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An acoustic prion assay

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ABSTRACT

An acoustic prion assay has been demonstrated for sheep brain samples. Only five false positives and no false negatives were observed in a test of 45 positive and 45 negative samples. The acoustic prion sensor was constructed using a thickness shear mode quartz resonator coated with a covalently bound recombinant prion protein. The characteristic indicator of a scrapie infected sheep brain sample was an observed shoulder in the frequency decrease in response to a sample.

The response of the sensor aligns with a conformational shift in the surface protein and with the propagation mechanism of the disease. This alignment is evident in the response timing and shape, dependence on concentration, cross species behaviour and impact of blood plasma. This alignment is far from sufficient to prove the mechanism of the sensor but it does offer the possibility of a rapid and inexpensive additional tool to explore prion disease.

1. Introduction

Prions, the infectious pathogens that cause transmissible spongiform encephalopathies in animals, are proteins containing no genetic material. The normal or healthy form of the cellular prion protein (PrP^C) is found in mammalian tissue. The pathogenic form (PrP^{Sc}), found in diseased tissue, differs from the normal protein form only in the protein's conformation or shape [1]. Although the designation PrP^{Sc} is named for scrapie, the disease in sheep, it is used to designate any infectious prion protein. PrP^C and PrP^{Sc} have the same amino acid sequence. Pan et al. [2] determined the secondary structures of both PrP^C and PrP^{Sc}. PrP^C has 42% of its peptides folded in an α-helix configuration with little (3%) in the β-sheet form, while PrP^{Sc} has 30% in the α-helix form and 43% in the β-sheet form. The change to the β-sheet form appears to make the protein insoluble in detergent media and confers resistance to digestion by protease enzymes. A resistant fragment, consisting of 50% in the β-sheet form, retains its infective capacity [3].

The conversion of PrP^C to PrP^{Sc} occurs *in vivo* as PrP^{Sc} acts as a template, binding and refolding PrP^C [1]. Other co-factors such as chaperone proteins may be involved. The conversion has also been carried out *in vitro* [4]. Here, PrP^{Sc} was incubated with uninfected brain homogenate which provided PrP^C as well as possible unidentified co-factors. The PrP^C appeared to bind to the PrP^{Sc}, which should it be prompted to refold, would increase the size of the initially introduced PrP^{Sc} oligomers.

The present work presents the proof of principle for an acoustic assay technique using a thickness shear mode sensor (TSM). The primary objective of this paper is to demonstrate the capacity to distinguish scrapie positive (clinically infected) and negative sheep brain samples. The premise of the technique in this work is that the TSM surface, coated with a recombinant sheep PrP^C, will bind sample proteins, which may lead to conformational shifts on the surface. A secondary objective of the paper is to explore whether the TSM response is consistent with this premise. If this is valid, the TSM technique may offer further insight into the disease.

To achieve the primary objective, two criteria must be satisfied. First, the response to positive test samples must be a reproducible resonant frequency and/or energy dissipation signal that is significantly higher than the background fluctuation of these signals, and second, the response to positive test samples must be distinguishable from the response to negative samples.

The secondary objective builds on the operating principles of TSMs. The TSM is a piezoelectric crystal wafer that oscillates through the entire crystal with motion parallel to the crystal faces and resonates at a frequency primarily determined by the thickness of the crystal. There are several mechanisms that shift the resonant frequency of a TSM device [5]. The first is a mass response first quantified by Sauerbrey [6]. A mass rigidly attached to the crystal surface stores kinetic energy, reducing the velocity and hence the frequency required to match the kinetic energy to the elastic energy stored in the shear deformation of the crystal. This match is required for resonance.

The second mechanism is the interaction between the crystal surface and a surrounding fluid medium. Kinetic energy is transferred to the fluid, but unlike rigidly attached mass, only some of the energy is returned to the crystal. Kanazawa and Gordon [7] showed that the viscous energy is lost as a shear wave propagates into the liquid but the

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viscous effect is confined to within a few hundred nanometers from the crystal surface. Again the resonant frequency is reduced and the energy loss appears as an equivalent resistance which may be measured electrically.

The third mechanism, which provides the basis for this acoustic prion assay, involves the coupling between the crystal surface and the fluid [5]. The match between the polarity of the fluid and the hydrophobicity of the surface determines the strength of the kinetic energy coupling between the two. The weaker the coupling, the less the fluid viscosity will reduce the resonant frequency of the crystal.

Following the premise outlined above, we hypothesize that the overall response of this TSM sensor configuration, when exposed to a positive sample, is a combination of three mechanisms. Ideally, the PrP^{Sc} in positive samples would trigger a change in the recombinant prion protein coating the TSM sensor, resulting in a more hydrophobic surface as α-helices convert to β-sheets, leading to a change in surface hydrophobicity and a measurable resonant frequency shift. This would occur with a background frequency reduction as other proteins and cell debris bind to the coated surface.

2. Materials and methods

The TSM sensors used in this assay were made with 9 MHz AT-cut quartz crystals manufactured by Laptech Precision Inc. (Bowmanville, Ontario). The 13.5 mm diameter crystals were glued to replace the bottoms of 11 mm glass vials using food grade RTV-108 silicone cement (MG Chemicals, Surrey, BC). The vial with an attached crystal is shown in Fig. 1. The gold electrodes, deposited onto both flat surfaces of the crystal, were 5 mm in diameter. This diameter allowed sufficient clearance from the glue joint to prevent any observable interference with the crystal oscillations. Choosing a larger crystal diameter relative to the glass vial provided a flange for clamping the sensor into a test fixture.

The gold electrodes were connected *via* the test fixture, to an oscillator, a PLO-10i (Inficon, East Syracuse, NY) which phase locked the output to the series resonant frequency of the crystal. The frequency and motional resistance were continuously measured and 10 s averages were recorded using a custom-built frequency counter and analog conversion system.

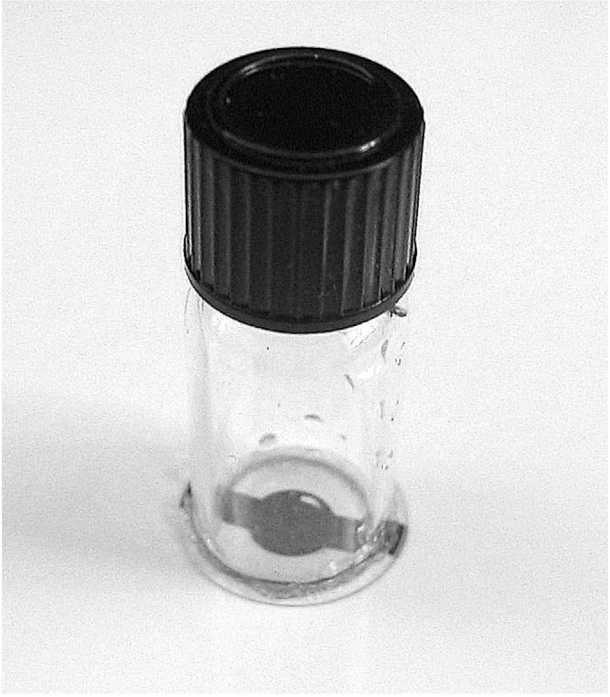

Fig. 1. Test vial with attached crystal.

The preparation of the crystal surfaces required linking, activating and coating procedures which were adapted from Lyle et al. [8]. The linking reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. The linking procedure required that inside surfaces of test vials were cleaned with the following sequence of reagents: 10% nitric acid, water, acetone and ethanol. Under a nitrogen atmosphere the gold surfaces inside the test vials were coated with 11-mercapto undecanoic acid (11-MUA) by soaking in a 10 mM solution of 11-MUA in ethanol at room temperature. After 24 h, this solution was removed, the vials washed with ethanol, dried with nitrogen and capped. The vials were then refrigerated until used.

At the time of each trial, one vial was opened, installed in the test fixture and connected to the oscillator. The activating step involved adding 350 μL of 15 mM *N*-hydroxy succinimide (NHS) in water to 350 μL of 75 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) in water, mixed at room temperature and then placed in the vial for 1 h at 37 °C. The activator solution was then removed and the vial was washed with water. This left succinimidyl ester groups on the 11-MUA linker.

The coating step involved adding 200 μL of a recombinant sheep PrP^{C} solution to the vial for 1 h at 37 °C. The PrP^{C} coating solution was prepared by adding 1 mL of 10 mM acetate buffer at pH 4.0 to 100 μg of the recombinant protein as supplied by the manufacturer (AJ Roboscreen GmbH, Leipzig). Exposed NH_2 groups on the recombinant protein formed amide bonds with the bound succinimide ester. After the 1 h coating step, the vial was washed with acetate buffer. Buffered homogenate was then mixed as described below, added to the vial and frequency and resistance data were collected with the vial incubated at 37 °C.

Brain sample homogenates were generated by identical methods for both positive and negative samples. Scrapie positive samples were taken from sheep showing clinical signs of the disease and the infection was confirmed by both ELISA and immuno-histochemical examination [9]. In summary, 1 g of fresh brain tissue was cut into three portions. Each portion was separately weighed (350 mg ± 40 mg) and added to a BioRad grinding tube together with 1.75 mL of 5% glucose solution and a "1/4" ceramic bead. A Fast-Prep Ribolyzer ground the mixture using one 45 s agitation cycle. The three portions were then recombined and mixed thoroughly. The combined homogenate was divided into 300 μL aliquots and stored at −70 °C until use.

Each brain sample was prepared from the frozen homogenate. 175 μL of this brain homogenate was thawed at 4 °C for 2–3 h, and added to 125 μL of buffer [4] containing 0.5% Triton X-100 and 0.05% sodium dodecyl sulphate in phosphate buffered saline at pH 7.4. 50 μL of COMPLETE™ protease inhibitor (Roche Diagnostics GmbH, Germany) was also added at this step to block any protease enzymes present in the brain samples.

3. Results and discussion

3.1. Proof of principle

Fig. 2 provides the raw frequency shifts for three normal and three scrapie infected brain samples. The large pulses (at the zero, one and 2 h marks) are artifacts occurring when the fluid in the crystal vial was replaced. Over the first hour, the crystal surface was activated with the EDC/NHS linker. Over the second hour the surface was coated with the recombinant PrP^{C}. At the 2 h mark, the recombinant protein coating solution was removed, the brain sample was added and the data recorded for several hours. The frequency shifts for all samples have been normalized to a zero based on the frequency obtained with the recombinant PrP^{C} solution (between 1 and 2 h).

There are two evident differences between the normal and scrapie sample responses. The first is the overall magnitude of the frequency shift and the second is the different shape of the response curves. The scrapie samples lead to a noticeable shoulder in the response curves.

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