Development and implementation of a synthetic oligomeric internal quality control sample for oligomer-based diagnostic assays

Marlene Pils[1,2], Alexandra Dybala[1,3], Fabian Rehn[1,2], Lara Blömeke[1,2], Detlev Riesner[1,3], Dieter Willbold[1,2,3] and Oliver Bannach[1,2*]

[1] attyloid GmbH, 40225 Düsseldorf, Germany

[2] Institute of Biological Information Processing (Structural Biochemistry: IBI-7), Forschungszentrum Jülich, 52428 Jülich, Germany

[3] Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany

*Correspondence to: Dr. Oliver Bannach, attyloid GmbH, Merowingerplatz 1a, 40225 Düsseldorf, Germany, o.bannach@attyloid.com, Tel.: +49-15126594700

Abstract

Objectives

Protein misfolding and aggregation are pathological hallmarks for a variety of neurodegenerative diseases. In Alzheimer's disease (AD), the soluble and toxic amyloid-β (Aβ) oligomers are a promising biomarker candidate in AD diagnostics and drug development. However, accurate quantification of Aβ oligomers is still hampered by limited reproducibility, assay sensitivity and specificity. Previously, we have introduced surface-based fluorescence intensity distribution analysis (sFIDA) featuring absolute specificity and sub-femtomolar detection limits for single particles, such as oligomers and aggregates. In this study, an internal quality control (IQC) sample was developed to improve standardization, quality assurance and routine applicability of sFIDA technology.

Design and methods

After establishing a standard aggregation protocol for monomeric $A\beta_{1-42}$, oligomers were characterized using Thioflavin T fluorescence spectroscopy and atomic force microscopy. Subsequently, the applicability of the oligomers in sFIDA was analyzed by investigating validation parameters and the use of a control chart for monitoring the IQC performance was exemplified based on three Aβ oligomer concentrations.

Results

We developed a protocol that allowed us reproducible preparation of an Aβ oligomer-based IQC sample, yielding a monodisperse size distribution of 2.65 nm as determined by atomic force microscopy. In the sFIDA assay, detection limits of femtomolar oligomers, high assay selectivity and dilution linearity over 5-log units were demonstrated. A Shewhart chart was implemented to monitor the performance of IQC sample over time.

Conclusion

Development and implementation of an oligomer-based IQC sample is another important step towards routine application of oligomer-based *in vitro* diagnostics in neurodegenerative diseases.

Keywords

Alzheimer's disease, Amyloid-β peptide, Oligomer-based diagnostics, Internal quality control, oligomers, diagnostics, biomarker, Shewhart chart

1 Introduction

Alzheimer's dementia (AD) is a progressing brain disease causing an increasing deterioration of mental abilities. AD is mainly characterized by misfolding and aggregation of amyloid-β (Aβ) peptides and Tau proteins into amyloid plaques and neurofibrillary tangles [1]. For decades, these deposits were considered as the major cause for disease onset and progression. However, it is now increasingly recognized that the soluble oligomeric species formed during the aggregation process are the major neurotoxic species in AD [2–4]. Consequently, these oligomers represent a widely used drug target as well as a promising biomarker candidate for early AD diagnostics. The minute amounts of oligomeric Aβ in body fluids such as cerebrospinal fluid (CSF, aM-fM) [4] as well as excessive concentrations of Aβ monomers and matrix components require extremely sensitive and specific quantitation technologies [5]. Moreover, oligomer quantification is challenging due to heterogeneous and transient nature of the oligomers [3].

Previously, we have developed the surface-based fluorescence intensity distribution analysis (sFIDA) technology as an oligomer-specific quantitation method with single particle sensitivity [6–8]. While the biochemical setup of the sFIDA assay is similar to sandwich ELISA, the readout is microscopy-based featuring sub-femtomolar sensitivity [5]. In sFIDA, Aβ species are captured on a glass surface by an N-terminal anti-Aβ-antibody and afterwards Aβ oligomers are detected by two different fluorescence labelled antibodies. Since capture and detection antibodies are competing for the same or overlapping epitopes, monomeric Aβ is not detected [9]. To count the number of oligomers in a sample, the glass surface is imaged by dual-color total internal reflection fluorescence microscopy (TIRFM). To reduce background noise, a cutoff, which is a pre-defined intensity value, is applied and only signals above the cutoff are evaluated as the sFIDA readout. Moreover, signal co-localization of both fluorescently labelled detection antibodies increases specificity and directly correlates with the Aβ oligomer concentration in the sample [9].

Recently, we have introduced and characterized stable silica nanoparticles (SiNaPs) coated with Aβ peptides as calibration standard, which improved assay comparability and the standardization process of oligomer-based diagnostic methods [10]. Additionally, an internal quality control (IQC) sample is required to standardize oligomer-based assay towards routine measurements [11]. The major objectives of quality assurance are to minimize variability arising from analytical, biological, and clinical sources to provide confidence in diagnostic test results, as well as informing the user of unacceptable or declining assay performance [12;13]. For monitoring the day-to-day (between-run) precision and accuracy, the implementation of an IQC sample is highly recommended [11]. While several assay manufacturers simply use dilutions of the calibration standard as an IQC, this approach is inappropriate for a number of

reasons. For example, if there is any deterioration in the calibration standard materials, the IQC sample will deteriorate as well [11;13]. Especially when using SiNaPs both as calibration standard and as IQC sample, respectively, such an effect can possibly arise. While natural oligomers can undergo structural changes and epitope masking in response to changes in buffer conditions or matrix effects [14;15], SiNaPs are very robust due to their artificial, silica-based nature. Hence, deterioration of assay performance may remain undetected for a considerable time. For the assessment of between-run precision, IQC readouts may be recorded and compared by suitable quality assurance tools like control sheets, Shewhart charts or Cusum Charts, respectively [11–13]. The scope of this work was to develop and characterize an Aβ oligomer-based IQC, and implement it in the sFIDA assay to demonstrate its applicability for oligomer diagnostic methods.

2 Material and methods

2.1 Formation of $A\beta_{1-42}$ oligomers

The aggregation protocol shown in this study was established by considering findings of other researchers in the field of Aβ aggregation [16–18]. 5 µg of $A\beta_{1-42}$ (Bachem AG, Bubendorf, Switzerland), lyophilized in 1,1,1,3,3,3-Hexafluor-2-propanol (HFIP, Sigma-Aldrich, St. Louis, USA), was solved in 5 µl dimethylsulfoxid (DMSO, Sigma-Aldrich, St. Louis, USA) briefly mixed, spun down and agitated for 10 minutes at 650 rpm (Eppendorf Thermomixer, Hamburg, Germany) at RT. Finally, the monomeric sample was diluted in PBS (Sigma-Aldrich, Missouri, USA) to a concentration of 10 µM containing 0.04% NaN_3 (AppliChem GmbH, Darmstadt, Germany), again briefly mixed, centrifuged, and agitated for 16 h at 650 rpm at RT to promote oligomerization.

2.2 Characterization of oligomers

2.2.1 Thioflavin T fluorescence spectroscopy

Aggregation kinetics of monomeric $A\beta_{1-42}$ into oligomers were monitored using thioflavin T (ThT, ultrapure, Eurogentec Ldt, Seraing, Belgium) assay. Upon binding to β-sheet rich deposits such as the cross β-sheet quaternary structure of amyloid fibrils, the cationic benzothiazole dye shows enhanced fluorescence with excitation and emission at 440 nm and 480 nm, respectively. In this study, a six-fold determination of the aggregation reaction was determined. To this end, 5 µM of ThT was added to freshly prepared $A\beta_{1-42}$ monomer solution (see 2.1) and the solution was applied on 96-well low binding plate (Corning, New York, USA) with 100 µl per well. As blank, three replicates of PBS containing 5 µM ThT, 0.04% NaN_3 and 5% DMSO were included in the measurement. The wells were incubated for 16 h at RT, and every 5 minutes the ThT fluorescence signal was recorded using a ClarioStar plate reader (BMG Labtech GmbH, Ortenberg, Germany). Background correction of the readouts was

obtained by subtraction of the mean blank values from the six sample signals. Finally, the blank corrected signals were normalized against the plateau values and plotted against the time.

2.2.2 Atomic force microscopy

Atomic force microscopy (AFM) generates detailed surface information at a nanometer scale and it was chosen for the size determination and distribution of the synthesized Aβ oligomers. A volume of 10 µl of the Aβ oligomer preparation was loaded onto a mica slide and incubated for 30 minutes at RT in a closed petri dish. The 10 µM stock of the oligomers was diluted to 1 µM in PBS beforehand. In order to prevent drying artifacts, a wet tissue was added to the petri dish. Additionally, 10 µM of solved monomeric Aβ (see 2.1) was prepared analogously as a control. The slide was washed 3x with 100 µl ddH_2O and subsequently dried with N_2 gas. The samples were measured using NanoWizard III (JPK BioAFM, Bruker Corporation, Billerica, USA) with an OMCL-AC160TS cantilever (Olympus Corporation, Tokyo, Japan) in intermittent contact mode (AC mode) in air. For size determination, three images (2x2 µm with a resolution of 512x512 pixels) were recorded with a frequency of 0.5 Hz. Assuming a globular shape of the oligomers, the height profile of a total number of 1300 oligomers was further analyzed with ImageJ using the “Find Maxima” tool. The determined height was equated to the size of the oligomers.

2.3 sFIDA

As described in chapter 1, sFIDA uses a biochemical setup which is similar to sandwich ELISA. However, the well surface is scanned by dual-color TIRF microscopy and a single particle readout is obtained. A detailed description of the assay procedure including synthesis of calibration standard, labelling of detection antibodies as well as image-data acquisition and analysis is presented in Supplement Section A1.

2.4 Analytical validation

2.4.1 Detection limits and Quantification limits

For the calculation of the Limit of Blank (LoB) and Limit of Detection (LoD), 24 blank control (BC) samples were analysed, and parameters were calculated according to Armbruster *et al.* [19] using Eq. **1** andEq. **2**.

Eq. 1 $$\mathbf{LoB} = \mathbf{mean\ sFIDA\ reaodut}_{\mathbf{BC}} + \mathbf{1.645} \times \mathbf{standard\ deviation}_{\mathbf{BC}}$$

Eq. 2 $$\mathbf{LoD} = \mathbf{mean\ sFIDA\ readout}_{\mathbf{BC}} + \mathbf{2} \times \mathbf{standard\ deviation}_{\mathbf{BC}}$$

Using the calibrated sFIDA readouts of the Aβ oligomer dilution series, the linear working range was defined by calculating the lower and the upper endpoint as well as the dilution linearity. Therefore, the concentrations that significantly differed from the next lower concentration were determined with one-sided Mann-Whitney U test using a confidence interval of 5%. Before

calculating of dilution linearity, background correction was performed by subtracting the BC value from each IQC sample value. Subsequently, the percent dilution linearity of each dilution step was calculated using Eq. 3. Within the working range, the mean dilution linearity should be between 80-120% and coefficient of determination should be higher than 0.95 to be accepted.

Eq. 3 $$\textbf{dilution linearity}\ [\%] = \frac{\textbf{observed concentration}}{(\textbf{previous observed value/dilution factor})} \times \mathbf{100}\%$$

For the assessment of intra-assay variability (within-run precision), the CV% of the four replicates of the same sample within the same run were calculated.

2.4.2 Analytical selectivity

The selectivity of sFIDA indicated by percent signal reduction (Eq. 4) was carried out by measuring IQC-13 (10 nM monomer concentration) sample in different assay setups. To exclude unspecific binding to blocking agent used, capture control was performed where capture antibody was omitted. To exclude autofluorescence events, the assay was performed without detection probes. In addition, the cross-reactivity of anti-Tau antibodies against the produced Aβ oligomers were also tested. To evaluate the insensitivity of sFIDA against monomeric Aβ species, 10 nM of freshly diluted monomeric $A\beta_{1\text{-}42}$ was applied. Simulating matrix effects, IQC-13 was spiked in bovine CSF and the sFIDA readouts, generated using 0.05% cutoff based CSF-blank were compared to the equal concentration in dilution buffer.

Eq. 4 $$\textbf{signal reduction}\ [\%] = (\mathbf{1} - \frac{\textbf{observed readout}}{\textbf{expected readout}}) \times \mathbf{100}\%$$

2.4.3 Establishment of QC-tool

In this study, a Shewhart chart, the most widely used tool for IQC [11], was used to monitor the readouts of 20 replicates of IQC-10, IQC-8 and IQC-6. To this end, observed particle concentrations were plotted as absolute values against the number of analyses. Using the mean and the standard deviation of the 20 observations, the lower and upper control limits (LCL/UCL) and action limits (LAL/UAL), respectively, were calculated according to Eq. 5 an Eq. 6 and were integrated into the control chart.

Eq. 5 $$\textbf{LCL/UCL} = \textbf{mean} \pm \mathbf{2} \times \textbf{standard deviation}$$

Eq. 6 $$\textbf{LAL/UAL} = \textbf{mean} \pm \mathbf{3} \times \textbf{standard deviation}$$

In general, values within the control limits are considered as satisfactory. Even if values are located between control and action limits, it is still accepted as long as it does not affect more

than 10% of the measured values. However, if one result of a sample occurs outside the action limit, or nine consecutive results create a trend (decreasing or increasing) or lie on one side of the central line, operator's invention becomes necessary [20]. Between-run precision is considered satisfactory if all results are unbiased, lie within the warning limits and the mean CV% of the 20 replicates are below 20%.

3 Results

The aim of this work was to develop and characterize an Aβ oligomer-based IQC, and implement it in the sFIDA assay. The first part of the results describe the characterization of the IQC sample, which was prepared using the aggregation protocol established in chapter 2.1. In the second part, implementation of the IQC sample in sFIDA was investigated by different validation parameter and finally, the use of Shewhart chart for monitoring the IQC performance was exemplified based on three IQC samples.

Aβ oligomer-based IQC sample displays high reproducibility and homogeneity

Formation of Aβ oligomers was monitored via Thioflavin T (ThT) kinetic analyses, indicating the conversion to cross-β-sheet structures, which are characteristic for aggregation of amyloid proteins. After a lag time of approximately 30 minutes, the ThT signal increased and reached a plateau value after 4 h. The plateau was constant within the experimental duration of 16 h. In total, six replicates were monitored, and the mean of the normalized ThT signals was determined (Figure 1). Minor standard deviation of the mean indicates reproducibility of the oligomerization process among the replicates. For additional characterization of the oligomers, AFM was performed to determine size distribution and morphology of the oligomers. AFM analyses revealed homogeneous and globular shaped particles (Figure 2a) indicating that the observed ThT signal was not due to formation of high molecular weight fibrils. In contrast, no structures were observed in the monomer control containing a 10-fold higher protein concentration. Size distribution analysis revealed that the median height of all 1,300 oligomers was 2.65 nm at a minimum size of 1.07 nm. Only 2% of the detected oligomers were ≥ 5 nm.

Application of Aβ oligomer-based IQC sample in the sFIDA assay

After characterization, we investigated the applicability of the prepared Aβ oligomers as IQC sample for the sFIDA assay. We prepared a 3.16-fold concentration series of the oligomers in dilution buffer and subjected each sample to sFIDA analysis in quadruplicate determination. The given molar concentrations referred to monomer concentrations ranging from 100 nM (IQC-15) to 0.01 pM (IQC-1). Based on the determined SiNaPs calibration curve (y=5.08*x-0.25), particle concentrations of Aβ oligomers of each IQC sample were calculated from the sFIDA readouts. Individual sFIDA readouts and calibrated particle concentrations for each IQC sample are listed in Supplement Section A2. Figure 3 shows the IQC performance in sFIDA, reflected by a 5-log dynamic range and an analytical sensitivity down to sub-femtomolar

particle concentration (LoB: 0.25 fM, LoD: 0.28 fM). LLoQ, ULoQ and an acceptable dilution linearity (acceptance range of 80-120%, see 2.4.1) defined the working range of the used IQC samples. The two quantification limits were identified using one-sided Mann-Whitney U test with a confidence interval of 5% (Supplement Section A2) and were set to a particle concentration of 0.36 fM (IQC-3) and 197 pM (IQC-14). Within this range, a mean percentage dilution linearity of 107% was determined (Supplement Section A3). Interestingly, a coefficient of determination of 0.73 indicated improvable dilution linearity. However, after exclusion of IQC-14 from the dilution series which yielded nearly the same readout than IQC-13, the coefficient of determination is increased to 0.99. Consequently, the percent dilution linearity also changed, although the value of 109% was subsequently still accepted. Furthermore, within this linear working range between 0.36 fM-196 pM, a mean CV% of 18.4% was observed, indicating acceptable intra-assay variance for single particle analysis.

sFIDA features high analytical specificity for Aβ oligomer-based IQC sample

By testing different assay controls, we confirmed that sFIDA is highly specific for aggregated Aβ species and robust against false positive signals due to matrix interferences and cross-reactivities. Figure 4a shows sFIDA readouts of IQC-13 applied on different assay setups. For all controls, a signal reduction of almost 100% was observed (Supplement Section A4a). It was shown that Aβ oligomers can only be detected if they have been captured on the assay surface via anti-Aβ antibodies but not in the absence of capture antibodies. Moreover, false positive signals generated by autofluorescence of chemicals and buffers could not be detected. Neither the use of anti-Tau antibodies as detection probe nor monomer (Figure 4b) as target caused false-positive signals due to cross-reactivities. Furthermore, after spiking the Aβ oligomers in CSF (Figure 4c, Supplement Section A4b), only negligible matrix effects were observed indicated by a minor signal reduction of 0.2%. In addition, matrix components in the CSF-blank reduced background signal more efficiently than the dilution buffer, indicated by a signal reduction of approximately 67% (CSF-blank vs BC). Consequently, the signal-to-noise ratio between IQC-13 and the respective blank was three times higher in CSF than in dilution buffer.

Shewhart chart as reliable QC tool for monitoring IQC performance

Control charts are an extremely valuable tool to monitor assay performance and can be utilized by any laboratory [20]. In this work, we demonstrated the use of a Shewhart chart to monitor the performance of three IQC samples in sFIDA (for respective TIRFM-images showing co-localized pixel above cutoff value see Supplement Section A5) by integrating 20 measurement points into each chart. Afterwards, charts were interpreted analogously to defined rules (2.4.3). All three IQC samples showed low inter-assay variability as the calculated mean CV% of the respective 20 observations was below 20% (IQC-6 = 16.2%, IQC-8 = 16.5%, IQC-10 = 17.6%). As illustrated in Figure 5, none of the IQC samples exceeded the action limit. In

addition, neither IQC-6 nor IQC-8 exceeded the control limits, but one value of sample IQC-10 occurred between control and action limit. Since this was the only deviation, assay performance was still considered acceptable within the predefined range. Also, no ascending or descending trend of nine consecutive observation points was seen in any of the IQC samples. Even if no out-of-control situation could be determined in any of the IQC samples, IQC-6 in particular should be monitored further, as a general downward progression became apparent. Moreover, an out-of-control situation may possibly arise in the foreseeable future if the next three observation points also occur between central line and the lower control limit. Operator´s invention may also be necessary for IQC-10, as it indicated a slight cyclic behavior after observation point 1 and 11.

Discussion

In the present study, we have developed a homogeneous and reproducible Aβ oligomer-based IQC sample for sFIDA and established a reliable QC tool for monitoring assay performance.

We characterized formation of Aβ oligomers by using ThT kinetic assay and AFM. ThT analyses showed comparable kinetics within six replicates suggesting high reproducibility of the oligomerization process. Oligomers are in thermodynamic equilibrium with monomers and larger aggregates, such as amyloid fibrils. For *in vitro* produced oligomers it was shown that most of the oligomers can dissociate into monomers within a few hours whereas only a small amount of oligomers convert to fibrils [21]. The oligomers produced for this study, however, do not dissociate within 16 h as indicated by a plateau ThT readout and therefore the IQC sample is likely to be stable during the assay procedure. Moreover, the conversion to fibrils can be excluded because no fibrillary species are detected either by AFM or sFIDA. Further characterization by AFM revealed homogenous, globular-shaped oligomers with an average size of 2.65 nm, whereby it should be noted that AFM analyses reveal the size of a dried oligomer instead of the hydrodynamic size.

In this study, the sensitivity of the assay was increased compared to our previously published data [5] and the detection of very small oligomers at sub-femtomolar concentrations, down to 0.28 fM (LoD), was possible, which qualifies the assay for biofluid-based diagnostics [4]. Compared to other oligomer-specific Aβ assays, sFIDA ensures high assay sensitivity. Even though a direct, exact comparison is not possible due to different assay setups, used calibration standards or different calculations for detection limits, we took a closer look at the femtomolar sensitivity ranges of different assays (see Table 1). Hence, the developed sFIDA assay achieved a sub-femtomolar detection limit, which is mandatory especially for the detection of Aβ oligomers in CSF that are present only in very low concentrations. Only the assay described by Savage *et al*. [22] (0.08 fM) or the commercial ELISA assay from Immuno-Biological Laboratories Co. Ltd. (IBL) [23] (31.4 fM), which also claims to detect single

particles, could offer a sensitivity in the low femtomolar concentration range, which is presumably due to the combined use of N-terminal and oligomer-specific assay antibodies. In contrast, all listed homotypic assays, using the same antibody for capture and detection, showed insufficient sensitivity levels between 91-720 fM. However, these assays [24–26], as well as the sFIDA assay of this study, are insensitive towards monomers due to the biochemical principle of epitope competition.

Here, we introduced a Shewhart chart to monitor the performance of three different IQC samples using the particle concentration of 20 different replicates for each chart. According to the defined rules for chart interpretation [20], no out-of-control situation was detected in any of the IQC samples, however, some consecutive measured points may indicate a trend or cyclic behavior. Especially for those assays subjected to quality management, such as the sFIDA technology, troubleshooting for such events or in case of out-of-order situations can be accelerated significantly, as possible causes related to the operator, instruments or lot numbers of assay components are regularly documented.

Based on the experiments performed in this study, we demonstrated that the Aβ oligomer-based IQC samples are reproducible, homogeneous and well applicable in the sFIDA assay. However, as our studies were performed in an artificial sample matrix, further validation including extensive recovery and parallelism studies should be obtained from authentic biological sample matrices like plasma or CSF [27]. Especially for CSF, the validation should be straight forward, since for IQC-15 we determined a signal loss of only 0.2% due to matrix effects. In contrast, the use of plasma could make validation more difficult, as strong matrix effects like interference between Aβ and human serum albumin or between circulating human antibodies with the assay antibodies are expected [15;28;29]. In addition to matrix effects, sample stability and sample tubing can also influence measurement signal [30;31]. As in future routine applications of sFIDA technology, the IQC sample should ideally be available as a ready-to-use kit component. Hence, the influence of bench-top stability and long-term stability of the IQC sample should be investigated by testing different sample tubes, storage temperatures and durations as well as the effects of repetitive freeze-thaw-cycles [27;32].

Conclusion

Since protein misfolding and aggregation are pathological hallmarks for a variety of neurodegenerative diseases, it is obvious to establish further IQC samples to improve the routine application of oligomer-based diagnostics such as sFIDA. Although for transition from research use only to *in vitro* diagnostics (IVD) the corresponding regulatory requirements must also be taken into account [9], the Aβ-oligomer-based IQC sample implemented in this study can be considered as another important step driving the standardization, routine application,

and ultimately the registration of sFIDA technology as a diagnostic tool for AD diagnostics for the IVD market.

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Authors contributions

MP developed the sFIDA assay. AD, MP and LB established aggregation protocol. AD and MP performed characterization and validation studies and analyzed the data. FR helped carry out the statistics. MP and AD wrote the manuscript together with OB. OB, DW and DR supervised the project. All authors participated in the discussion of the data, provided critical feedback and contributed to the manuscript.

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DW and OB are founders and shareholders of attyloid GmbH. DW and DR are members of attyloid's supervisory board. These had no influence of the interpretation of the data. All other authors declare no competing interests related to this work.

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