

Use of the FLUOstar OPTIMA Microplate Reader to Monitor Amyloid Formation

Sarah Shammass and Ann-Christin Brorsson
Department of Chemistry and Cavendish Laboratory, University of Cambridge

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- Abberant protein aggregation implicated in several degenerative diseases
- Protein aggregation kinetic followed with FLUOstar OPTIMA
- Custom-made script allows for timed ex situ experiments

Introduction

Amyloid fibrils are implicated in a number of diseases, known as amyloidosis, including type II diabetes and Alzheimer's disease. Each disease is associated with the misfolding of a particular protein into linear aggregates (fibrils) that accumulate in the body's organs as plaques¹. Various different species may be formed during the aggregation process, including linear precursors known as protofilaments, which often appear in the early stages.

Although only a handful of proteins are implicated in amyloid-related diseases there is evidence that amyloid formation could be a generic property of the polypeptide chain i.e. during appropriate (partially destabilising) conditions, "any" protein will form amyloid fibrils². Despite this property it is clear that the amino acid side chains contribute to the stability and the morphology of amyloid aggregates, as well as the kinetics of their formation³. In this study various mutants of an amyloid forming protein V have been designed to investigate the effect of the mutation on the aggregation behavior.

One group of fluorescent dye's which have been shown to bind to amyloid fibrils are the anilinothalene sulphonic acid's (ANS)⁴. These dye's are not "amyloid-specific"; they bind to proteins via exposed hydrophobic patches on the surface, and are traditionally used to demonstrate the presence of unfolded or partly folded proteins. In this study the dye bis-ANS has been used to follow the aggregation process of protein V and mutants to give information about the kinetics and the hydrophobic properties of the formed amyloid aggregates.

In this report we demonstrate that the BMG LABTECH FLUOstar OPTIMA microplate reader (Fig.1) can monitor amyloid formation by performing fluorescence kinetics studies. We have made particular use of the script mode feature, allowing us to use a simple procedure for making timed ex situ measurements. The model protein V that has been used in this study is associated with amyloid disease, and our result has enable us to gain information about the intrinsic determinants of protein aggregation which could be of significance in understanding the underlying biochemical mechanism of amyloid pathogenicity.



Fig. 1: BMG LABTECH's FLUOstar OPTIMA multidetection microplate reader

Materials and Methods

1. Aggregation kinetics monitored by bis-ANS fluorescence

To prevent any interference of the aggregation process by including dye in the reaction mixture, ex situ measurements were made (aliquots of the aggregation mixture being removed at various time-points and added to a buffered solution of bis-ANS (obtained from Sigma-Aldrich, UK, Ord. No. 49058) for fluorescence measurements). This approach means that each well in the well-plate represents a single measurement in the kinetic assay. It is therefore not possible to use the standard kinetic evaluation program from BMG LABTECH which records temporal changes in the fluorescence signal from the same well only. Instead we have designed a script using BMG LABTECH's Script mode that allows us to accurately probe the fluorescence properties at each time-point.

The script runs a series of standard fluorescence protocols in a defined order allowing fluorescence measurements for consecutive wells. Additionally, it moves the wellplate in and out at appropriate times to assist sampling, and records the reaction time for each sample. By selecting the data to be exported to a CSV file, the results of the consecutive measurements (and the corresponding reaction times) can be recorded in a single file which can be opened and analyzed by a range of commercial and freeware programs.

Procedure

Buffer A: 10 μ M bis-ANS in 50 mM sodium phosphate buffer pH 7.4
Buffer B: 50 mM sodium phosphate buffer pH 7.4

- Protocols, one for each well measured, should be prepared
The protocols themselves are all identical apart from the well number. The instrument settings are as shown:
 - Type: Fluorescence Slow kinetics
 - Excitation filter wavelength: 400nm
 - Emission filter wavelength: 500nm
 - Temperature: 30°C
 - No shaking
 - Select CSV file format – also select the following options:
 - append data to the end of file (rather than replacing previous data)
 - output filename as <ID1>
 - report style such as Short Header which outputs the relevant variables e.g. our script uses <ID2> to represent the reaction time
 - Insert the plate into the FLUOstar OPTIMA
 - Open script
 - Edit script: choose a filename for the CSV output i.e. <ID1>
 - Save and run script (all actions are prompted by script from now on)
 - Pipette 45 μ L of A and 5 μ L of B into the first well as a control.
 - First measurement is made.
 - Pressing enter starts a countdown. After 30 s a beep sounds – this is a prompt to start the aggregation by adding buffer B to protein V to a protein concentration of 30 μ M.
 - Pipette 45 μ L of A and 5 μ L of aggregation mixture into the next well
 - Measurement is made
- Repeat x-xi until sufficient data has been collected, and then stop script.

2. Final Bis-ANS fluorescence intensities for the different mutants

Standard concentration curves were constructed by using the same procedure as in 1. Various volumes (instead of 5 μ l) of mature fibrils (30 μ M) were added to 45 μ l of buffer A. The concentration of bis-ANS was kept constant by addition of buffer B to a final mixture volume of 50 μ l.

Results and Discussion

Figure 2 shows the kinetic plot of the aggregation process for two mutants of protein V (X and W). At time = 0 s protein X has a very low fluorescence signal, this increases until it reaches a fairly stable level at around 7000 s. The increase likely represents conversion of the monomer into mature fibrils with hydrophobic surfaces. The fluorescence with protein W increases over the same timescale as protein X, however the signal for protein W is consistently around 2500 a.u. higher than the signal for protein X. The non-zero initial fluorescence of protein W suggests the presence of aggregates, formed rapidly within the dead time of the experiment, that are able to bind bis-ANS. That these are not formed for protein X is interesting since it is generally thought that early oligomers represent the toxic species in amyloid diseases (rather than the fibrils)^{5,6}.

The higher fluorescence signal found for Protein W after stabilization of the bis-ANS signal suggests that the mature aggregated species formed from this mutant are also more hydrophobic than species formed from protein X. Differences in the hydrophobic nature between aggregated species are also interesting to study since a hydrophobic species could have the potential to mediate toxicity by interacting with the cell membrane in vivo.

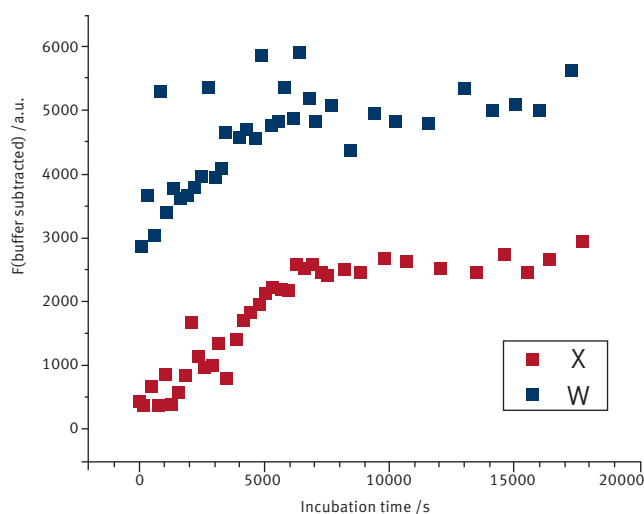


Fig. 2: Kinetics of aggregation of proteins X and W monitored by ex situ bis-ANS fluorescence.

The bis-ANS binding of protein V and another mutant Y was also investigated at longer times. Proteins were left under aggregating conditions for 3 weeks allowing fibril formation. Aliquots were then removed to create a standard concentration curve for each protein (see Figure 3).

Linear fits to the data were made and the gradients and R values for each protein are shown in Table 1. A good fit was obtained for protein V, however that for protein Y was poor despite a reasonably high fluorescence intensity. This may reflect a very heterogenous population of species (observed by TEM, data not shown). Protein Y also has a lower fluorescence signal than protein V, indicating that less hydrophobic surfaces are exposed.

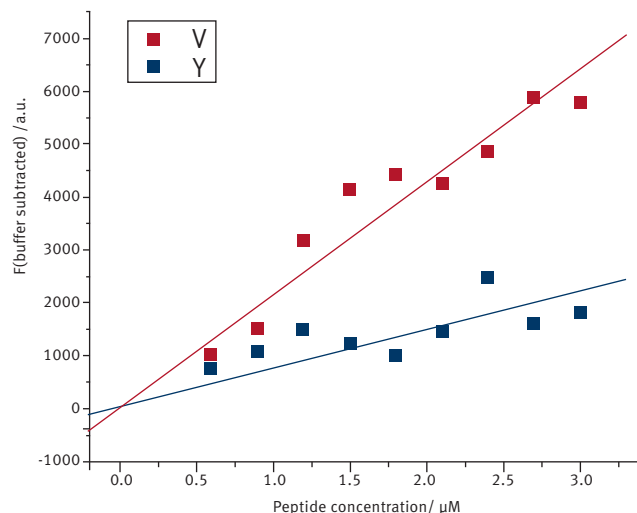


Fig. 3: Bis-ANS fluorescence intensity as a function of protein concentration (bis-ANS concentration is constant between samples) for the proteins V and Y.

Table 1: Linear fits for each standard curve of fig. 3

Protein	Gradient	R
V	2135 ± 91	0.95
Y	740 ± 76	0.72

Conclusion

In this report we have demonstrated that the FLUOstar OPTIMA can be used to follow protein aggregation kinetics using fluorescence measurements. In particular we have shown that using the script mode it is possible to make timed ex situ measurements fairly simply, creating an output into a single CSV file.

References

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Germany: **BMG LABTECH GmbH** Tel: **+49 781 96968-0**

Australia: **BMG LABTECH Pty. Ltd.** Tel: **+61 3 59734744**
 France: **BMG LABTECH SARL** Tel: **+33 1 48 86 20 20**
 Japan: **BMG LABTECH JAPAN Ltd.** Tel: **+81 48 647 7217**
 UK: **BMG LABTECH Ltd.** Tel: **+44 1296 336650**
 USA: **BMG LABTECH Inc.** Tel: **+1 919 806 1735**

Internet: **www.bmg-labtech.com** info@bmg-labtech.com