

Real-time detection and size distribution analysis of aggregates of amylin applying confocal fluorescence spectroscopy

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Abstract

In this report we demonstrate the ability of the ConSense™ analyzer to detect and analyze the formation of aggregates of proteins in a model system of amylin and the fluorescent dye Nile red. Measurements with the ConSense quickly provide information on the presence and the size distribution of aggregates in the samples.

Introduction

Aggregation of molecules often becomes a serious issue in drug development and demands a high sensitivity for measurements at high dilution. This is especially true when it comes to sample conservation and drug formulation.

Aggregate formation depends on a whole set of parameters such as temperature, pH, ionic strength and the concentration of the protein. Thus, the ability to quickly detect aggregates of proteins is an important tool in the development of formulations.

Materials and method

Amylin is a 37 amino acid polypeptide. The related amyloid beta (A β) and its aggregates are known to be the main component of plaques in Alzheimer disease. Thus, the aggregation mechanisms of amyloids are quite well studied.

In this study we use the dye Nile red to stain amylin aggregates at concentrations from 0.02 nM to 10 μ M. In the absence of amylin aggregates the dye shows only little fluorescence and, hence, Nile red is well suited for this purpose.

The concentration of Nile red was 100 nM, well below the maximum water solubility of approx. 1 μ M.

Nile red shows excitation and emission maxima at 540 and 640 nm respectively.

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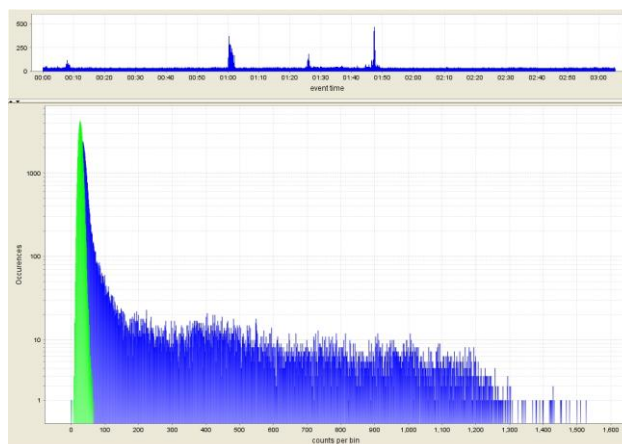


Fig.1 Intensity trace (top) of a mixture of 100nM Nile red and 10 μ M amylin, building aggregates (blue) vs. Nile red without amylin, Aggregates with intensities of 500 counts per second can be observed. The photon counting histogram (below) shows the high abundance of passages with high intensity.

A stock solution of amylin was freshly prepared, mixed with Nile red and diluted with PBS buffer to obtain the final concentration. The mixture was allowed to react for one hour at room temperature.

Instrumentation

Measurements were conducted with the confocal ConSense Analyzer using the 532 nm laser at 160 μ W. The read time was 60 seconds. The samples were presented in fluIT's 14 channel standard glass chips.

Results and discussion

Fig. 2 displays the different aggregation states for amylin concentrations of 10 μ M, 100 nM and 0.05 nM. The mean brightness gives a measure for the amount of Nile red integrated into the aggregate and also the size of the aggregate itself.

The three aggregate populations are very different from each other and high brightness aggregates only appear in the micro molar amylin concentration range (blue data).

Also the 100 nM amylin concentration shows some aggregation (red data set) when compared to the 0.05 nM concentration.

This indicates that oligomers of amylin already form at rather low concentrations and quickly under the chosen conditions. This observation emphasizes the importance of amyloid oligomers for the formation of aggregates.

The ConSense thus provides a simple and rapid method to detect aggregates online and to investigate the mechanism of aggregation.

Additionally, it gives information on the size distribution based on the observation of individual aggregates rather than averaged parameters for aggregation.

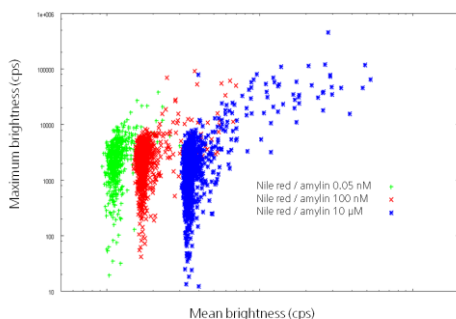


Fig. 2: Plot of the molecular brightness of amylin aggregates against the mean brightness. The mean brightness gives an indication for the amount of Nile red molecules attached to each aggregate and therefore also for the size of the individual aggregates.

The raw data traces of nanomolar amylin samples (Fig.3) show differences in the occurrence and the intensity of events from amylin oligomers. Further work has to be carried out to resolve and quantify these differences.

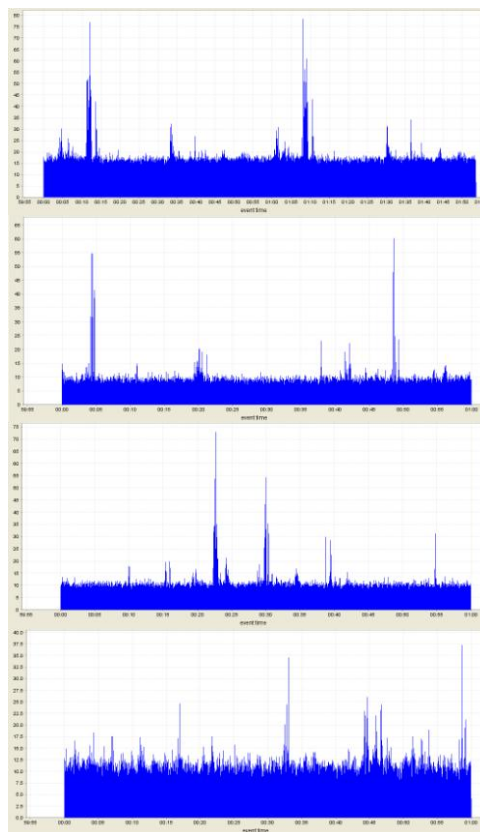


Fig. 3: Intensity traces of mixtures of 100 nM Nile red with 100, 12.5, 0.8 and 0.05 nM amylin (read from top to bottom). Each peak indicates the transit of an agglomerate through the confocal detection volume. Frequency, intensity and width of the peaks reflect the number of agglomerates and their individual sizes. Maximum intensities of peaks were 80, 65, 70 and 32 counts per seconds respectively (scale is changing).