SINGLE PARTICLE DETECTION
A Diagnostic Tool for Particle Associated Diseases like Alzheimer's Disease and Creutzfeldt-Jakob Disease

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Abstract: Neurodegenerative diseases like Alzheimer's disease (AD), prion diseases and others are progressive and lethal. High-molecular weight aggregates of the Amyloid-β-peptides (Aβ) or of the misfolded prion protein (PrP) are found in patients afflicted by AD or prion diseases, respectively. Despite of many attempts, neither a therapy for recovery, nor an early diagnosis at preclinical stages are available. Psychological tests and imaging approaches not directly related with a secure disease marker are in use only for late stages of the disease. The Creutzfeldt-Jakob-disease (CJD), a human prion disease, is caused by accumulation of aggregates consisting of an abnormally shaped version of PrP. CJD is diagnosed with certainty only by neuropathology *post mortem*. In this study a multidisciplinary development of a novel mode of single particle counting of immobilized Aβ and PrP aggregates as the most direct biomarkers for Alzheimer's disease and Prion diseases, respectively, is introduced. For ultrasensitive detection of aggregates, the suitable instrumentation as well as data acquisition and data analysis are developed using single molecule detection and advanced laser scanning fluorescence techniques. In the novel assay development effort biochemistry, detection and analysis were improved to detect single aggregates immobilised on a surface. First results show the improvement of single particle detection of PrP-aggregates of TSE-afflicted cattle and hamsters as well as synthetic Aβ-aggregates.

1 INTRODUCTION

In many neurodegenerative diseases e.g. prion diseases, Alzheimer's disease, Parkinson's disease, Huntington's Disease, protein aggregates are formed in the very beginning or in the progress of disease (Lee et al., 2005). Up to now it is not known, if these aggregates are causative agents or symptoms of the respective disease, but many studies show, that the aggregates or even oligomers of the according proteins are neurotoxic and therewith a reason of neurodegeneration. (Selkoe, 2003)

To understand the progression of these diseases, as well as disease associated or causative mechanisms and to monitor potential therapeutically approaches an ultrasensitive tool to quantify these disease related aggregates is required. A challenge for the analytic system is to reliably count only those aggregates or oligomers that consist of the specified protein or peptide. Monomeric molecules need to be clearly distinguished because they are present in healthy organism as well.

We developed a new method, which is able to count single protein aggregates bound by a capture-antibody to a surface (surface-FIDA) (Birkmann et al., 2007). Our new test system is based on fluorescence correlation spectroscopy (Eigen and Rigler, 1994). It is quantifying the number and size of aggregates simultaneously labelled by two different antibodies for dual colour fluorescence intensity distribution analysis (2D-FIDA) (Birkmann et al., 2006). Only aggregates and oligomers but not monomeric proteins are counted. To increase the sensitivity, particles were concentrated in the two-dimensional space by immobilizing it to capture antibodies on the surface of the slide. Laser beams are scanning the surface systematically, so even single particles are detected (Birkmann et al., 2007).

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We report on the successful use of surface-FIDA as diagnostic tool for prion diseases. The infectious agents of prion diseases are composed primarily of the pathogenic isoform of the prion protein designated PrP^{Sc}, which is generated by a conformational change of the cellular isoform PrP^{C}. In contrast to its cellular isoform, the pathogenic isoform PrP^{Sc} forms insoluble aggregates. Hitherto accredited prion tests use the PK-resistance of PrP^{Sc} as a marker for the disease. Because of varying portions of disease related aggregated PrP, which is not PK-resistant, these prion tests offer only a limited sensitivity. Therefore prion protein aggregate detection, which does not rely on PK-digestion, is favourable for sensitive diagnosis. It allows detection of both, PK-resistant and PK-sensitive PrP^{Sc} aggregates.

Up to now, we could successfully verify the novel test system for correct diagnosis of Scrapie-infected hamsters as well as BSE-infected cattle in the clinical stages of diseases (Birkmann et al., 2007). Furthermore, we were able to detect PrP aggregates in the cerebrospinal fluid of cattle of BSE-infected cattle for the first time (Birkmann et al., 2007). During the next steps we will adopt the highly sensitive test system for diagnosis of human prion diseases like Creutzfeldt-Jakob disease and other aggregate related diseases, especially Alzheimer's disease.

In this study we apply surface-FIDA to different disease associated aggregates. First we show the single aggregate detection of prion protein aggregates purified from brain homogenates of Scrapie-infected hamsters and BSE-infected cattle to demonstrate the principal of surface-FIDA to detect single particles. In the second part of the work we show the transfer of surface-FIDA to the detection of single Aβ aggregates as diagnostic approach for Alzheimer's disease. Therefore we compared the detection of Aβ aggregates in solution with the application of surface-FIDA.

2 MATERIALS AND METHODS

2.1 Fluorescence Labelling of Antibodies

Antibodies R1 were kindly provided by S.B. Prusiner, UCSF, USA (Williamson et al., 1998). Antibodies 12F10 and Saf32 were obtained from SpiBio (Massy Cedex, France); antibody D18 was obtained from InPro (San Francisco, USA). For the detection of Aβ aggregates, antibodies 6E10 (Sigma Aldrich, Hamburg, Germany), 8G7, 19H11 and 4G8 (Calbiochem) were purchased.

Antibodies were labelled in free amino groups via reactive succinimidyl ester groups of Alexa-633 and Alexa-488 (Molecular Probes, Oregon, USA). For labelling, approximately 50 µg antibodies were incubated with 5 µg dye in carbonate buffer, pH 8.4 in a total volume of 100 µl for 1 hour. Conjugates were separated from free dye by gel filtration via NAP5-column (Pharmacia) with 10 mM TBS, pH 7.2 and 0.2 M NaCl as elution buffer. Labelled antibodies were stored in the dark at 4°C.

2.2 Fluorescence Correlation Spectroscopy

In fluorescence correlation spectroscopy (FCS) the fluorescence intensity is recorded in a very small volume, i.e. in the femtoliter range. Measurements were performed with the instrument FCS Olympus IX 50 (Evotec OAI, Hamburg, Germany) with a beam scanner unit in dual-colour mode with an Argon ion laser (excitation wavelength 488/514 nm) and a helium-neon laser (excitation wavelength 633 nm). The beam scanner unit allows the scanning of the sample for aggregates. In practice the detection volume is moved through the sample in horizontal and vertical dimensions. The beam scanner was used by moving 1 mm in one direction a rectangular deviation of 100 µm with a frequency of 50 Hz and an integration time of 50 µs. A piezo element was integrated in the optic of the FCS Olympus IX 50, which allowed a precise z-positioning of the focus in the 100 nm range.

2.3 Surface-FIDA

The surface-FIDA assay was carried out as described by earlier (Birkmann et al., 2007).

Briefly, 0.25 – 1 µg capture antibody was adhesively bound to poly-D-lysine activated glass surfaces. After blocking the unspecific binding sites with 10 % fetal calf serum, potentially present aggregates were bound to the capture by incubating 20 µl of a sample for at least two hours at 4 °C. After washing twice with PBS buffer (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4, pH 7.4), the fluorescence labelled detection antibodies were applied (0.1 µg/µl) and incubated for 1 h at 20 °C. After five washing steps with PBST (PBS with 0.1 % (w/w) Tween 20) and two washing steps with PBS, the measurements were carried out.

2.4 Preparation of Synthetic Aß-aggregates

Aβ(1-42) was purchased from JPT Peptide Technologies (Berlin, Germany). For aggregate preparation Aβ was dissolved in DMSO to 400 µM, diluted to 66 µM in PBS (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4) and incubated for five days at 37 °C. Aggregate formation was monitored using Thioflavin T (ThT) assay. For that, 10 mM ThT (Sigma, Hamburg, Germany) was added to the samples. Fluorescence was monitored with a microplate reader at excitation and emission wavelengths of 440 nm and 490 nm, respectively (Polarstar Optima, BMG, Offenburg, Germany).

For surface-FIDA, the aggregates were diluted 1:10 in pooled CSF of healthy people. CSF was obtained by Biochemed Services, Winchester, USA).

3 RESULTS

3.1 Methodical Setup

The new optical method for detection of protein aggregates is based on fluorescence intensity distribution analysis (FIDA). For detection of pathologic protein aggregates single molecule detection (SMD) combined with quantification of single aggregates immobilised on a relatively large surface was employed to achieve high sensitivity and specificity.

The new method, therefore, is called surface-FIDA. It is able to count single protein particles bound to a capture-antibody on the surface (fig. 1a) (Birkmann et al., 2007). Specific protein-particles are labelled simultaneously by two different antibodies for dual colour fluorescence intensity distribution analysis (2D-FIDA). Among the capture and both detection antibodies, at least two antibodies bind the same epitope. Thus, only aggregates but not monomers are counted. A laser beam scans the two-dimensional surface systematically in a double-meander mode. Thus, even single protein-particles are detected (fig. 1B). By utilising two lasers simultaneously two different fluorescence labels can be crosscorrelated. Only if the different labels are bound to the same aggregate both labels occur to the same time in small detection focus. A typical distribution of a coincident signal of double labelled aggregates is shown in fig. 1C.

3.2 Detection of Pathological Protein Particles with Surface-FIDA

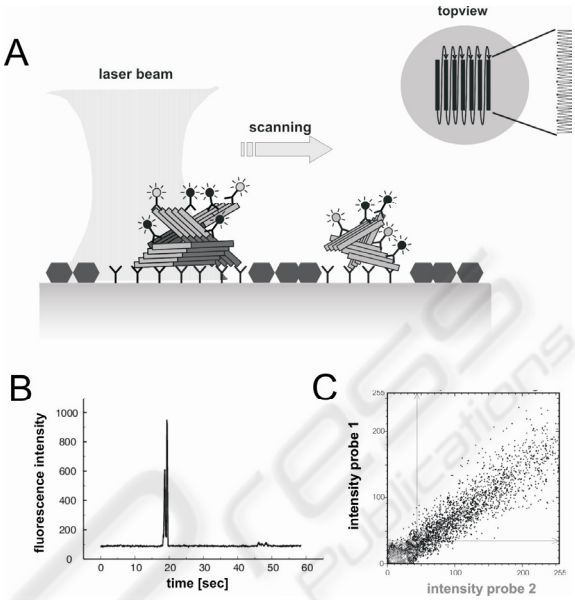

Figure 1: A) Scheme of surface-FIDA; B) fluorescence peak caused by the labelled aggregate: principal of particle counting; C) 2D-FIDA:Two probes with different fluorescence labels were used; Only simultaneous binding of both probes to the aggregates results in the specific diagonal signal distribution as shown in the plot

To observe, if the surface-FIDA setup is able to detect single aggregates different types of protein aggregates were tested.

3.2.1 PrP-particles Purified from Brain of Scrapie Infected Hamsters

Prion Protein aggregates were purified from brain homogenates of Scrapie infected hamsters in the clinical state of disease by NaPTA precipitation (Safar et al., 1998). The antibody R1 (Williamson et al., 1998) served as capture. The antibodies D13 and R1 were fluorescence labelled and utilized as detection probes. Same treated brain homogenates of healthy hamsters were used as control samples. The results of 2D-surface-FIDA in different distances to the surface are shown in fig. 2. In the samples of Scrapie infected hamsters at all distances between 10 µm and 20µm fluorescence peaks with high fluorescence intensity in both channels could be detected. At distances below 10 µm background signal in the control sample rise and in distances above 20 µm the signals of the Scrapie samples descended (data not shown).

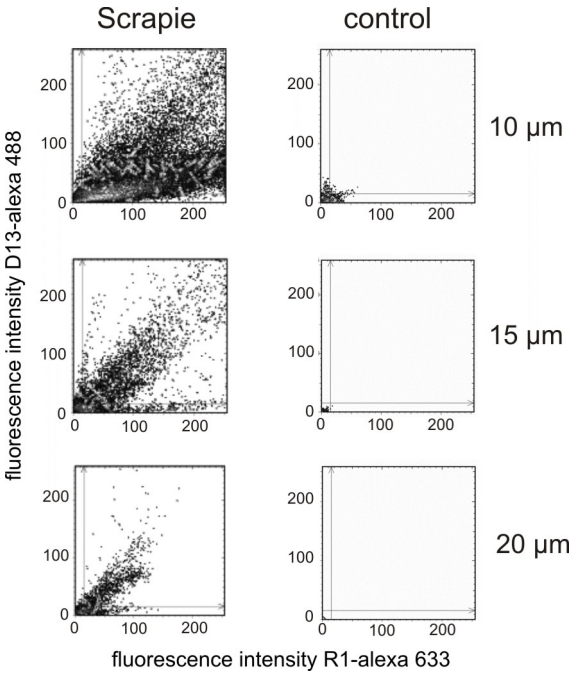

Figure 2: 2D-surface-FIDA of PrP-aggregates purified from brain homogenate of Scrapie infected hamsters and same treated brain homogenate of a healthy control in different distances to the surface 10-20 µm.

3.2.2 PrP-particles Purified from Brain of BSE Infected Cattle

Prion Protein aggregates were purified from brain homogenates of BSE infected cattle in the clinical state of disease by NaPTA precipitation (Safar et al., 2002). The antibody Saf32 (Krasemann et al., 1999) served as capture. The antibodies 12F10 and Saf32 were fluorescence labelled and utilized as detection probes. Same treated brain homogenates of healthy cattle were used as control samples. The results of 2D-surface-FIDA at different distances to the surface are shown in fig. 3.

3.2.3 Synthetic Aβ-Aggregates

As a first proof of principle, synthetical Aβ aggregates were used in the assay described above. First measurements were done in solution without immobilizing the aggregates. The antibodies 6E10 (N-terminal epitope) and 8G7 (C-terminal epitope) were fluorescence labelled and used as detection probes. Experiments were done in PBS. In the negative control, 0.2 % SDS was used to prevent Aβ aggregation, as monitored by ThT assay (data not shown). As expected, only aggregated Aβ resulted in fluorescence peaks as can be seen in fig. 4a.

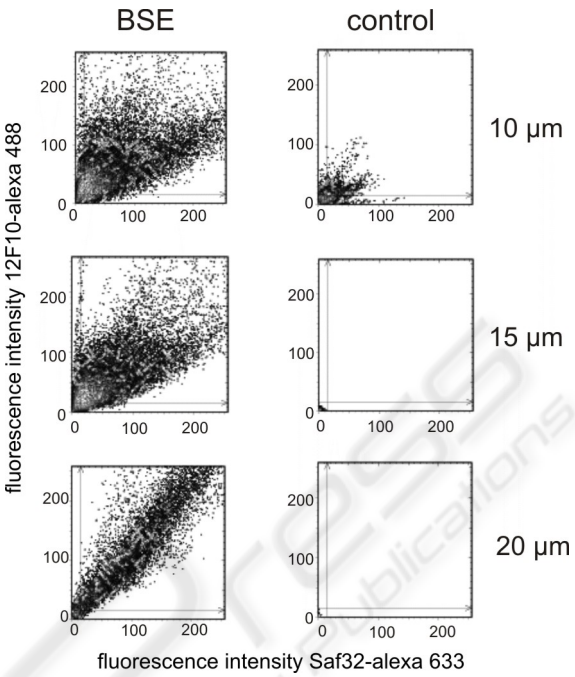

Figure 3: 2D-surface-FIDA of PrP-aggregates purified from brain homogenate of BSE infected cattle and same treated brain homogenate of a healthy control.

In a next step, Aβ aggregates diluted 1:10 in CSF to meet realistic conditions were immobilized on the surface of the slide. Antibody 4G8 (binding to amino acids 1-17 of Aβ) served as capture. The antibodies 6E10 and 19H11 were fluorescence labelled and served as detection probes. As both antibodies bind to the N-terminal part of Aβ, a simultaneous labelling of Aβ monomers was excluded. As controls, only CSF without additional Aβ aggregates was used in the immobilization procedure. The results of 2D-surface-FIDA are shown in fig. 4b. The measurements were done at 5 µm distance to the surface.

4 CONCLUSIONS

The proof of principle for the use of surface-FIDA to detect aggregates was shown for natural PrP-aggregates purified from brain of Scrapie infected hamsters, BSE infected cattle and for synthetic Aβ aggregates diluted in CSF.

Single particle counting as diagnostic tool is more sensitive as compared to measuring the integrated signal of all or many particles. “Single particle counting” allows measuring of multiple

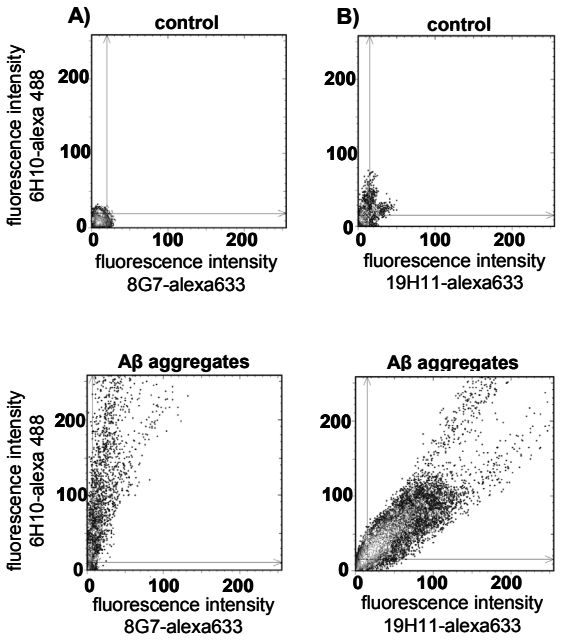

Figure 4: A) 2D-FIDA of synthetically prepared Aβ aggregates in solution (concentration 3,3 µM). Aβ, kept from aggregation by 0.2 % SDS, was used as control. B) 2D-surface-FIDA of synthetically prepared and in CSF diluted Aβ aggregates (concentration 6µM). CSF without additional Aβ aggregates was used as control. Measurements were done at 5 µm distance to the slide surface.

parameters of the individual particles are recorded like size, number of epitopes, different epitopes on the same particle etc. and those parameters can be used for improvement of specificity.

When the detection of single particles was carried out in suspension using the dual colour fluorescence intensity distribution analysis (2D-FIDA) (Birkmann et al., 2006), it was done in a small volume taken from a much larger sample volume by moving the laser detection focus through a cuvette. Diffusion of the particles and scanning of the volume were superimposed so that it was difficult to account quantitatively for all particles in the sample. Therefore the immobilisation of the particles on a surface had a major impact of the sensitivity of the whole assay, because it allows searching for the particles in a systematical way.

In the near future, we will develop surface-FIDA into an ultrasensitive diagnostic assay for particle associated disease, especially CJD and Alzheimer's disease. Such an assay will allow early diagnosis of AD and CJD using a minimally invasive approach in the living patient. In addition, such a diagnostic tool will be crucial for on line monitoring of disease progression and progress of a therapeutic approach.

Table 1: Sensitivity and Specificity Characteristics of surface-FIDA.

sensitivity	specificity
Concentration of particles on two dimensional surface	Simultaneous binding of three probes (one capture, two detection probes)
Single particle detection	Adjustable washing steps
Reproducible and complete counting of all aggregates by surface scanning	Detection of protein aggregates only, no monomers

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