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Review

Capillary electromigration based techniques in diagnostics of prion protein caused diseases

Transmissible spongiform encephalopathies are a group of fatal neurodegenerative diseases with long incubation time. This group includes Creutzfeld-Jakob disease, kuru, scrapie, chronic wasting disease, and bovine spongiform encephalopathy. Sensitive and specific detection of abnormal prion protein as “a source agent” of the above-mentioned diseases in blood could provide a diagnostic test or a screening assay for animal and human prion protein diseases diagnostics. Therefore, diagnostic tests for prion protein diseases represent unique challenge requiring development of novel assays exploiting properties of prion protein complex. Presently, diagnostic methods such as protein misfolding cyclic amplification, conformation-dependent immunoassay, dissociation-enhanced lanthanide fluorescent immunoassay, fluorescence correlation spectroscopy, and/or flow microbead immunoassay are used for abnormal prion protein (PrP^{Sc}) detection. On the other hand, using of CE for PrP^{Sc} detection in body fluids is an attractive alternative; it has been already applied for the blood samples of infected sheep, elk, chimpanzee, as well as humans. In this review, assays for prion protein detection are summarized with special attention to capillary electromigration based techniques, such as CE, CIEF, and/or CGE. The potential of the miniaturized and integrated lab-on-chip devices is highlighted, emphasizing recent advances of this field in the proteomic analysis.

Keywords:
Alzheimer / CE / Neurodegenerative disease / Prion protein / Transmissible spongiform encephalopathies DOI 10.1002/elps.201200208

1 Introduction

Transmissible spongiform encephalopathies (TSEs) are infectious diseases that cause progressive degenerative disorders of the central nervous system. Sixteen different variants of prion disease have been reported, nine in humans and seven in animals [1–4], listed in Table 1. Since there is a number of neuropathological similarities and genetic links between Alzheimer and prion diseases [5], it is not surprising that the coexistence of Alzheimer disease pathology in Creutzfeld-Jakob disease (CJD) has been reported [6].

According to seeding-nucleation model, the agent that causes these diseases is an abnormal prion protein (PrP^{Sc}) catalyzing the conversion of normal prion protein (PrP^{C}) molecules into PrP^{Sc} [1]. The PrP^{Sc} is a conformational isoform of the PrP^{C}. This conformation change, from the α-helix in PrP^{c} to the β-sheet of the PrP^{Sc}, significantly affects the protein function. Generally, PrP^{C} is a membrane-bound glycoprotein found in the central nervous system of all mammals and avian species. The monomeric form of PrP^{C} has molecular mass of approximately 27 kDa. The protein is tethered to the outside surface of cellular membranes by a glycosylphosphatidylinositol anchor at its C terminus. NMR studies on recombinant human PrP^{C} demonstrated that the C-terminal region adopts a globular fold that is largely helical, but with a small two-strand β-sheet. Similar structures are found for hamster and mouse prion proteins. N-terminal region, up to approximately residue 120, is unstructured and flexible in solution. A hallmark of this region is the so-called octarepeat domain composed of tandem repeats of the fundamental eight-residue sequence PHGGGWGQ. In most species, including humans, four or five repeat segments are found. Interestingly, the octarepeat domain is among the most highly conserved regions of the prion protein. There are currently no high-resolution structures for PrP^{Sc}, but recent electron-crystallography experiments suggest that residues 89–175 refold into the β-helix [7].

At present, there is no evidence that a nucleic acid is involved in structural changes of PrP^{C} into PrP^{Sc}. The PrP encoding gene occurs in normal and in spongiform

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Abbreviations: CJD, Creutzfeld-Jakob disease; **IHC**, immunohistochemistry; **PrP**, prion protein; **PrPC**, normal prion protein; **PrPSc**, abnormal prion protein; **TSE**, Transmissible spongiform encephalopathies; **vCJD**, variant of CJD

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Table 1. The list of prion protein related diseases

	Abbreviation	Host
Animal prion protein disease		
Transmissible mink encephalopathy	TME	Mink
Scrapie	-	Sheep
Chronic wasting disease	CWD	Cervids
Bovine spongiform encephalopathy	BSE	Cattle
Exotic ungulate spongiform encephalopathy	EUE	Nyala, Kudu
Feline spongiform encephalopathy	FSE	Cats
TSE in nonhuman primates	NHP	Lemurs
Human prion protein disease		
Sporadic Creutzfeldt-Jacob Disease	sCJD	Human
Familial Creutzfeldt-Jacob Disease	fCJD	Human
Variant Creutzfeldt-Jacob Disease	vCJD	Human
Iatrogenic Creutzfeldt-Jacob Disease	iCJD	Human
Fatal familial insomnia	FFI	Human
Sporadic fatal insomnia	sFI	Human
Variably protease-sensitive prionopathy	VPSPr	Human
Gerstmann-Sträussler-Scheinker Syndrome	GSS	Human
Kuru	-	Human

encephalopathy affected brain. Missense mutations in the human Prnp gene (prion protein gene) are responsible for inherited prion diseases. In uninfected animals, Prnp encodes a glycophosphatidylinositol-anchored protein denoted PrP^C; in prion infections, PrP^C is converted to PrP^{Sc} by templated refolding. In spite of the fact that Prnp is conserved in mammalian species, attempts to verify interactions of putative PrP-binding proteins by genetic means have proven frustrating: the ZrchI and Npu lines of Prnp-ablated mice (Prnp(0/0) mice) lacking PrP^C remains healthy throughout development. This indicates that PrP^C plays a role that is not apparent in a laboratory setting or that other molecules have overlapping functions. However, the protein undergoes a posttranslational alteration that truncates a host PrP^C at the N-terminus and causes it to be resistant to protease digestion in diseased brain [8]. After this modification, the protein aggregates into rod-shaped fibrils in the brains of infected animals.

The specific function of PrP^C in healthy tissues remains unknown. However, several intriguing lines of evidence have emerged recently suggesting that PrP^C may exert a cytoprotective activity, particularly against internal or environmental stresses that initiate an apoptotic program [9, 10]. Moreover, recent data indicate that PrP^C may play a critical role in the pathogene-sis of Alzheimer disease. A feedback loop has been suggested in the nor-mal brain, where PrP^C exerts an inhibitory effect on β-secretase BACE1 to decrease both amyloid-β and amyloid intracellular domain production. In turn, the amyloid intracellular domain upregulates PrP^C expression, thus maintaining the inhibitory ef-fect of PrP^C on BACE1. In Alzheimer disease, this feedback loop is disrupted and the abundant amyloid-β oligomers bind to PrP^C and prevent it from regulating BACE1 activity [5].

It has been recently clarified that the prion protein binds copper in vivo, and the interaction between PrP^C and copper requires the highly conserved, N-terminal octarepeat domain [7]. It was found that the amino-terminal domain of PrP^C exhibits five to six copper-binding sites (Cu(II)) presented as a glycine chelate. At neutral pH, binding occurs with positive cooperativity, with binding affinity compatible with estimates for extracellular, labile copper. Two lines of independently derived PrP^C gene-ablated (Prnp(0/0)) mice exhibit severe reductions in the copper content of membrane-enriched brain extracts. Similar reductions in synaptosomal and endosome-enriched subcellular fractions have been also observed in this model. Prnp(0/0) mice are also characterized by altered cellular phenotypes, including a reduction in the activity of copper/zinc superoxide dismutase and altered electrophysiological responses in the presence of copper excess. These findings indicate that PrP^C can exist in a Cu-metalloprotein form in vivo [11].

It should not be missed out that the connection between prion proteins and other ions, such as manganese [12], zinc [13], iron [14], and calcium, have been discussed [15]. Aluminum is also associated with neurodegenerative diseases; it promotes a specific beta-amyloid (1–42) aggregation, thus leading to marked toxic effects on neuroblastoma cells [16].

2 Analytical methods for prion protein detection

The diagnosis of infectious diseases is relatively a well-established area. It can be done either by the immune reaction or by the amplification of a nucleic acid that is specific to the prion protein using PCR [17]. However, in TSEs, the infectious agent has the same sequence as a naturally occurring protein and thus no immune reaction is observed. Moreover, there is no nucleic acid to be amplified. Therefore, the diagnostics of prion protein caused diseases represents a sort of challenge [18]. In case of human diseases, diagnosis is based almost exclusively on clinical examination and the disease is then considered as probable depending on the extent to which the clinical symptoms fit the standard guidelines. Currently, PrP^{Sc} is the only disease-specific analyte commercially used for identification of prion diseases [19]. From the clinical point of view, the most sensitive and specific method of diagnosing TSE is unquestionably experimental infection in laboratory animals. The animal is injected with a homogenate prepared from the suspicious tissue and appearance of clinical signs is followed. The disease development is then confirmed after dissection using classic techniques (histology, immunohistology, Western Blot). These methods are too laborious and time-consuming to be used for routine high-throughput screening [20]. Recently, new postmortem tests have been introduced enabling rapid screening of the suspicious samples. Currently five commercial tests are approved by the European Commission for bovine spongiform encephalopathy detection (Prionics-Check Western test, Enfer test, CEA/Biorad test, Prionics-Check LIA test, and conformational-dependent immunoassay). All these tests are based on immunodetection of the pathological PrP^{Sc} isoform; four of them use

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proteolysis to distinguish PrP^C from misfolded PrP^{Sc} [18]. It has to be noted that none of these tests is able to identify infected animal at the presymptomatic stage and therefore the risk of the infectious agents entering the food chain is not completely eliminated.

Also some commercial assays (TeSeE™ CJD ELISA and TeSeE™) are available to detect PrP^{Sc} in cerebral and lymphoid tissues of TSE patients. These two assays have been compared by Ugnon-Cafe et al. [21] using samples from 54 variant of vCJD (vCJD) affected patients and 51 controls. Authors concluded that these tools were rapid and robust for routine in vitro human TSE diagnosis and characterization. CJD could be also diagnosed during the patient's lifetime by detection of PrP^{Sc} in the tonsil. A pilot study was undertaken to look at the feasibility of testing for vCJD in deceased donors using tonsillar tissue. Obtaining tonsillar tissue in the immediate postmortem period was limited by the presence of rigor mortis. Tonsillar tissue was suitable for routine analysis for the presence of prion protein associated with vCJD in deceased tissue donors. In spite of the fact that palatine and lingual tonsil tissue could be obtained in pairs, it was possible, in the majority of cases, to set aside an intact sample for confirmatory testing if required [22].

The main problem with the diagnosis based on the PrP^{Sc} detection is that the pathological form of PrP is abundant only at late stages of the disease in a brain. However, infectivity studies have shown that prion proteins occurred in low amounts in peripheral tissues, such as lymphoid organs and blood, already at early stages of the disease during the presymptomatic period. Another challenge for diagnosis and surveillance is that hosts can incubate infectious prion proteins for many months or years. During this period, they exhibit no overt clinical symptoms. Incubation period for some human prion diseases can be as long as 40 years [23]. The incubation time of the first human transmissible spongiform encephalopathy "Kuru" described by Professor Gajdusek in the late 1950s was estimated within the range from 21 to 40 years [24, 25].

In order to avoid the use of antibodies, several spectroscopic methods such as multispectral ultraviolet fluoroscopy [26], fluorescence correlation spectroscopy [27, 28], magnetic resonance spectroscopy [29–31], or FTIR [32, 33] have been employed. The main disadvantages of these methods include requirements of expensive and sophisticated equipment as well as skilled operator. Recently also Raman scattering spectroscopy using gold nanorods 3D supercrystals was used for prion protein detection [34]. Last but not least, sensitive MS based method of quantitating the prion proteins in a variety of mammalian species has been presented [35].

Identification of cell lines highly sensitive to prion protein infection led to the development of cell-based titration procedures aiming at replacing animal bioassays, usually performed in mice or hamsters. However, most of these cell lines are only permissive to mouse-adapted prion proteins strains and do not allow titration of prion proteins from other species. In the study of Arellano-Anaya et al. [36], it has been shown that epithelial RK13, a cell line permissive to mouse and bank vole prion protein strains and to natural prion protein agents from sheep and cervids, enables a robust and sensitive detection of mouse and ovine-derived prion proteins. Notably, the cell culture work is strongly reduced as the RK13 cell assay procedure designed here does not require subcultivation of the inoculated cultures. It was also shown that prion proteins effectively bind to culture plastic vessel and are quantitatively detected by the cell assay.

A new in vitro amplification technology, designated "RT quaking-induced conversion," has been described for detection of the abnormal form of prion protein (PrP^{Sc}) in easily accessible specimens such as cerebrospinal fluid. RT quaking-induced conversion method can be applied to other prion diseases, including scrapie, chronic wasting disease, and bovine spongiform encephalopathy, and is able to quantify prion protein seeding activity when combined with an end-point dilution of samples [37]. Solid-state matrix can be used for capturing and concentrating disease-associated prion proteins. Coupling of this method with direct immunodetection of surface-bound material enabled to distinguish 10^{-10} dilution of exogenous vCJD prion protein infected brain from a 10^{-6} dilution of normal brain (mean chemiluminescent signal, 1.3×10^5 for vCJD versus 9.9×10^4 for normal brain, showing an assay sensitivity for vCJD of 71.4% and a specificity of 100%) [38].

2.1 Immuno-based method for prion protein detection

Generally, the majority of analytical diagnostic methods rely on the proteolytic removal of endogenous PrP^C prior to detection of PrP^{Sc} (Fig. 1). PrP^{Sc} is relatively resistant toward proteolytic degradation whereas PrP^C is entirely digested by proteinase K. Identical treatment leads to removal of a variable number of N-terminal amino acids in case of PrP^{Sc}. This results in appearance of three distinct bands, corresponding to the di-, mono-, and unglycosylated form of PrP, upon Western blotting. Several techniques have been developed to detect PrP^{Sc} in brain tissues, including Western blot or other immunoblot methods [39–41]. Quantitative Western blot analysis revealed the highest expression of PrP^C in cerebellum, obex, and spinal cord. Intermediate levels were detected in thymus, intestine, nervous, heart, and spleen, and lower levels in lung, muscle, kidney, lymph node, skin, pancreas, and liver [42]. Western blotting coupled with gel electrophoresis is one of the immunodetection methods successfully used for detection of PrP^{Sc} in tissue extracts [43, 44]. After denaturation of the tissue extract by heating with SDS, it is analyzed by PAGE and the denatured protein is transferred to a solid support and detected with an enzyme-labeled antibody, often of goat, rabbit, and mouse. The specificity of Western blotting is based on the fact that proteolysis with proteinase K characteristically alters the molecular mass (approximately 5 kDa) of the PrP^c, due to the partial degradation of the N-terminal part of the protein [20]. Immunohistochemical analysis detected intense cellular-specific PrP^C staining

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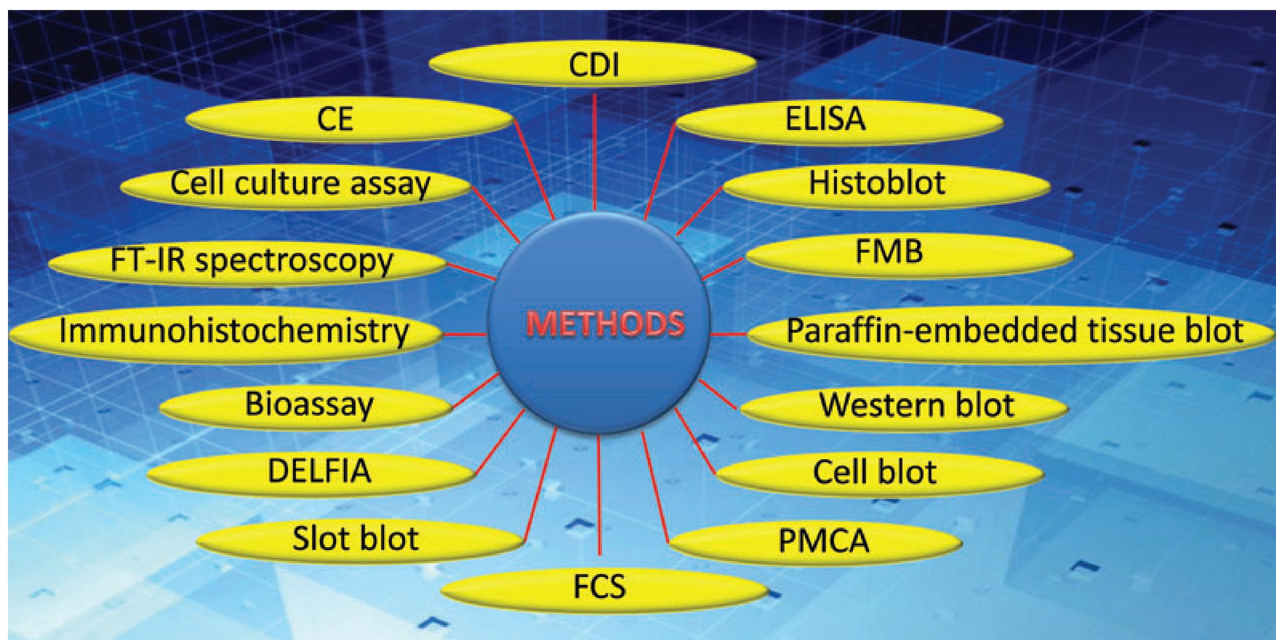

Figure 1. Methods used for prion protein detection: CE, cell culture assay, FTIR spectroscopy, immunohistochemistry, bioassay, dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA), slot blot, fluorescence correlation spectroscopy (FCS), protein misfolding cyclic amplification (PMCA), cell blot, Western blot, paraffin-embedded tissue blot, flow microbead immunoassay (FMB), histoblot, ELISA, current density imaging (CDI).

in neurons, thymocytes, and lymphocytes. PrP^{C} was also detected in the enteric wall, pancreatic islets of Langerhans, myocardium, pulmonary alveolar sacs, renal glomeruli, and dermal epithelial cells [42].

Circumvention of the protease digestion step might theoretically result in the increasing sensitivity of PrP^{Sc}-based detection methods and thus make these methods more amenable to high-throughput technologies [45–47]. Also a method using Western blot assay with precipitation of streptomycin sulfate has been reported, improving the PrP^{Sc} detection sensitivity and providing the potential for specific, rapid, and flexible determination of low PrP^{Sc} levels in the specimens not only from the central nervous system, but also from peripheral organs or fluids [48]. Employing of PrP^{Sc} stock sample into various mimic specimens, including normal hamster brain homogenate, human cerebrospinal fluid, and urine, demonstrated that streptomycin precipitation markedly increased the detection sensitivity of PrP^{Sc}, regardless low concentration or large volume. In addition, PrP^{Sc} from human brain tissue of fCJD was efficiently precipitated with streptomycin sulfate. Another approach that has proven usefulness is the in situ detection of PrP by immunohistochemistry (IHC). In most IHC procedures, the brain tissue sections are treated to destroy PrP^{C} with formic acid rather than with protease, since formic acid also enhances the PrP^{Sc} immunoreactivity [8]. A suitable IHC procedure was developed using brain tissue from hamsters that had been inoculated with the transmissible mink encephalopathy agent. Tissue samples were fixed in PLP (periodate, lysine, paraformaldehyde) that contained paraformaldehyde at a concentration of 0.125%. Before application of the IHC technique, tissue sections were deparaffinized and treated with formic acid simultaneously to enhance PrP^{Sc} immunoreactivity and to degrade PrP^{C}. Primary antibody was obtained from a rabbit immunized to PrP^{Sc} extracted from brains of mice with experimentally induced scrapie. Brains from 21 sheep with histopathologically confirmed scrapie were examined by IHC. In all of these brains, PrP^{Sc} was widely distributed throughout the brain.

One of the attempts of prion protein diagnosis includes the production of conformational PrP^{Sc}-specific antibodies as described by Korth et al. They prepared 15B3 antibody that specifically precipitates bovine, murine, or human PrP^{Sc}, but not PrP^{C}, suggesting that it recognizes an epitope common to prion proteins from different species [49]. This approach may even eliminate the protease digestion because of specificity of antibodies. Recent experiments in this area have shown that formation of β-sheet structures in prion proteins was connected to the increased solvent accessibility of the tyrosine residues. Based on these results, animals were immunized with synthetic peptides rich in tyrosine and several antibodies recognizing PrP^{Sc} (and not PrP^{C}) were produced [50]. Similarly, a mAb against a carboxy-terminal PrP synthetic peptide [51] as well as anti-DNA antibody [52] have been developed. Even though these antibodies have been successfully tested, this work has no commercial outcome at present. On the other hand, protein misfolding cyclic amplification has a great potential and it is certainly the most promising approach from the viewpoint of developing a blood test. It mimics pathological processes and is similar to PCR; PrP^{Sc} is

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incubated in the presence of PrP^{C} excess to initiate conversion to PrP^{Sc} aggregates that are subsequently dispersed by sonication to encourage the formation of new aggregates. The quantity of PrP^{Sc} formed depends on the number of expansion/sonication cycles performed [53–56]. To date, no commonly available test can give a reliable diagnosis using a readily available sample from a living animal or person, such as blood or urine [20]. Therefore also nonprion biomarkers are searched to increase the diagnostic possibilities [23, 57].

2.2 CE of prion proteins

Electrophoretic methods represented by gel electrophoresis are commonly used for detection of prion proteins, especially in combination with Western blot. Recently, TGGE was used to find that sensitivity to scrapie is associated with polymorphisms in three codons of prion protein gene: 136, 154, and 171. The TGGE method was used to detect point mutations in these codons responsible for sensitivity or resistance to scrapie [58–61]. In another recent study, the authors utilized recombinant human PrP as a probe in combination with 2DE and MALDI-TOF MS for identification of heterogeneous nuclear ribonucleoprotein A2/B1 and aldolase C as novel interaction partners for PrP [62]. Comparison of one-dimensional gel electrophoresis and immunoblotting and two-dimensional immunoblot demonstrated serious differences and evidenced that a qualitative difference in glycans contributes to prion protein diversity [63].

Since 1992, when first reports on ACE were published, this technique has emerged as a useful and sensitive technique for studying bimolecular noncovalent interactions and for determining binding and dissociation constants of formed complexes. Numerous interactions including protein–ligand [64], peptide–metal [65], protein–protein [66], antibody–antigen [67], and enzyme–drug [68] have been examined using ACE. The subset of techniques known as ACE is a group of CE immunoassays defined as methods in which antibodies or antibody-related substances are used as selective binding agents for chemical detection [69].

Generally, two types of immunoassays (i) heterogeneous and (ii) homogeneous can be distinguished. In heterogeneous immunoassays, antibodies are immobilized on a solid support and interact with antigen at the boundary layer. Subsequently, the unbound antibodies and other components can be easily removed. In homogenous immunoassays, antibodies interact with antigens in solution. Heterogeneous as well as homogeneous immunoassays can be further subdivided into competitive and noncompetitive techniques. In competitive mode, the antigens of interest compete with exogenous labeled antigens for a limited, precisely defined, number of antibody-binding sites. Thus, the generated signal is inversely proportional to the antigen concentration. In noncompetitive mode, antigens are captured by an excess of antibodies and are detected after subsequent binding of a second set of labeled antibodies that bind to the antigen at a different epitope. This forms a “sandwich” immunoassay where the signal is proportional to the antigen concentration [70]. In homogeneous immunoassay based on CE, the immune complex and free antibodies are discriminated based on their electrophoretic mobilities [71].

Several procedures using CE for prion proteins have been developed by Schmerr et al. [72–82]. In the first work of this group, brain tissues of scrapie-infected as well as healthy sheep were used to prepare PrP^{Sc}, which was subsequently reacted with a rabbit antibody specific for a peptide of the prion protein. The immunocomplex formation was observed for the samples from scrapie-infected brain, but not for samples from normal brain. Moreover, when a fluorescein-labeled goat anti-rabbit immunoglobulin was used as a second antibody, the detection of immunocomplex formation was enhanced both by the immunological technique and by using LIF for detection. CE can be used to show immunocomplex formation when PrP^{Sc} occurs in sheep brain [72]. In the following paper, the competition between fluorescently labeled synthetic peptide and prion proteins from infected brain tissues was used to increase the sensitivity of the detection [73]. Later, faster and better resolved separation of the immune complexes from the unbound peptide was achieved using 200 mM Tricine (pH 8.0) in comparison to phosphate or borate buffer systems. As increasing amounts of unlabeled peptide were added to the assay, a concentration-dependent reduction in the immune complex peak was observed. The assay could detect less than 10.0 fmol of unlabeled peptide. Using these optimized conditions there was a quantitative difference in the competition of preparations from scrapie-infected sheep brain and normal sheep brain [74]. Following study from the same group compared SDS gel CE to conventional SDS-PAGE and Western blot to detect the monomer of this aggregated protein. In infected sheep brain samples, but not in healthy sheep brains, a major peak at a molecular mass of 19.2 kDa and a minor peak with a leading shoulder were observed. The molecular mass determined for this protein was in good correlation with that estimated on Western blot (22.4 kDa). The equivalent amount of brain sample in the capillary was similar to 50 μg. The amount of brain sample was 100 times less than that needed for Western blot for sheep samples [75]. A new method—competition immunoassay using flourescein-labeled synthetic peptides (amino acid positions 142–154 and 155–178) from PrP^{Sc} and free zone CE with LIF was presented in 1998 [76]. Antibodies were prepared to each synthetic peptide and used in the competition assay. The fluorescent-labeled peptides bound to the antibody were separated from the unbound peptides. When PrP^{Sc} extracted from infected sheep brain was added to the assay, approximately 135 pg of PrP^{Sc} could be detected; however, there was little or no competition observed when using extracts from normal sheep tissue [76]. In 1999, Schmerr et al. suggested a way of detection of prion proteins in blood. A peptide from the carboxyl terminal region, amino acid positions 218–232, was labeled with fluorescein during the synthesis of the peptide at the amino terminus. Antibodies that have been produced from this peptide were affinity purified and used in a CE immunoassay. The amount of fluorescein-labeled peptide in the

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capillary was 50 amol. Blood was obtained from healthy sheep and elk, from sheep infected with scrapie, and elk infected with chronic wasting disease. Buffy coats and plasma were prepared by a conventional method. The abnormal prion protein was detected in fractions from blood of infected animals but not in the blood of healthy animals [77]. The emergence of a new environmentally caused vCJD has stimulated the research on a practical diagnostic screening test. The immunocompetitive CE assay has been reported to detect disease-specific, proteinase-resistant prion protein (PrPres) in the blood of scrapie-infected sheep. Thus, this method was applied to blood from CJD-infected chimpanzees and humans. The threshold of detection achieved with immunocompetitive CE was 0.6 nM of synthetic peptide corresponding to the prion protein (PrP) C-terminus, and 2 nM of recombinant human PrP under the optimized conditions. However, the test was unable to distinguish between extracts of leucocytes from healthy and CJD-infected chimpanzees, and from healthy human donors and patients affected with various forms of CJD shown in Table 1 [83]. Extraction method based on interaction of antibodies specific to fluorescently labeled synthetic peptides and protein A Sepharose has been described. After elution, the amount of fluorescent peptide that was captured versus the total amount placed in the assay was evaluated by CZE-LIF. Of the three peptides used in this evaluation, it was found that the recovery was approximately 25–35% [78]. Also noncompetitive immunoassay for prion protein was established. FITC-labeled protein A (FITC-PrA) was used as a fluorescent probe to tag mAb through noncovalent binding of FITC-PrA to the Fc region of the antibody. The FITC-PrA-Ab was incubated with the analyte, prion protein, under optimized condition, forming the immunocomplex FITC-PrA-Ab-PrP. The complex was separated and analyzed by CE. The addition of carboxymethyl-β-cyclodextrin in the running buffer as dynamical coating reagent improved the reproducibility and the resolution. The complex was isolated in less than 1 min with theoretical plates of 3.8×10^4. The estimated detection limit for PrP was 6 ng/mL. The method was successfully applied for testing blood samples from scrapie-infected sheep [79]. The application of carboxymethyl-cyclodextrin as a buffer additive suppressing the analyte adsorption and enhancing separation selectivity in the CE as well as mAbs were used in the next work by the same authors [80]. The amount of both free and fluorescein-labeled peptide bound to antibody (immunocomplex) was determined by CE-LIF. In the presence of PrP, the peak height ratio of the immunocomplex and the free peptide was altered compared to control (Fig. 2A and B). These changes were directly proportional to the amount of PrP present. The reaction times of the antibody with either the peptide or the recombinant PrP was significantly improved (less than 1 min) using mAbs as compared to polyclonal antibodies (16–18 h). The results of the blood assay were consistent with scrapie status of the sheep as determined postmortem by Western blot analysis [80]. The method evaluated for its performance in the preclinical diagnosis of bovine TSEs has been described by Jackman et al. [81]. The blood samples from scrapie-infected sheep aged 7–12 months and of the scrapie-susceptible PrP genotypes Val-Arg-Gln/Val-Arg-Gln and Val-Arg-Gln/Ala-Arg-Gln were analyzed and the abnormal PrP was found. These results correlated with the postmortem diagnosis of scrapie. The sheep were preclinical and appeared normal at the time of testing, but later died with clinical disease approximately 12 months after the testing. In older animals and in those with clinical signs, a smaller percentage of animals was tested as positive. The application of an immunocapillary electrophoresis method developed for blood from patients with CJD is described. The test was evaluated by using clinical blood specimens from patients with variant ($n = 5$) or sporadic ($n = 4$) CJD and patients initially suspected of having CJD who were given an alternative diagnosis ($n = 6$). In this context, the immunocapillary electrophoresis assay was specific, but incompletely sensitive (55%). The method was unable to detect abnormal prion protein in variant CJD brain or spleen reference materials due to its loss during the extraction process [82]. Summary of electrolytes and detection limits is given in Table 2.

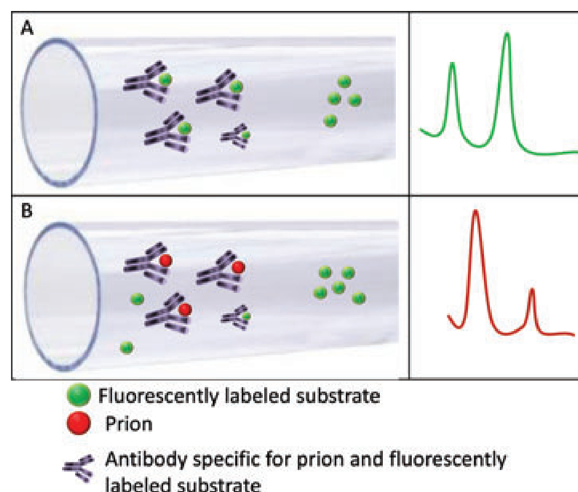

Figure 2. The principle of immune detection of prion protein using CE. (A) Fluorescently labeled substrate binds antibody specific for prion protein. (B) In the presence of prion protein, this protein binds antibody specific for prion protein and the signal for complex of fluorescently labeled substrate with antibody specific for prion protein decreases.

It is of interest to note the lack of widespread popularity of CE in the fields of PrP^{Sc} detection and prion disease diagnosis. Although the first report of PrP^{Sc} detection by CE appeared over 15 years ago, the use of this and similar methodologies has not dominated the field of prion protein analysis. This is probably caused by the dominancy of commonly used immunochemical methods that are well established over decades and therefore difficult to compete with.

However, we believe that advances in CE focused particularly on microfluidic devices and development of portable instruments enabling rapid, simple, and low-cost analyses would turn the attention back to this powerful analytical

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Table 2. LOD and electrolytes used for determination of prion proteins in various types of real samples

Sample	Electrolyte	LOD	REF
Sheep brain	200 mM Tricine (pH 8.0)	10.0 fmol of unlabeled peptide	[74]
Sheep brain	SDS gel CE	50 μg	[75]
Sheep blood	0.6% CM-CD in 25 mM TAPS at pH 8.8.	80 ng/mL (or mass detection limit 1 pg)	[80]
Sheep brain	200 mM Tricine, pH 8.0, containing 0.1% *n*-octylglucoside and 0.1% BSA	135 pg PrP^{Sc}	[76]
Sheep and elk blood	250 mM Tricine, pH 8.0, containing 0.1% *n*-octylglucoside and 0.1% BSA	50 amol	[77]
Chimpanzee and human blood	250 mM Tricine, pH 8.0, containing 0.1% *n*-octylglucoside and 0.1% BSA	0.6 nM of synthetic peptide 2 nM of recombinant human PrP	[83]
Sheep blood	Carboxymethyl-beta-cyclodextrin	For rPrP was 6 ng/mL	[79]

technique. Even though numerous obstacles still have to be overcome. In some cases, analysis of complex biological samples by CE might be problematic requiring sample pretreatment procedures to be involved. However, application of modern isolation procedures such as extraction of target molecule by magnetic particles as well as hyphenation of sample pretreatment and analytical processes into the one miniature device based on "lab-on-chip" concept will increase the chances of CE for routine applicability.

Furthermore, it is important to highlight the sensitivity issue. For diagnostic purposes, only "yes/no" information is required to distinguish between healthy and infected animal: according to the hypothesis only a molecule of infectious prion protein is able to convert other prion molecules and initiate the disease. Routinely used methods based on prion protein detection are required to utilize procedures for amplification of PrP^{Sc} in the sample either by cell cultivation, protein misfolding cyclic amplification, or the use of an RNA ligand based adsorbent that improve the detection limits of several hundred-fold [84]. There is also another approach to PrP^{Sc} determination that involves development of high sensitivity methods with extremely low limits of detection. We believe that analytical techniques such as CE, MS, and/or other spectroscopic methods may contribute not only to the identification of diseased individuals but also to better and more detailed understanding of the disease development, progress, and treatment efficiency.

3 Future perspective

Expanding interest is focusing on inexpensive, portable, high-throughput, and sensitive integrated diagnostic devices, since traditional tools are labor-, cost-, and time-consuming and also have limited potential for usage in resource-limited settings. Efforts illustrate the great potential of miniaturization in developing point-of-care devices for molecular diagnostics. Recent advances in CE are focused primarily on microfluidic devices [85] due to the numerous advantages such as extremely short time of analysis, exceptionally low amount of sample required, and portability of the instrumentation. Also in the area of protein analysis, the chip-based CE is of a great interest. Due to the requirements for the detection sensitivity, LIF detection is the most commonly applied. The combination of lab-on-chip and fluorescent labels is of great interest for the protein detection. From the group of fluorescent labels quantum dots as a part of modern research area are attracting a great attention as excellent fluorescent labels with outstanding optical properties. These nanoparticles are made up of 100–100 000 atoms with typical range of diameter from 1 to 10 nm. They have broad excitation spectra, narrow, tunable, and symmetric emission spectra, and exceptional photostability. Moreover, their surface is suitable for chemical modification thus enabling the specific interaction with a wide range of the target molecules. Quantum dots have been already employed for prion protein analysis [86] and opened numerous new possibilities in the field of in vivo imaging.

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