sheep and goats in 1732 [1]. An inoculation study performed in 1935 demonstrated the transmissibility of scrapie between animals. The first transmissible human prion disease, Kuru, was discovered in 1957 in the Fore natives of Papua, New Guinea. Kuru was proven to be transmissible in 1965 when D. Carleton Gajdusek and colleagues transmitted Kuru to a chimpanzee [2]. At that time Kuru was thought to be caused by a slow, unconventional virus based on its long incubation period. The causative agent was believed to be a virus until 1982 when Stanley B. Prusiner described a small proteinaceous infectious particle (prion) associated with TSEs, which was resistant to inactivation by most procedures that modify nucleic acids [4]. The failure to detect a polynucleotide associated with the infectious prion particle created a new concept in biology, the prion hypothesis. The prion hypothesis postulates that the disease-causing isoform propagates itself using the normal isoform of the same protein as a substrate [4].

The prion theory has recently been applied to other neurodegenerative diseases that are associated with misfolded proteins; two examples are Alzheimer disease and Parkinson’s disease. Despite the considerable advances in our understanding of prion biology, the underlying pathological mechanism and whether there are other cellular cofactors is still unknown. The list of human prion diseases now includes kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), sporadic and familial fatal insomnia, and variable protease-sensitive prionopathy (VPSPr).

Though the normal cellular isoform of the wild-type (wt) prion protein, PrPc, and the disease-causing isoform, PrPSc, share a common sequential pattern of posttranslational modification, but they are structurally distinct. This was established using a variety of biochemical and mass spectroscopic techniques. The conformational structure and biochemical differences between PrPc and PrPSc [5] are shown in table 1:

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The structural changes are accompanied by alterations in the biochemical properties. In contrast to PrP<sup>c</sup>, PrP<sup>Sc</sup> is:

1. Insoluble in non-denaturing detergents,
2. Partially resistant to protease resulting in the formation of a protease resistant core called PrP<sub>27–30</sub> [6,7] although it is becoming increasingly clear that prion-infected tissues contain also insoluble but protease-sensitive isoforms of PrP which involved in prion diseases [8,9].
3. Clustered into fibrils of prion rods (that resemble purified amyloid) under the combined action of proteases and detergents [10].

The biochemical properties of prions serve as the basis for several relatively rapid and sensitive diagnostic assays for prion diseases. Some tests depend on the protease resistance of PrP<sup>Sc</sup> to proteolytic digestion, such as proteinase K, while other tests are based on the polymerization of PrP<sup>Sc</sup> into fibrils following extraction in non-ionic detergents [11]. However, in most cases, the ability to measure prion infectivity using a biological assay is limited by the low amount of infectious agent.

The test used to quantify the infectious prions in a sample is the animal bioassay or incubation time interval bioassay, [12] which detects prion infectivity titer based on the incubation period. The incubation period is the time between infection and clinical disease onset. Quantification of infectivity can therefore be determined by end-point dilution when half of the animals for a given dilution onset. Quantification of infectivity can therefore be determined by end-point dilution when half of the animals for a given dilution

### Table 1: Structural Difference between PrP<sup>c</sup> and PrP<sup>Sc</sup>

| Property                          | PrP<sup>c</sup> | PrP<sup>Sc</sup> |
|-----------------------------------|-----------------|-----------------|
| Disulfide bridge                  | Yes             | Yes             |
| Molecular mass after deglycosylation | 16 kDa [rPrP(90–231)] | 16 kDa (PrP 27–30) |
| Glycosylation                     | 2 N-linked sugars | 2 N-linked sugars |
| Glycoforms                        | Multiple         | Multiple         |
| Secondary structure               | Dominated by α-helices | Rich in β-structure |
| Sedimentation rate                | Consistent with monomeric species | Multimeric aggregated species |
| Accessible epitopes               | 109–112         | 225–231         |

PrP<sup>Sc</sup> accumulation can be detected in the cells. Consequently, the development of a highly sensitive, specific, and rapid biochemical test for early diagnosis of transmissible spongiform encephalopathy became essential. Additionally, since there are many unanswered questions about prion biology, such a tool might also aid in understanding the intrinsic mechanism by which prion replication occurs and whether other factors are involved in this extraordinary phenomenon.

### Protein Misfolding Cyclical Amplification (PMCA)

The first cell-free model of the PrP<sup>c</sup> to PrP<sup>Sc</sup> conversion assay was developed in 1994. Protease-sensitive PrP<sup>Sc</sup> was metabolically labeled with 35S and isolated from cultured cells; the PrP<sup>Sc</sup> on the other hand, is pretreated with 3M-guanidinehydrochloride (GndCL) before incubation with the labeled PrP<sup>c</sup>. After incubation for 48 hours with the PrP<sup>c</sup>, proteinase K (PK) was added to eliminate the full length 35S PrP<sup>c</sup>. Following this, the 35S PrP<sup>c</sup> that had been converted to PrP<sup>Sc</sup> and was resistant to PK digestion was analyzed by SDS-PAGE and autoradiography [16]. PK resistant 35S bands were only observed when PrP<sup>c</sup> was added to the incubation and the amount was greatly reduced when the PrP<sup>Sc</sup> was pretreated with 6M GndCL indicating that the conversion to the PK resistant form requires the presence of some native PrP<sup>Sc</sup>. Using the in vitro prion protein conversion assay, various studies examined the relationship between PrP<sup>Sc</sup> and infectivity; that is, the mechanism of the conversion reaction and the factors that could inhibit or stimulate conversion. Using the in vitro prion protein conversion assay Bessen and colleagues [17] showed that 2 different strains of hamster TE6 can produce 2 distinct sets of PrP<sup>c</sup> from the same PrP<sup>c</sup> proving that the conversion is strain specific. Further, different strains have distinct packing arrangements and the conformation of the newly formed PrP<sup>c</sup> polymer is determined by the pre-existing PrP<sup>c</sup> seed.

To conclude, the in vitro prion conversion was selective and required the presence of pre-existing PrP<sup>c</sup> that acts as a template to direct the formation of new prions. However, the sensitivity of the prion protein detection using the cell-free conversion method is limited by the relatively low efficiency of PrP<sup>Sc</sup> formation. Thus, an improved technique was developed for the in vitro amplification of PrP<sup>c</sup> called protein misfolding cyclical amplification, PMCA. PMCA is conceptually analogous to the polymerase chain reaction used to amplify DNA [18].
The Saborio group developed a technique using PrP(SC) that was extracted from healthy Syrian hamster brain homogenates and PrP(SC) extracted from infected Syrian hamster brain homogenates followed by acryllic process alternating incubation and sonication. The first incubation step induces formation of PrP(PSc) polymers using a large amount of normal PrP(PrP(C)) and minute amounts of PrP(SC). In the second phase the PrP(SC) polymers are dispersed into smaller fragments by sonication, generating new catalytic units to initiate (or “seed”) the continued formation of new PrP(SC) molecules. In this way, at the end of each successful cycle the number of “seeds” is increased in an exponential fashion, and thus the propagation of prions is accelerated. The quantity of PrP(SC) aggregates formed in a particular sample depends on the number of amplification cycles performed. To increase PMCA efficiency and its application, an automated programmable plate sonicator was developed [19] in which the tubes were positioned on an adaptor placed on the plate holder of a microsonicator and programmed to perform cycles of 30 min of incubation at 37 °C followed by a 40-s pulse of sonication set at 60% power. In early studies [20] amplification was modest (up to 60-fold), but using an automated programmable plate sonicator enabled amplification of several million fold, vastly improving sensitivity over standard detection methods [21].

Serial automated PMCA (saPMCA) has been applied successfully to replicate the misfolded proteins in variety of brain samples from experimental and natural TSEs of various animals [22]. saPMCA not only enables detection of PrP(SC) in blood samples of scrapie-affected hamsters, which contain extremely low amounts of PrP(SC), but also amplified undetectable quantities of PrP(Sc) from the very early stages of infection in presymptomatic hamsters and cattle brain tissue to an extent that was easily detected by Western blot. Even though PMCA technology has been adapted to amplify prions from a variety of species, the application of PMCA to humans is limited by the availability of normal human brain tissue to use as a substrate for PMCA [23].

PrP(Sc) generated by in vitro PMCA is indistinguishable from brain-derived PrP(SC) in terms of its biochemical and structural properties, and more importantly, it is associated with infectivity [24], producing a disease with characteristics identical to those produced by brain-derived prions. Thus, serial PMCA is able to generate in vitro a conversion product that closely mimics the PrP(SC)-generated auto catalytically in vivo. Taken together, these results clearly indicate that PMCA is a valuable tool for studying many aspects of prion biology.

**Applications of Protein Misfolding Cyclic Amplification**

The PMCA technique has numerous successful and reproducible applications in research and diagnostic aspects of prion disease. A particularly valuable application is the development of highly sensitive biochemical detection of PrP(SC), which constitutes the best biological marker for TSE diagnosis. We know that detection of a low amount of PrP(SC) in tissues other than brain is a challenge for early disease diagnosis. Serial PMCA is capable of detecting as little as a single molecule of oligomeric, infectious PrP(SC), generating millions of infectious units that enhance the sensitivity several billion times over the standard assays that are used to detect prions [19]. Accordingly, serial PMCA has been successfully applied to the detection of PrP(SC) in blood samples of both symptomatic scrapie-affected hamsters [21] and pre-symptomatic scrapie-affected hamsters [25]. However, direct amplification of blood derived prion protein associated with transmissible spongiform encephalopathies (PrPTSE) by PMCA has proven difficult, mostly due to the exhaustion of the substrate PrP(C) or other factors that may be necessary for amplification [18]. To overcome this, recombinant bacterial PrP(C) is used as a source of PrP(C) instead of brain homogenate [26], improving the speed and practicality of the assay. Another study showed that addition of cofactors such as synthetic polyadenylc acid RNA molecules, lipids, or metal ions could facilitate the production of infectious prions from recombinant PrP [27,28].

Highly sensitive and specific noninvasive tests that detect prions in samples of body fluids would be a very useful biochemical tool for identifying populations at risk, for reducing iatrogenic transmission of CJD, and in early diagnosis of the disease. Recent studies have demonstrated that PMCA can be used to detect PrP(SC) in blood and urine as well as cerebrospinal fluid samples from patients with various transmissible spongiform encephalopathies [29,30,31]. PMCA has been extended beyond the detection of minute quantities of prions to estimating the concentration of PrP(SC) present in samples [32].

Investigating factors that inhibit or induce prion propagation can also be accomplished using PMCA. A recent study using PMCA [33] reported that unglycosylated, anchorless recombinant full-length human PrP is a strong inhibitor of human prion propagation resulting in blocking the interaction of brain PrP(C) with PrP(SC), indicating that glycosylation and the glycoporphatidylinositols anchor are important in mediating the conversion of PrP(C) into PrP(SC). Moreover, recombinant PrP from other species also inhibits amplification of human prions suggesting that the interference of the conversion reaction at the molecular level is the basis for the species barrier. Even though a detailed understanding of the molecular aspects underlying the species barrier and strain phenomena are currently unclear, PMCA has been applied to compare and predict species barrier effects by combining PrP(SC) and PrP(C) from different sources in distinct quantities and quantitatively evaluating the efficiency of the conversion [34]. Thus, PMCA mimics in vitro the central pathogenic process of the disease process, provides a suitable tool for evaluating the activity of drug candidates for TSE treatment, and could be used to assess the efficacy of those drugs in clinical trials [18].

Despite the progress that PMCA has facilitated in prion research, the fact that the amplification process to detect prions relies on sonication, makes it difficult to control. In addition, PMCA results in a high percentage of false negatives and false positives, and it detects some strains more readily than others [35]. In fact, it was recently shown that the PMCA reaction can spontaneously generate prions in samples without a seed [36]. As a result, there...
was a need to develop an accurate, high throughputs diagnostic that is automated and can be easily used in a routine diagnostic lab.

Establishment of Quaking-Induced Conversion (Quic), RT-Quic And Qrt-Quic

A new practical prion assay, quaking-induced conversion (QuIC) has been developed. QuIC uses recombinant prion protein; rPrP-sen produced in bacteria as a substrate for seeded polymerization and an automated system for shaking tubes, replacing the sonication step, in amplification rounds [37]. Since bacterially expressed rPrP-sen can be produced rapidly in high purity, using the rPrP-QuIC method solves the difficulty of using the brain PrP-sen as the amplification substrate. Moreover, the fact that rPrP-sen can be easily mutated allows investigation into the role of specific sequences or amino acids in the conversion reaction and strategic labeling with probes simplifies and accelerates studies on the detection of prions [26].

For further simplification, the sonication step was replaced by periodic shaking, which enhances the interaction between rPrP-sen and PrPSEN. Shaking also promotes the fragmentation of PrP-res polymers. For comparison, the PMCA reaction successfully detected PrPSEN from a $10^{-5}$–$10^{-6}$ dilution of $10\%$ vCJD brain homogenate (the PrPSEN content was not reported) in 24 hrs whereas the QuIC assay detected $10^{-7}$–$10^{-8}$ dilutions of $10\%$ vCJD BH in 10 hrs [37]. The QuIC assay correctly discriminated between normal and scrapie-infected CSF samples in both hamster and sheep prion disease models. QuIC is a relatively fast and sensitive assay that can be applied as a diagnostic screening method to detect low levels of PrPSEN in biological samples to such as blood, CSF and lymphoid tissues. However, since the detection of specific protease-resistant prion-seeded products by immunoblotting is the final step in the assay, there is a time restriction on the productivity of the technique.

To further improve the utility of QuIC, a new technique called Real Time Quaking-Induced Conversion, RT-QuIC, was developed by modifications that enhanced the diagnostic capacity of QuIC in the ante mortem evaluation of suspected CJD. As in the amyloid seeding assay, ASA [35], polymerization of recPrP into amyloid fibers can be detected by a fluorescence shift in the dye Thio-flavin T (ThT). RT-QuIC combined the QuIC assay and a fluorescent ThT readout to facilitate high throughput applications. The RT-QuIC assay provides rapid and highly sensitive discrimination of prion-infected and uninfected brain tissues, nasal lavages, and CSF [38]. The RT-QuIC assay in a test of cerebrospinal fluid for the diagnosis of sporadic Creutzfeldt-Jakob disease achieved 87% sensitivity and 100% specificity, while in another study testing olfactory epithelium samples obtained from nasal brushings for diagnosing Creutzfeldt-Jakob disease the sensitivity was 97% with a specificity of 100% [39,40]. Thus, RT-QuIC provides a novel, practical method for the ante mortem diagnosis of human and animal prion diseases. Recently, a valuable noninvasive diagnostic tool called quantitative RT-QuIC (qRT-QuIC) was developed for monitoring disease progression and the effectiveness of anti-prion therapeutic approaches in animal studies and human clinical trials of prion diseases [41,42]. Based upon the quantitative correlation between prion seed concentration and the lag time to the start of the conversion reaction, qRT-QuIC allows quantification of prion infectivity in tissues, body fluids, and excreta. Standard curves were generating by using immunoblots to detect PrP 27-30 and correlating that with the seed amount. Then, undetermined amounts of PrP 27-30 seeds in a sample can be directly calculated based on the calibration curve. The quantitative aspect of qRT-QuIC suggests that it can provide a reliable assessment of anti-prion therapy in vivo in order to follow the effects of therapy on progression of prion diseases. Moreover, since qRT-QuIC provides an ultra-sensitive method for quantifying pathological amyloid aggregate seeds, this technique may also be applicable to other disease-associated proteins rich in β-pleated structures that bind ThT and that show seeded aggregation. However, further studies are required on a large number of human urine samples to assess the reducibility and applicability to human patients.

Physical and chemical factors influencing the quality of the RT-QuIC assay and recombinant prion protein (rPrP) substrate stability, such as salt and substrate concentrations, substrate storage, and pH need to be standardized for optimal analytical performance before the assay can be widely adopted [43]. In addition, the RT-QuIC reaction can be inhibited by inhibitor-laden samples such as plasma, whole blood, and tissue-contaminated samples. To overcome this problem, “enhanced QuIC” (eQuIC) was developed. In this assay, a prion/PrPSEN immunoprecipitation (IP) step is combined with the RT-QuIC prion amplification assay [44]. It has been reported that some antibodies are exquisitely selective for aggregated, insoluble forms of PrP, but not soluble PrPSEN, even when present in vast excess suggesting that it recognizes an epitome common to prions from different species [45,46]. Although (15B3) an antibody that recognizes the disease associated form of the prion protein and the motif-grafted antibodies cannot distinguish between infectious and non-infectious aggregates of PrP, the ability to discriminate against soluble PrPSEN makes it a suitable tool for diagnostic, therapeutic, and investigational purposes. Furthermore, the capacity of these antibodies to recognize multiple, aggregated forms of PrP in addition to PrPSEN allows detection of the most pathologically relevant forms of PrP and diagnosis of a much wider variety of prion disorders, possibly at earlier stages when it may lack infectivity and protease-resistance. Adding an immunoprecipitation (IP) step to RT-QuIC, which separates prions from inhibitors, markedly improved the sensitivity and applicability of the assay for the detection of prions in dilute, inhibitor-laden fluids such as blood plasma.

eQuIC differs from other assays that have serial amplification steps as in PMCA, rPrP-PMCA and QuIC by the two-stage substrate addition. The fresh rPrPSEN substrate replacement during the incubation period dramatically improved the assay time and sensitivity, although the mechanistic basis for this is not yet clear. The capability of eQuIC to detect prions in plasma samples raises the possibility that this assay could be used to improve not only prion disease diagnosis in humans and animals, but also to screen the blood supply for prion contamination.
Discussion

In addition to TSEs, several other diseases share prion like propagation resulting in alteration of the normal protein folded in its native conformation into insoluble proteins rich in β-sheet structure that are partially resistant to proteolysis and have an enhanced tendency to aggregate. The newly formed abnormal proteins have toxic properties that contribute to tissue damage and organ dysfunction. This group of diseases includes neurodegenerative diseases such as Alzheimer’s, Parkinson’s, and Huntington’s collectively referred to as protein misfolding disorders [23]. Although the distribution of protein aggregates is characteristic for each disease, the process of propagating misfolded proteins in these diseases follows the same seeding-nucleation mechanism, a mechanism by which infectious proteins can be transmitted. Several studies have suggested that misfolded amyloid beta (Aβ) can be transmitted by a prion-like mechanism following inoculation of brain extracts from patients with Alzheimer’s disease (AD brain tissues) into several animal models [47]. The Aβ deposits were induced in a time- and concentration-dependent manner after inoculation of AD brain tissue [48]. The accumulation of amyloid plaques increased progressively with the time after injection of the AD samples, and the Aβ lesions were observed in brain areas far from the injection site, which, without exposure to this material, would never have developed these alterations [49].

Similar to TSEs, the seeded-nucleated polymerization in Alzheimer’s disease exhibits an initial nucleation lag phase, a slow process in which monomers nucleate to form amyloid fibers. There is no detectable amyloid during the slow phase, but once the solution is nucleated, oligomers grow rapidly by integrating protein monomers into the growing polymer. Therefore, protein aggregation can be accelerated by addition of preformed seeds, fragmented amyloid fibrils, into a solution containing the monomeric protein [35,50].

As described previously in the Amyloid Seeding Assay (ASA), the seeding ability of a sample can be monitored using thioflavin T (ThT), a fluorescent dye, to detect and quantify the presence of amyloid oligomers in a biological sample. Accordingly, the principle of ASA has been applied to AD as a Kinetic Aggregation Assay to quantify seeding Aβ aggregates, which play a central role in AD pathogenesis, present in a given sample [51]. Similarly, PMCA was recently applied to detect misfolded Aβ oligomers in the cerebrospinal fluid (CSF) of AD patients. Aβ-PMCA enables detection of as little as 3 fmol of Aβ oligomers in a given sample. Further, it distinguished AD patient from non-AD patient with high sensitivity and specificity, suggesting that PMCA could be optimized for use as a highly sensitive and specific biochemical test for diagnosis for AD [52]. Since PMCA has the limitation of the sonication step described above, it was replaced in this study by shaking, which enhanced detection of AD due to the fragile nature of Aβ aggregates that makes them sensitive to disruption by sonication. Despite the promising results with the current Aβ-PMCA technology, there is variability between studies that needs to be addressed using a large number of samples to evaluate reproducibility. Further, since this study was performed on cerebrospinal fluid (CSF) samples of AD patients, adaptation of Aβ-PMCA to detect Aβ oligomers in other samples such as blood, urine, and nasal samples of AD patients would be essential for the development of a noninvasive biochemical test for more-routine testing. However, the promising applications of PMCA and ASA to

![Figure 1: Schematic drawing showing the principle of Quaking Induced Conversion (QuIC) in prion-like diseases. Monomers, e.g. Amyloid-β, α-synuclein, SOD, Huntingtin or any other prion like proteins, is converted into an aggregate form by repeated cycles of incubation and vigorous shaking in the presence of an oligomer template.](image-url)
the diagnosis of AD raises the possibility of using RT-QuIC, which is basically an advanced technique combining both tests, as a potential new means for the early, rapid and specific diagnosis of AD. RT-QuIC is safer, faster and more practical than other tests. Thus, its application to AD would lead to a wide-range of opportunities in clinical applications as well as in basic research labs. It could be used to explore the physical and chemical factors influencing AD leading to a better understanding of the molecular basis of the pathogenic process. The fact that RT-QuIC can be easily performed with reproducible results, offers a great opportunity for its use in routine monitoring of AD patients. More importantly, the utility of RT-QuIC in screening can be evaluated by applying it in a longitudinal study of asymptomatic carriers of familial AD for presymptomatic detection of AD. Nevertheless, more work needs to be done to validate the technique for use on AD patient samples and to assess its reproducibility. Then, the chemical and physical factors influencing the reaction need to be evaluated in order to develop a standardized method that can be adopted as a routine diagnostic test. The assay can be further improved by adding quantitative assessment equivalent to qRT-QuIC that would facilitate evaluating the effectiveness of new Alzheimer therapies.

In summary, from the molecular point of view, the prion protein and prion-like proteins share a number of characteristics. Therefore, the principles of well established prion diagnostic tests that have been accepted as efficient valuable biochemical assay might be applied to amplify and study the abnormal folding of these proteins as well.

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