Research in a Diagnostic Method for Light Chain Amyloidosis

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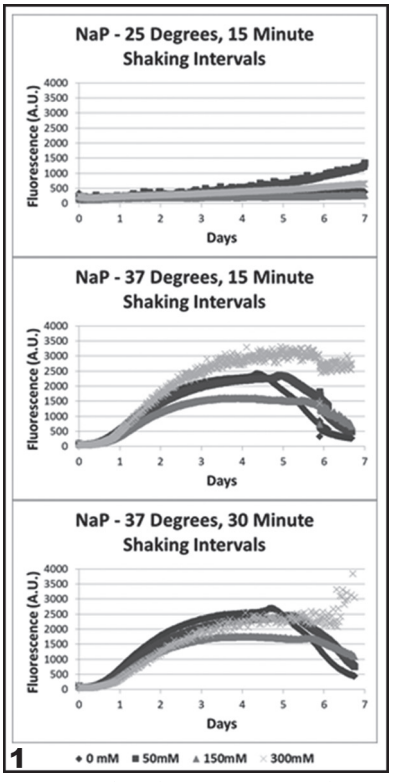
Abstract:

Multiple myeloma (MM) is a disease where a plasma B cell clone is excessively replicated in the bone marrow. Systemic light chain amyloidosis (AL) is a complication in some MM patients in which excess light chain (LC) proteins from the plasma B cell clones deposit as amyloid aggregates in various organs, mainly the heart and kidney. The diagnosis of AL and its differentiation from MM is challenging and traditionally is done through invasive tissue biopsies [1]. Early handling of AL is important for effectively treating the condition, so simple and effective diagnostic methods are desired. Our goal was to develop a technique for AL diagnosis based on LC proteins from MM patients excreted in urine using an *in vitro* aggregation assay. First, *in vitro* amyloid fibril formation was optimized in order to create fibrils. Buffer pH, NaCl concentration, and temperature were varied in aggregation assays, and the aggregation kinetics were monitored using the amyloid-specific dye Thioflavin T (ThT) [2]. Samples were also shaken during aggregation, and the time intervals between shaking were also varied. This was done to alter the formation of amyloid seeds, which act as bases for LC proteins add to [3]. Amyloid aggregate morphologies then needed to be confirmed, which was done through atomic force microscopy (AFM) and transmission electron microscopy (TEM). This was performed and conditions that generated LC aggregates with fibrillar structures within a week were found.

Methods:

Aggregation Assays. Aggregation assays were performed in a 96-well plate and monitored by ThT fluorescence with a Tecan Infinite F200 multimode reader. The aggregation buffers consisted of either 50 mM Glycine (pH 2.8) or sodium phosphate buffer (NaP, pH 7.2). The buffers contained either 0, 50, 150, or 300 mM NaCl. They also contained 10 mM dithiothreitol, 20 μM ThT, and 20 μM LC protein extracted from AL patient urine. LC proteins were aggregated in three separate sessions – one in 25°C that was shaken every 15 minutes, another in 37°C that was shaken every 15 minutes, and the last one in 37°C that was shaken every 30 minutes. Fluorescence was read at an excitation of 436 nm and an emission of 485 nm.

Imaging. AFM images were gathered from protein adsorbed to mica using tapping mode on a Bruker Dimension Atomic Force Microscope (AFM) and Etalon HA_NC/15 tips. The solutions adsorbed to mica were diluted 1:4 with either glycine or NaP. Images with



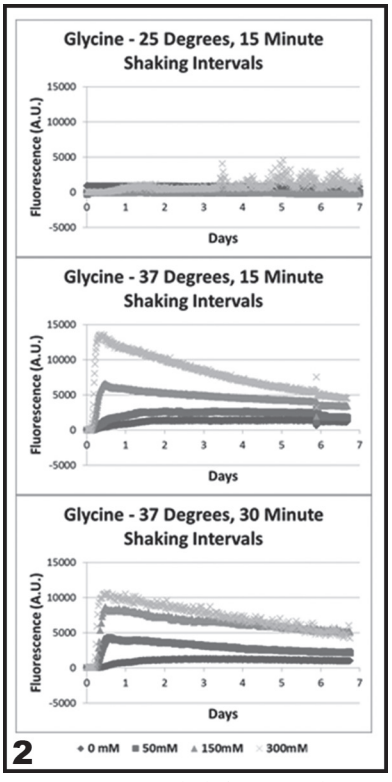
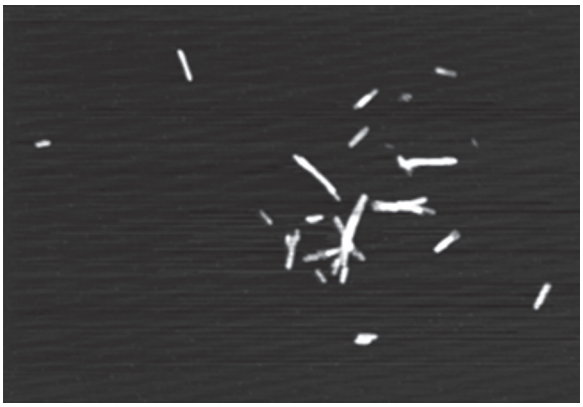


Figure 1: *Plots showing LC seven day aggregations in NaP with different conditions.*
Figure 2: *Plots showing LC seven day aggregations in glycine with different conditions.*



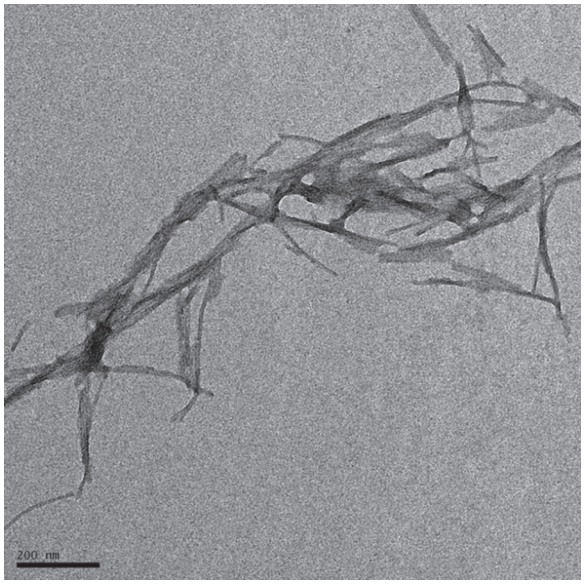


Figure 3, above: *AFM image confirming amyloid formation. The image is of an area 3.3 μm wide.* ***Figure 4, right:*** *TEM image of LC amyloids. Scale bar is 200 μm wide.*

transmission electron microscopy (TEM) were taken from protein solution adsorbed to Ted Pella 200 mesh carbon coated copper grids. Negative staining was originally performed with uranyl acetate replacement (UAR) stain. A switch to 4% uranyl acetate (UA) was made later on.

Results and Discussion:

Aggregation Assays. The plots shown in Figures 1 and 2 represent the aggregation assays performed with the LC proteins. It can be seen that ThT fluorescence did increase in the aggregations performed at 37°C, which indicates the formation of amyloid structures. The aggregations performed in NaP at 37 degrees slowly reached a maximum over the aggregations. The aggregation occurred much faster in glycine, followed by a slow decrease in florescence after peaking. The aggregation performed in NaP in 25°C did not peak, but did increase by the end of seven days. This slowed fluorescence increase when compared to the 37°C aggregations suggests that temperature alterations could be used to control aggregation. The difference in aggregation kinetics between shaking every 15 minutes when compared to 30 minutes was negligible. NaCl concentration had a positive correlation with fluorescence amplitudes and accelerated aggregation kinetics in glycine, but such a correlation was not seen in NaP.

Imaging. AFM and TEM were used to determine if aggregates with fibrillar morphologies were formed during the aggregations. Previously, LC amyloid formation was only observed after 24 day aggregation with our proteins. An image of our LC amyloid fibrils is shown in Figure 3. It is from the seven day aggregation sample performed in glycine that contained no NaCl, and was shaken for 5 s every 15 minutes at 37°C. TEM imaging of the proteins proved to be difficult at first, as UAR stain resulted in precipitate that appeared like fibrillar protein structures even on grids with no protein adsorbed. This discovery encouraged the switch to 4% UA that was used to identify fibrillar structures. A TEM image of our amyloid fibrils is shown in Figure 4.

Conclusions:

AL is currently difficult to diagnose without invasive medical procedures. Multiple conditions for *in vitro* aggregation of LC isolated from AL patient urine were investigated to determine if it could be used in AL diagnosis. Temperature and NaCl concentration were shown to affect aggregation, suggesting that control over LC amyloid aggregation is possible. AFM and TEM were used to show amyloids were formed during aggregation assays. The optimized aggregation protocol accelerated LC amyloid fibril formation to a few days instead of three weeks, encouraging its future utility as a diagnostic tool.

Acknowledgements:

I would like to thank Dr. Jan Bieschke, Kathrin Andrich, Nathan Reed, and Remya Nair of Washington University in St. Louis for all their help and the opportunity to work on this project. I would also like to thank Melanie-Claire Mallison of Cornell University, and the NNIN and the NSF for the invitation to participate in the NNIN REU program, and for funding under Grant No. ECCS-0335765.

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