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Protein misfolding cyclic amplification (PMCA): Current status and future directions

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ABSTRACT

Transmissible spongiform encephalopathies (TSEs) most commonly known as prion diseases are invariably fatal neurological disorders that affect humans and animals. These disorders differ from other neurodegenerative conformational diseases caused by the accumulation in the brain of misfolded proteins, sometimes with amyloid properties, in their ability to infect susceptible species by various routes. While the infectious properties of amyloidogenic proteins, other than misfolded prion protein (PrP^{TSE}), are currently under scrutiny, their potential to transmit from cell to cell, one of the intrinsic properties of the prion, has been recently *shown in vitro* and *in vivo*. Over the decades, various cell culture and laboratory animal models have been developed to study TSEs. These assays have been widely used in a variety of applications but showed to be time consuming and entailed elevated costs. Novel economic and fast alternatives became available with the development of *in vitro* assays that are based on the property of conformationally abnormal PrP^{TSE} to recruit normal cellular PrP^{C} to misfold. These include the cell-free conversion assay, protein misfolding cyclic amplification (PMCA) and quaking induced conversion assay (QuIC), of which the PMCA has been the only technology shown to generate infectious prions. Moreover, it allows indefinite amplification of PrP^{TSE} with strain-specific biochemical and biological properties of the original molecules and under certain conditions may give rise to new spontaneously generated prions. The method also allows addressing the species barrier phenomena and assessing possible risks of animal-to-animal and animal-to-human transmission. Additionally, its unprecedented sensitivity has made possible the detection of as little as one infectious dose of PrP^{TSE} and the biochemical identification of this protein in different tissues and biological fluids, including blood, cerebral spinal fluid (CSF), semen, milk, urine and saliva during the pre-clinical and clinical phases of the disease. The mechanistic similarities between TSEs and other conformational disorders have resulted in the adaptation of the PMCA to the amplification and detection of various amyloidogenic proteins. Here we provide a compelling discussion of the different applications of this technology to the study of TSEs and other neurodegenerative diseases.

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Abbreviations: TSEs, transmissible spongiform encephalopathies; PrP^{TSE}, disease associated misfolded PrP; PrP^{C}, cellular prion protein; PrP^{res}, proteinase K resistant misfolded PrP; PrP27–30, proteinase K resistant core of misfolded PrP; rPrP, recombinant PrP; PMCA, protein misfolding cyclic amplification; aPMCA, automated PMCA; saPMCA, serial automated PMCA; PK, proteinase K; Mo-vCJD, mouse-adapted variant Creutzfeldt-Jakob disease; 263K-BH, scrapie 263K-infected hamster brain homogenate; poly(A), polyadenylic acid; poly(rA), polyriboadenylic acid; poly(G), polyglutamic acid; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; CWD, chronic wasting disease; BSE, bovine spongiform encephalopathy; CNS, central nervous system; EV, extracellular vesicles; EVP, extracellular vesicle proteins; SP-SC, murine spleen-derived stromal cell cultures; CPD, citrate-phosphate dextrose; BH, brain homogenate; wt-MoBH, wild type-mouse brain homogenate; D_2O, deuterium oxide; RT, room temperature; PBS, phosphate-buffered saline; FVB, FVB/NCr mouse; CSF, cerebral spinal fluid; PE, phosphatidylethanolamine; α-Syn, alpha-synuclein; AD, Alzheimer's disease; PD, Parkinson's disease; Aβ, amyloid beta; GPI, glycosylphosphatidylinositol; sCJD, sporadic Creutzfeldt-Jakob disease; PrP^{CWD}, CWD-associated misfolded PrP; $rPrP\text{-}res^{poly(rA)}$, PK resistant rPrP generated by saPMCA in the presence of poly(rA); BC, buffy coat; WBC, white blood cells.

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1. Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases characterized by the accumulation in the brain and sometimes in the lymphoid tissues of conformationally misfolded prion protein (PrP^{TSE}) (Doi et al., 1988; Prusiner, 1982), which originates from a conformational transformation of a cellular membrane-bound prion protein (PrP^{C}) (Pan et al., 1993). PrP^{TSE} is considered to be the only (Castilla et al., 2005a; Deleault et al., 2007; Legname et al., 2004; Wang et al., 2010) or the major (Couzin, 2004; Dickinson et al., 1989; Weissmann, 1991) constituent of the infectious agent, the prion, whose misfolding and aggregation follows a nucleation-dependent polymerization mechanism (Jarrett and Lansbury, 1993). TSEs are characterized by a long, clinically silent incubation period that may exceed 50 years in humans (Collinge et al., 2006), followed by a short and aggressive clinical course of several months (Collinge, 2001). Owing to unusual infectious properties, prions have been the cause of two major epidemics in animals: (i) bovine spongiform encephalopathy (BSE) that has mainly affected cattle in the UK and France but did not spare other European countries and countries around the world, and which has been causatively linked to variant Creutzfeldt-Jakob disease that resulted in the death of 227 individuals (http://www.cjd.ed.ac.uk/data.html); (ii) chronic wasting disease (CWD), which spreads with unprecedented speed through cervid populations in North America presenting a new threat to other animal species, including ungulates, cattle and felines, and to humans. Given their transmissible nature, these diseases can be modeled by inoculation of infectious prions into different laboratory animals through various routes (Prusiner et al., 2004). However, the disadvantages of these time demanding and costly experiments lead to the development of several cell-based (Clarke and Haig, 1970; Clarke and Millson, 1976; Giri et al., 2006; Markovits et al., 1983; Milhavet et al., 2006; Raymond et al., 2006; Rubenstein et al., 1984; Taraboulos et al., 1990; Vilette et al., 2001) as well as cell-free *in vitro* assays (Atarashi et al., 2008; Deleault et al., 2003; Kocisko et al., 1994; Saborio et al., 2001).

More than a decade ago, protein misfolding cyclic amplification (PMCA) emerged as a technology designed to achieve sensitive levels of PrP^{TSE} detection by unlimited continuing replication of misfolded aggregates using a mechanism similar to DNA amplification by PCR (Saborio et al., 2001), where a template of PrP^{TSE} aggregates grows at the expense of a substrate (PrP^{C}) in a cyclic reaction (Fig. 1). Conceptually based on the nucleation-dependent polymerization model for prion replication, PMCA combines cycles of sonication that result in hydrodynamic shearing of PrP^{TSE} aggregates into smaller nuclei, and incubation, during which PrP^{TSE} molecules present in the nuclei imprint their abnormal conformation onto PrP^{C}, which is subsequently incorporated into growing PrP^{TSE} aggregates (Saborio et al., 2001). These cycles of sonication–incubation result in the exponential amplification of minute quantities of PrP^{TSE} present in a sample (Saá et al., 2006b; Saborio et al., 2001; Soto et al., 2002).

In this manuscript, we provide an overview of the PMCA technology from its origin and optimization to its widespread application to the study of TSEs and other neurodegenerative diseases caused by the accumulation of misfolded proteins.

2. Evolution of the PMCA technology: from early development to optimization

2.1. Protein misfolding cyclic amplification (manual PMCA)

In vitro conversion of purified radiolabeled PrP^{C} into PrP^{TSE} was first achieved by Caughey and colleagues by means of the cell-free conversion assay (Kocisko et al., 1994) which was later on used to investigate the molecular basis of TSE transmission between species and within species with different PrP genotypes (Bossers et al., 1997; Kocisko et al., 1995). However, the use of non-physiological conditions and the low yields of PrP^{TSE} of this assay limited its further applications. Several years later, the original PMCA system was developed using a Bandelin Sonoplus HD2070 sonicator equipped with a Titanium microtip MS73 (Bandelin Electronic, Berlin, Germany). Proof-of-concept experiments were performed by serially diluting a scrapie 263K-infected hamster brain homogenate (263K-BH), used as source of PrP^{TSE}, into a healthy hamster BH, source of PrP^{C} and other co-factors important for prion replication. Serial dilutions of 263K-BH were performed to mimic samples from various tissues and/or distinct phases of TSE that contain different levels of PrP^{TSE}. Aliquots of these samples were immediately deep-frozen at −80 °C, while the residual samples were subjected to various cycles of incubation and sonication. Thereafter, frozen and amplified samples were digested with proteinase K (PK) and the success of conversion was evaluated by Western blotting. This procedure resulted in a dramatic increase in PrP^{res} levels in amplified samples as compared to equivalent frozen controls where the PrP^{res} signal disappeared after a 3000-fold dilution of the infected brain. In contrast, PrP^{res} could still be detected in a 500,000-fold diluted 263K-BH after PMCA. Densitometric analysis estimated that >99% of PrP^{res} present in amplified samples was PMCA-generated (Saborio et al., 2001).

2.2. Serial automated PMCA (saPMCA)

In its original format, PMCA allowed an increase of sensitivity for PrP^{res} detection between 10- and 60-fold; and represented a technical breakthrough in prion research by providing a new *in vitro* system to investigate important aspects of prion biology such, as the molecular requirements for prion replication (Deleault et al., 2003; Lucassen et al., 2003; Nishina et al., 2004), the strain and species specificity of prion conversion (Lucassen et al., 2003; Soto et al., 2005), and to evaluate replication inhibitory drugs with a potential application in disease therapy (Barret et al., 2003). However, it was inefficient to sustain continuous PrP^{res} replication, thus preventing evaluation of the biological properties of newly generated molecules (Bieschke et al., 2004).

Since the level of PMCA-induced amplification is directly proportional to the number of cycles applied to the sample, the overall efficiency of the assay was limited by the cycles performed during a working day. To overcome this limitation, the system was automated by using a programmable sonicator equipped with a microplate horn (Q700, Qsonica LLC, Newtown, CT) which allowed continuous operation (Castilla et al., 2005a,b; Saá et al., 2005, 2006b). PMCA automation resulted in routine performance of more cycles with lower processing times, higher consistency by eliminating operator-dependent variability, and prevented loss of material and cross-contamination by removing probe intrusion into the sample. Moreover, higher throughput screening of samples was achieved by processing up to 96 samples at a time, making this technology amenable for large-scale biochemical detection (Castilla et al., 2006, 2005b; Saá et al., 2005, 2006a,b).

Amplification of 263K-BH PrP^{TSE} in 140 cycles of the newly automated PMCA (aPMCA) enabled the detection of PrP^{res} in a 6.6 million-fold dilution of the infected brain; which represented a sensitivity increase of 5 orders of magnitude relative to the manual PMCA (Castilla et al., 2005b). During the course of these experiments, it became evident that the conversion efficiency leveled off at approximately 140 cycles (70 h) and started to decrease after 150 cycles of PMCA. Because not all PrP^{C} present in the substrate had been converted into PrP^{res}, the reduced efficiency was likely related to decreasing levels of PrP^{C} or other brain cofactors needed for

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