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Prion protein detection in serum using micromechanical resonator arrays

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ABSTRACT

Prion proteins that have transformed from their normal cellular counterparts (PrP^c) into infectious form (PrP^{res}) are responsible for causing progressive neurodegenerative diseases in numerous species, such as bovine spongiform encephalopathy (BSE) in cattle (also known as mad cow disease), scrapie in sheep, and Creutzfeldt–Jakob disease (CJD) in humans. Due to a possible link between BSE and CJD it is highly desirable to develop non-invasive and *ante mortem* tests for the detection of prion proteins in bovine samples. Such *ante mortem* tests of all cows prior to slaughter will help to prevent the introduction of PrP^{res} into the human food supply. Furthermore, detection of PrP^{res} in donated blood will also help to prevent the transmission of CJD among humans through blood transfusion. In this study, we have continued development of a micromechanical resonator array that is capable of detecting PrP^c in bovine blood serum. The sensitivity of the resonators for the detection of PrP^c is further enhanced by the use of secondary mass labels. A pair of antibodies is used in a sandwich immunoassay format to immobilize PrP^c on the surface of resonators and attach nanoparticles as secondary mass labels to PrP^c. Secondary mass labeling is optimized in terms of incubation time to maximize the frequency shifts that correspond to the presence of PrP^c on the surface of resonators. Our results show that a minimum of 200 pg mL^{-1} of PrP^c in blood serum can be detected using micromechanical resonator arrays.

1. Introduction

Prions, discovered by Stanley Prusiner in 1982 [1], are proteins, which in mis-folded form are believed to be responsible for causing progressive neurodegenerative diseases in numerous species, such as bovine spongiform encephalopathy (BSE) in cattle (also known as mad cow disease), scrapie in sheep, and Creutzfeldt–Jakob disease (CJD) in humans. These are fatal diseases and currently untreatable. It is believed that these diseases are caused by the conversion of normal cellular prion proteins (PrP^c) into a mis-folded infectious form (PrP^{res}). Although the infectious form has the same amino acid sequence as the normal form, it is rich in beta-sheet structures as compared to the normal form which is rich in alpha helical structures [2].

The current state of technology to detect BSE relies upon the *post-mortem* detection of prions in homogenates of brain tissue removed from slaughtered cows. The numerous methods used to detect PrP^{res} in the brain homogenate are described below. Such *post-mortem* testing therefore is not suitable for routine screening of all animals, regardless of initial symptoms of BSE when animals cannot stand or walk. But, it has been shown that the animals not showing any symptoms of the disease could potentially be carrying the disease [3,4]. If these animals are not tested for PrP^{res}, they could conceivably make their way into the human food supply. Thus in order to improve the safety of human food, new technologies must be developed to allow *ante mortem* tests for PrP^{res} proteins in the body fluids of the cows. Blood is considered as the most appropriate body fluids, and is known to contain infectivity even before the onset of the clinical symptoms [5–7]. An important aspect of these *ante mortem* tests is the short detection time, so that all animals can be routinely screened prior to slaughter. Contraction of CJD in humans is not only attributed to the consumption of contaminated meat, but also to blood transfusions from an infected donor [5,8]. This is a serious problem, especially if an asymptomatic infected individual was not prevented from donating blood because of the lack of sufficiently sensitive screening tests [9–11]. Such presumed healthy humans could trigger the transmission of the disease to other humans through blood transfusion. Therefore the lack of routine testing of cattle is compounded by the lack of routine testing of human blood for transfusion, establishing a dangerous scenario whereby transmission of infectious prions from cattle to humans could lead to additional transmission of those infectious prions from one human to another via blood transmission.

There are several methods available for the detection of prion proteins in animals, namely, protein misfolding cyclic amplification,

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conformation-dependent immunoassay, dissociation enhanced lanthanide fluorescent immunoassay, capillary gel electrophoresis, fluorescence correlation spectroscopy, flow microbead immunoassay, etc. [12]. Most of these techniques are intended to detect PrP^{res} in the brain homogenate and thus will not be suitable for routine blood screening based tests. Saa et al. [13] developed a method to detect PrP^{res} proteins in the blood of presymptomatic animals. The number of PrP^{res} proteins in the blood of infected hamsters was amplified by mixing them with normal brain homogenate and running protein misfolding cyclic amplification (PMCA) to convert PrP^{c} (in the normal brain homogenate) into PrP^{res}. Seven rounds of 144 PMCA cycles were performed in order to sufficiently amplify the initial low numbers of PrP^{res} to the detection level. The technique was sensitive to detect as low as 20–50 molecules of monomeric hamster PrP, however, the time of cyclic amplification alone was 525 h (7× 75 h). Another study based on cytometric methods demonstrated analytical sensitivity in the range of 10 aM or 0.24 fg mL^{-1} of PrP^{res} for serum samples [14]. The cytometric test, however, requires at least a 20 h incubation time, followed by flow cytometry. Both of these methods are highly sensitive to detect extremely small amounts of infectious prions in blood, but until either of these methods can be automated and performed in a faster manner, they will prove to be too complicated and time consuming to be practical for routine rapid screening of all cattles prior to slaughter.

Micromechanical resonators are considered as suitable candidate to develop sensitive and rapid blood based tests to detect prion proteins. Resonators have been used to detect attograms of masses [15]. They are suitable to detect variety of biomolecules, as the resonators can be functionalized with a number of different biorecognition molecules (antibody, nucleic acid, and other protein). Resonators can be fabricated with a variety of materials or coated with a particular material as suitable for the functionalization chemistry. These devices resonate at a frequency which decreases as mass is added to the surface. This frequency shift is measured to detect the presence of mass on the surface. The sensitivity of these resonators to mass detection depends on size, shape, thickness and the fabrication material [16–22]. In order to detect biomolecules of smaller masses, mass amplification techniques are used. Amplification is achieved by using secondary mass labeling with massive particles binding to the target analyte already present on the surface of resonators [23]. These amplification techniques are not time consuming as compared to PMCA or cytometry used for prion protein detection, although the amplification is limited by the background noise due to non-specific binding. In addition to this, the array format of the resonators makes it possible to run multiple tests on the same sample to improve the statistics of detection as well as to reduce false positives and negatives.

In the current work, we demonstrate the potential of these devices for the direct detection of prion proteins in blood serum. We have fabricated an array of paddlever shaped resonators for the detection PrP^{c} in blood samples. Secondary antibodies and nanoparticles were used for the mass amplification to detect the presence of small amounts of PrP^{c}. A sandwich immunoassay was used to immobilize PrP to the surface of resonators and subsequently add nanoparticles. Kinetic studies of secondary mass labeling have also been performed to improve the sensitivity of the resonators for detection of PrP^{c}. Due to the limitation of biosafety level of our research lab, we were able to work on non-infectious full length prion protein (PrP^{c}) and not infectious prion protein (PrP^{res}). We project this work to be successfully used with PrP^{res} in real world based on the affinity of the antibodies used, which have been claimed to be equally sensitive for the detection of both PrP^{c} and PrP^{res} (based on the information provided by the antibodies supplier).

2. Materials and methods

2.1. Reagents

Two antibodies were used for the sandwich assay. One is conjugated to the surface of the resonators (capture antibodies) while another is used to attach nanoparticles as additional mass labels (detection antibodies). Both antibodies are monoclonal and are produced in mice against different epitopes of bovine prion protein. Prion proteins used in this work are histidine tagged recombinant proteins and are full-length mature part of bovine PrP (25–244) expressed in *E. coli* BL21 (Millipore Inc.). Capture antibodies are against amino acids 23–237 of bovine prion protein (Millipore Inc.) and detection antibodies are against amino acids 123–136 and 140–160 of bovine prion protein (Abcam Inc.). Bovine serum albumin was used to block the surface of the resonators and was purchased from Sigma. Streptavidin conjugated nanoparticles (R&D Systems, Minneapolis, MN) of diameter 150 nm were used as secondary mass labels. These particles were irregularly shaped particles with diameters ranging from 80 nm to 200 nm (as analyzed with SEM). Deprionized fetal bovine serum was obtained from BioRad Laboratories. BioRad obtained fetal bovine serum from Biowest, Rau. Della Caille, Nuaille, France and origination of the product is from Canada. Deprionization was performed by BioRad Laboratories. Aliquots were made from the stock and were stored frozen until used. Before use the aliquots were thawed and vortexed. 3-Aminopropyl triethoxysilane (APTES, Sigma) was used to functionalize the surface of resonators with primary amine groups. Glutaraldehyde was used as a homo-bifunctional cross-linker to attach antibodies on the silanized surfaces. Glycine solution prepared in deionized (DI) water was used for quenching. Both of these chemicals were purchased from Sigma. Detection antibodies were modified with biotin to attach 5–7 biotin molecules per antibody molecule by using NHS-PEO_4-biotin (Pierce Chemicals). The biotin per antibody was determined by using EZ biotin quantification kit (Pierce Chemicals).

2.2. Resonator fabrication and functionalization

Resonators were fabricated as described in our previous work [24]. Briefly, a 150 nm thick layer of low-stress nitride was deposited on a thermally oxidized silicon wafer. The silicon nitride device layer was then patterned using an anisotropic reactive ion etch. Chips were then dipped in hydrofluoric acid in order to remove the 1.5 μm thick sacrificial silicon dioxide layer under the resonators and release them from the substrate. Release allows them to move freely and resonate. Resonators in this work were cantilevered structures with a 3 μm × 10 μm paddle at the free end, also called paddlevers, with typical resonant frequencies of approximately 4.6 MHz and quality factors of about 7000 for bare devices.

In this study, APTES (10% solution in dry toluene) was used to silanize the surface of the resonators. Glutaraldehyde (5% solution in 10 mM borate buffer pH 8.0) was used as a cross-linker between amine groups on capture antibodies (50 μg mL^{-1} in PBS buffer) and the surface of the resonators. The conjugation chemistry to modify the surface of resonator with capture antibodies is shown in Fig. 1. After a wash with DI water (95 rpm for 4 min, two times), the devices were quenched with glycine (50 mM solution in DI water, 30 min) to block unreacted amine group binding sites on the surface of the resonators followed by a blocking with 1% BSA (w/v) in PBS buffer (30 min). Blood serum obtained from BioRad laboratories was spiked with different concentrations of prion protein. Excess of prion protein was removed by washing with DI water (95 rpm for 4 min, two times). This was followed by a 10 min blocking step. Detection antibodies (50 μg mL^{-1} in PBS buffer) were used to label prion protein on the surface of resonators (80 min