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HTTP://DX.DOI.ORG/10.5504/BBEQ.2013.0094 BE
BIOTECHNOLOGICAL EQUIPMENT

NEW APPROACHES TO RAPID SCREENING FOR PROTEIN AGGREGATES BY LIGHT SCATTERING

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ABSTRACT

This paper reports on a recent measurement innovation, the PAM Zero (Protein Aggregation Monitor), that combines state-of-the-art fibre optic technology with a novel measurement chamber to allow as little as 2 μL of protein solution to be analysed while retained in a pipette tip. This eliminates the need to waste scarce sample by dispensing into a cuvette or cell, reduces the need for dilution and prevents cross contamination. Software design has also focussed on making the user interface as simple as possible so that PAM Zero can be used by a bench scientist with minimal training to measure the amount of aggregate present in as little as 4 seconds, reducing the analytical load on central analytical facilities and saving time by rapid rejection of inappropriate samples from the research pipeline.

Results from a variety of early studies using PAM Zero and its pre-production prototypes to measure protein aggregation will be presented that demonstrate the benefits of this new method. The paper concludes with suggestions on how to enhance the measurement technology for use in protein structure research using an automated user interface and multivariate analysis algorithms to enable the prediction of suitable crystallisation conditions and accelerate structure determination.

Biotechnol. & Biotechnol. Eq. 2013, **27**(6), 4327-4331

Keywords: light scattering, protein aggregation

Introduction

Various light-scattering (LS) techniques, sometimes combined with molecular separation systems, have been used over the years to study macromolecules, viruses, bacteria, micro-organisms, and other nanoparticles (7, 8), but all suffer from a variety of drawbacks limiting their usefulness for the study of complex biological systems in early stage drug discovery research. These range from selecting concentration ranges that best suit the analysis theory used rather than measuring aggregation at the level required for other experiments, shear breakdown of aggregates in a size exclusion chromatography column, through to the need to consume significant amounts of scarce purified protein merely to determine aggregation levels, leaving insufficient for subsequent detailed analysis.

In addition to technical issues surrounding current LS methods, their use for routine analysis of protein aggregation, whether as a static screening technique or to follow reaction kinetics, has been restricted by cost and complexity. Moreover, they often require the creation of dedicated analytical facilities away from the lab bench and recruitment of high-level analytical personnel to interpret the data collected. Since the main reason for screening purified proteins is to accelerate the identification of candidate molecules and/or crystallisation conditions to reduce costly analytical bottlenecks, these limitations have severely constrained the use of LS in this application.

We have considered the innovation, design and development issues associated with the implementation of a novel instrument intended for the bio-pharmaceutical industry (6). Our analysis of the biopharmaceutical research lab workflows and the economics of drug development lead to a recognition that there was a market opportunity for a simple light scattering instrument that is low cost, small size, simple to use and uses minimal sample volume.

Fig. 1. Light-scattering evolution showing the traditional market leader (bottom), original AggreKem with built in PC (top), and PAM Zero (front). Reprinted with permission from White et al. (6), *Mechatronics and Manufacturing Engineering: Research and Development*, Woodhead Publishing Ltd., ISBN 0 85709 150 6.

This realisation resulted in the development of a detector called AggreKem. The AggreKem had a minimum sample volume of 5 μL and used fiber optic technology to keep overall footprint small. In addition, it was designed with a built-in Windows PC with custom software that enabled the aggregate content to be measured quickly without the need for a desktop PC and a computer keyboard (**Fig. 1**). Despite the fact that the AggreKem was very well received by the customer base when launched, in particular for screening protein solutions for the presence of aggregates prior to crystallization, the instrument did have some key limitations. The main one was the significant back pressure generated when injecting a sample because of the low internal diameter tubing used. This was making it difficult to achieve the minimum sample volume reproducibly and demanded increased sample volumes to be used.

Responding to this experience, the light-scattering detector was re-designed to produce the PAM Zero (Protein Aggregation Monitor) shown in **Fig. 1**. This retained the earlier fiber optic technology, but the flow path/sample injection system has been replaced with a simple port that enables the tip of a laboratory pipette to be inserted. This design allows the measurement to be performed within the sampling tip, effectively a zero-volume measurement since the 2 μL sample aliquot taken up in the pipette can be returned to a sample tube after analysis. The software was rewritten in JAVA to make it possible to be used with the multiplicity of operating systems now available in the lab, and data is collected using a simple USB connection. In addition, the ability to automate kinetic measurements has also been added. The net result is a hand-held, simple to use detector that uses minimal sample volume and is half the price of an AggreKem despite the enhanced capabilities.

Materials and Methods

In order to test PAM Zero design principles discussed above we used as protein samples Proliferating Cell Nuclear Antigen (PCNA), Retinoblastoma protein (Rb) and Amyloid β Protein Fragment 1-40 (Ab(1-40)). PCNA and Rb were purified as described earlier (2, 9) and Ab(1-40) was purchased from Sigma–Aldrich Co.

Protein samples were analysed using either the proof of concept demonstrator or a pre-production PAM Zero unit. In both cases, the software developed for the production detector was used to collect data and calculate the level of aggregation in each sample tested, expressed as the % Excess Scatter determined from the previously known protein monomeric MW and solution concentration, which was determined using an AstraGene UV/Vis Low Volume Spectrophotometer. AstraGene uses a similar "In-Tip" measurement technology to PAM Zero, allowing both measurements to be undertaken on the same 2 μL sample volume that is then either replaced in a vial for storage or transferred to another analysis system.

For all results contained within this paper, the measurement time was around 5 s, between 2 μL and 10 μL sample volumes were used, and each individual measurement was determined as the mean of 3 000 scattering measurements collected in a 1 s time interval. This average was subjected to proprietary data filtering algorithms to reduce the effect of background noise caused by low level nano-particulates in the buffer solutions that can skew LS measurements.

Results and Discussion

Aggregation screening prior to protein crystal growth

It has long been known that the presence of aggregates in protein solutions prior to concentrating for crystal growth is a strong indicator that the result will be a poly-crystal rather than the single crystal needed for structure determination by x-ray crystallography (3, 4, 5). There is, therefore, considerable interest in being able to develop a low-cost, rapid screen for the presence of aggregates in a given start solution that is capable of determining within a few seconds if the crystallisation conditions chosen are likely to work or not, rather than wait several days to find out.

For this evaluation, a PCNA protein sample was supplied that had been previously studied using x-ray crystallography after the correct crystallisation conditions had been determined by trial and error (1). It was dissolved in four different buffer systems typical of those used to start to identify the correct conditions by traditional methods, three at pH 8 and pH 7.5, and one at pH 3, deliberately chosen to promote aggregation. The concentration of each solution was nominally 5 mg/mL.

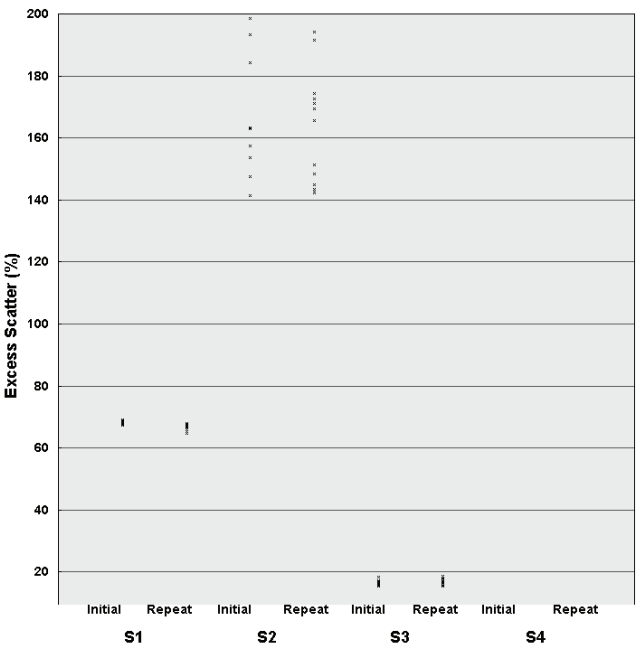

Fig. 2. Crystallisation conditions for PCNA – initial aggregate content.

A 10 μL aliquot of each protein/buffer combination was placed into the detector, then 20 measurements were collected at a measurement time of 1 s. To check on repeatability, once all four solutions had been tested, a fresh aliquot of each was taken and the measurements repeated. **Table 1** gives the mean

results calculated for each data set, while **Fig. 2** plots the individual results taken to provide an idea of repeatability.

TABLE 1

Crystallisation conditions for PCNA

Buffer Conditions	% Excess Scatter	
	Initial	Repeat
S1 - Hepes/NaCl - pH 8	68	67
S2 - Citric acid/NaCl - pH 3	195	185
S3 - Na_3Cit/Hepes/NaCl - pH 8	16	17
S4 - PEG3350/Tris·HCl - pH 7.5	1	1

PAM Zero correctly identified S4 as the buffer used to successfully grow a single crystal for structure determination. The entire experiment (excluding initial sample preparation) took approximately 5 min to complete.

Aggregation inhibitor efficiency testing

There is a lot of interest currently in the study of how to slow down long-term protein aggregation in the body as a treatment for various degenerative diseases. Research has focused on the use of inhibitor molecules to achieve this goal coupled with advances in the early diagnosis of such conditions with the aim of delaying the onset of visible symptoms and cognitive impairment.

Because of the difficulty/cost involved in isolating and purifying amyloid proteins for testing, coupled with the low MW (~4 KDa) of the monomer unit, traditional test methods involve the use of fluorescence measurements, which requires complex sample preparation to attach suitable tags. As well as the time taken to prepare such samples, researchers are concerned about the effect attaching a fluorescent tag to the protein molecules has on the way aggregate formation occurs. Also, while the detection method is very sensitive, most fluorimeters require large sample volumes (well in excess of 100 µL) to be prepared that cannot be re-used. PAM Zero overcomes these problems, so tests were performed to check on whether it had the sensitivity and speed to be used as an alternative screen for the measurement of inhibitor efficiency.

A series of samples of Ab(1-40) were supplied, having been mixed with different proprietary inhibitors then incubated at 37 °C for 24 h to induce aggregation. A control sample of pure Ab(1-40) was prepared according to the same protocol and supplied for comparison. Each sample solution supplied was measured in triplicate using PAM Zero, and % Excess Scatter measured for each. Because the solutions were all pre-aggregated, the start M_w for the amyloid protein was not accurately known in this case, so the results measured using PAM Zero were normalised to the control sample that was set at 100 % aggregated. In this way, the ability of each candidate inhibitor to reduce the rate of aggregation could be quoted in terms of a reduction from the 100 % control level. The results obtained are plotted in **Fig. 3**.

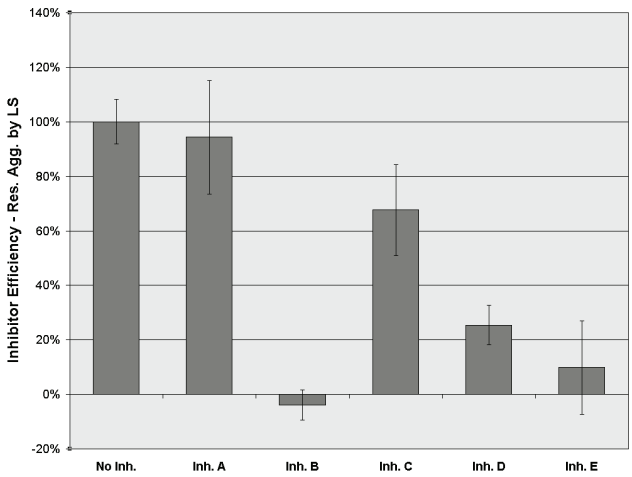

Fig. 3. Comparison of amyloid protein aggregation in the presence of inhibitors measured by PAM Zero.

Total analysis time (excluding sample preparation) was 3 min. Inhibitor B and E were clearly the best candidates for further detailed research, eliminating the need to progress further with A, C and D.

Since there was no need to attach fluorescent tags, the only sample preparation involved in this study was to incubate for 24 h to initiate aggregation. LS was clearly demonstrated to have significant benefits over traditional methods, while the speed of measurement demonstrated potential for using PAM Zero to follow the kinetics of aggregation reactions in real time – something that is very difficult to achieve and costly using a fluorimeter.

Aggregation kinetic studies on amyloid proteins

To test the findings above, a sample of Ab(1-40) mixed with Inhibitor B was incubated at 37 °C for 24 h to induce aggregation, then monitored by LS for 6 h to determine the rate of disaggregation due to the inhibitor. The PAM Zero software was modified to measure the LS signal from the solution as a function of time without calculating the %Excess Scatter. In this way, simple reaction rate curves could be produced to show the rate of either aggregate growth or, as in the present study, disaggregation due to the presence of an inhibitor molecule.

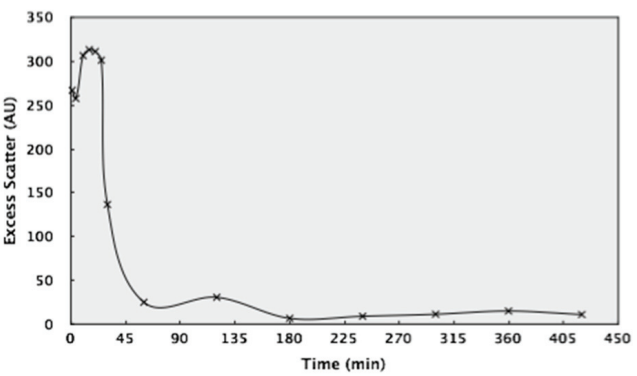

Fig. 4. Dis-aggregation kinetics of Ab(1-40) mixed with an inhibitor.

The results obtained for the amyloid protein sample are shown in **Fig. 4**. For this study, the time interval used was 5 min for the first hour, then hourly, though the PAM Zero software can measure at intervals of 10 s for monitoring rapid reactions. The LS results showed that initially, there was a slight increase in the scattering probably caused by the formation of a few large, loosely entangled aggregate clumps during mixing that rapidly broke up. When viewed using fluorescence detection, which measures the total number of fluorescent molecules rather than their average size, this was not observed and the overall curve produced suggested that the inhibitor was not as efficient as was actually the case when a size-weighted (i.e. LS) measurement technique is used. Thus, LS is not only faster, cheaper, and less wasteful of sample, it also provides finer detail as to the kinetic mechanisms that assist the researcher in designing new treatments.

Protein aggregation QC screening: comparison between PAM Zero and traditional Size Exclusion Chromatography (SEC) methodologies

Final area of applicability for a rapid, simple, low-cost detector that uses minimal sample is in Quality Control (QC) screening of protein solutions where age-induced aggregation can shorten shelf life. This last study was designed to evaluate the performance of PAM Zero as an alternative/adjunct to the traditional, but time-consuming, HPLC/SEC methods routinely used in modern biopharmaceutical QC test protocols.

A series of model aggregated protein samples were prepared in a PBS buffer by mixing Rb monomer with known percentages of a deliberately aged (aggregated) solution of the same protein. A range of samples containing 1 % to 10 % aggregated protein in the monomer solution were provided as well as pure monomer (0 %) and pure aggregate (100 %).

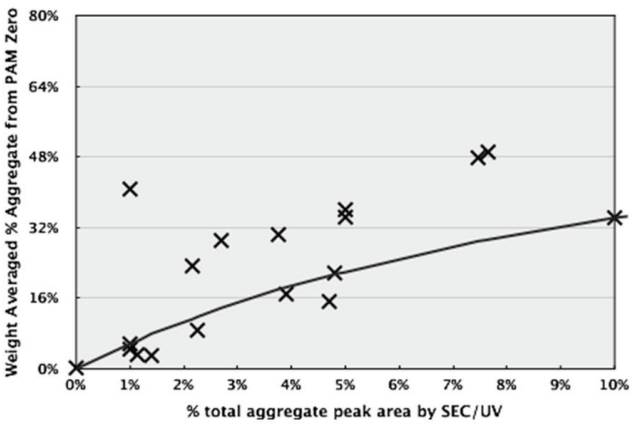

Fig. 5. Pass/Fail test by PAM Zero.

Each of the mixed samples had been analyzed using a traditional calibrated protein SEC/UV separation system to determine the overall aggregate concentration and oligomeric distribution. For each SEC analysis, the run time was 20 min and 100 µL of sample was injected. Each sample was then measured using a PAM Zero and AstraGene UV spectrophotometer to determine the concentration and weight average molar mass (M_w) of each sample provided. As it was difficult to directly relate the average aggregation levels determined by PAM Zero without any size separation to isolate the oligomers present with the SEC results supplied with the samples, it was decided to convert both sets of results to percent aggregate values by dividing the sample M_w by LS with the monomer M_w also determined by PAM Zero. The results were then plotted as Weight Average Aggregate Level (LS) versus Number Average content (SEC) as shown in **Fig. 5**.

The solid line was calculated using standard molar-mass averaging algorithms using the dimer/trimer/tetramer etc ratios contained in the 100 % sample and the amount of the aggregated fraction mixed with the monomer. This gives the theoretical relationship between M_n derived values from SEC/UV and the M_w response determined by LS. Despite the different measures of percent aggregation derived from the two techniques, the results obtained for the majority of the tested samples (65 %) were found to lie along the theoretical curve, confirming the relationships used were valid for subsequent screening by PAM Zero alone. The remainder of the tested samples all showed evidence of additional aggregation, resulting in light-scattering measurements higher than predicted from the mixing calculation used. It was subsequently discovered that these had been subjected to additional aging by being kept at room temperature for 1 day prior to testing, rather than at 4 °C. Alternative relationships could be calculated to fit the additional aggregation in these samples, but these are not shown.

As a result of the experiments undertaken, it is clear that considerable time savings could be produced in a QC lab undertaking batch sampling of protein solutions to monitor shelf life if, instead of running a test protocol only involving SEC/UV, a simple set-up protocol is run in the first instance by SEC/UV that would identify the correct PAM Zero measure of average aggregation by LS to use as the Pass/Fail criterion, then switch all batch testing to the PAM Zero instrument. Using this study as an example, if 19 samples are run in triplicate for each batch of samples required by the laboratory protocol, the analysis time and sample usage in each case would be as follows: for HPLC/SEC – analysis time = 19 h, sample volume destroyed = 5.7 mL, while for PAM Zero – analysis time = 24 min, sample volume destroyed = 0 µL.

Conclusions

This study demonstrates the capabilities of the PAM Zero instrument to analyse aggregation content and aggregation kinetics of biomolecules. The results show that the instrument offers an easy way for crystallographers to screen samples prior to crystallization in order to accelerate structural work. The presence of aggregates that could lead to polycrystal formation can be detected within seconds. In addition, PAM Zero can be used for direct measurement of aggregation phenomena, without the need for fluorescent labelling. The instrument is

able to plot reaction curves in real time without any complex sample preparation.

The main advantages of using PAM Zero over other methods are the lower sample volumes, reduced operating costs, faster analysis, enhanced efficiency and the small size of the instrument. Moreover, PAM Zero software is platform independent and can be attached to any Windows, MAC or LINUX PC via a USB connection that can integrate the data analysis software.

Disclosure

PAM Zero is a product of Norton Scientific Inc.

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