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The Formation of Nematic Liquid Crystal Phases by Hen Lysozyme Amyloid Fibrils

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Amyloid fibrils are a polymeric β-sheet aggregate of protein, consisting of fibrils that are on the order of a few micrometers long, 10−20 nm wide, relatively stiff, and nonbranching. Although formed by some proteins in the process of several diseases, a large number of proteins unrelated to disease has also been shown to form amyloid fibrils, prompting the suggestion that the ability to aggregate into these fibrils is one generic to all polypeptide chains.[1] Amyloid fibrils can be regarded as long and rigid. In this aspect they are comparable to synthetic polymers. These, as well as biological polymers such as DNA and polysaccharides, are often known to form liquid crystal phases.[2] These relatively ordered phases form because of the geometric properties of the molecules, rather than any specific physical or chemical properties—in Onsager theory, for example, the aspect ratio and volume fraction are the most important variables.[2] We show here that preformed amyloid fibrils of hen lysozyme can form similar liquid crystal phases. This behavior fits in well with our recent suggestion that the apparently ubiquitous nature of amyloid fibrils suggests that the exact molecular detail may be of less importance to aggregation than in native protein interactions and that a more generic description using polymer physics might accurately describe the formation and properties of amyloid fibrils.[3,4] The protein hen lysozyme is known to form amyloid fibrils when incubated at low pH and elevated temperatures[5] (see Supporting Information). Incubation of the protein at relatively high concentrations (10 mM or 14.3% w/v) resulted in substantial yields of amyloid fibrils but, undesirably, the solutions gelled after 2 days. Polarized optical microscopy images revealed that the gels contained large numbers of small birefringent domains, indicating the presence of liquid crystal phases[2] (not shown). It is assumed that the high protein concentration leads to a large number of fibrils forming, resulting in a dense fibril network and the solution gelling. This does not allow the fibrils to align over long distances, evidenced by the appearance of many small liquid crystal domains: a "liquid crystal glass". Seeded fibril formation at a lower concentration (1 mM) and subsequent filtering of the solution to remove nonfibrillar protein allowed the formation of a 1.4 mM stock solution of amyloid fibrils. This stock solution was diluted to various concentrations from 0.3 to 1.4 mM and left to stand at room temperature for 3 days. Initial experiments were done at pH 2, where the protein carries a net positive charge (+19). In Figure 1 are shown polarized light images of the vials with the fibril solutions.

Figure 1A shows solutions of lysozyme in the absence of any added NaCl. Clearly, liquid crystal phases start to form at about 0.6 mM, where the solution first contains clearly "lit up" patches. As the concentration is increased, the patches become brighter and more widespread. Although the fibrils were aligned at higher concentrations, the solutions became noticeably more viscous. It

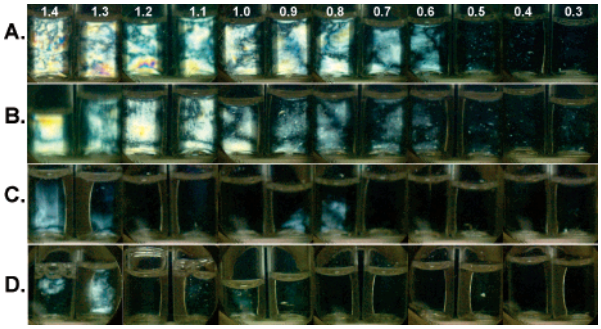

Figure 1. Glass vials with hen lysozyme fibril containing solutions at pH 2.0 imaged between crossed polars. Concentrations are indicated (in mM). Shown are fibrils in the absence (A) and in the presence (B) of 0.1 M NaCl and freeze−thawed fibrils in the absence (C) and in the presence (D) of 0.1 M NaCl. Vials were approximately 1 cm wide. The small domains occasionally seen at lower concentrations in panels C and D are presumed due to uneven fibril fracturing in the different samples.

is unclear why the expected lowering of the viscosity[2] is not observed. Further work into the viscosity and rheo-optical behavior of these solutions is underway. In the presence of 0.1 M NaCl (Figure B), the liquid crystal content of the solutions similarly increases with concentration. Now, however, the onset of a clear liquid crystal phase is not seen until an apparently higher concentration of 0.6−0.7 mM. Confirmation of the presence of amyloid fibrils in these liquid crystal states is shown in Figure 1 of the Supporting Information.

For all concentrations, shaking the solutions results in the formation of short-lived flow-induced liquid crystal phases with increasing persistence at higher concentrations. To confirm the expected importance of the fibril aspect ratio, the solutions were plunge-frozen in liquid nitrogen, which is known to break the amyloid fibrils into shorter fragments (Supporting Information, Figure 2). As expected, the formation of liquid crystal phases by the shorter fibrils in the absence (Figure 1C) and presence (Figure 1D) of 0.1 M NaCl is much less favorable than for the fibrils that had not been frozen. Liquid crystal phases now only form at concentrations above 1.2 mM. Shaking the solution will briefly result in flow-induced birefringence, indicating alignment of the fragmented fibrils is still possible. Their shorter aspect ratio, however, prevents this from occurring spontaneously until the higher concentration.

In an attempt to study the importance of charge, solutions containing amyloid fibrils were studied at different pH values, from pH 2 to pH 12. It was observed that as charge on the molecule decreased, smaller liquid crystal domains formed. This is likely due to the decreasing charge−charge repulsion leading to easier and faster entanglement, thus disfavoring long-range alignment (Supporting Information, Figure 3).

Samples of a 1 mM fibril solution, prior to freeze-thawing, were taken and pipetted into quartz 1 mm path length cuvettes. The

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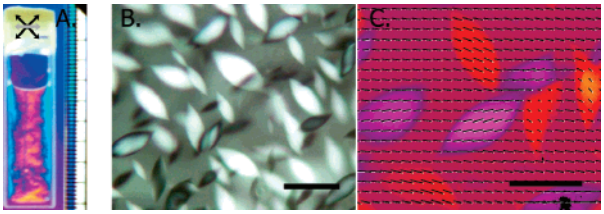

Figure 2. (A) Image of a cuvette with non-freeze−thawed fibrils at 1 mM. Orientation of the crossed polars is shown by black arrows, the fast axis of the waveplate is shown by a gray arrow. On the right is shown a scalebar with gradations in mm. (B) Polarized light image of the tactoids formed by freeze−thawed fibril solutions at 1.3 mM is shown. Scale bar is 100 μm. (C) Tactoids image taken on a 'Metripol' setup is shown. The lines indicate the alignment of the fibrils. Colors also indicate alignment, from purple (horizontal, 0°) to green (vertical, 90°) to red (horizontal, 180°). Scale bar is 100 μm.

solutions were instantly birefringent owing to flow alignment. After several days, order was seen to persist along the length of the cuvette. Figure 2A shows a cuvette, placed between crossed polars with an added waveplate at 45° to the polars. The addition of the waveplate results in the appearance of colors, indicating alignment of birefringent species. Using the orientations previously determined,[4] in the picture shown, blue indicates a vertical orientation, while yellow denotes horizontal alignment. It can be seen, therefore, that in the center of the cuvette the fibrils are aligned with the meniscus, while nearer the edges of the cuvette the fibrils are aligned parallel to the edges. The order persists over a distance of approximately 3 cm (Figure 2A).

To closer investigate the liquid crystal domains that form, solutions of non-freeze−thawed fibrils at 0.6 mM were placed in dimpled slides and covered with a cover glass. Both in the absence and in the presence of NaCl, birefringent areas could be observed by optical microscopy. The domains do not look markedly different, although there is a tendency for the domains to be smaller in the presence of 0.1 M NaCl (not shown). Interestingly, only nematic (uniaxially aligned) domains could be observed. This is unlike the work of Aggeli et al. who observed nematic domains and spherulites.[6] It is possible that their use of a short peptide, rather than a full-length protein, gave rise to more well-defined amyloid fibrils, resulting in an increased ability to aggregate. Only very occasionally could spherulites be seen in our solutions, but it is thought that these are a consequence of concurrent random aggregation and fibril formation, rather than being formed by phase separated amyloid fibrils. They therefore resemble the spherulites formed by bovine insulin.[3]

Freeze−thawed fibril samples at 1.3 mM were similarly placed in dimpled slides and covered with a cover slip. Only weakly birefringent domains could be observed. These domains appeared indistinguishable from those observed in non-freeze−thawed solutions. Near the edge of the cover slip, however, different structures could be discerned (Figure 2B). These look similar to the "tactoids" formed by F-actin.[7] The tactoids consist of individual, lens shaped, birefringent objects up to 100 μm in size. The tactoids were imaged using a Metripol setup, consisting of a mobile polarizer with fixed quarter waveplate and analyzer. This setup allows a quantitative analysis of sample birefringence and includes information about orientation of the constituent molecules (Figure 2C).[8] The images show that the fibrils are aligned roughly parallel to the long axis of the tactoids, similar to those formed from actin.[7] The mechanism by which they form, however, is still unknown.

Recently, work has started to exploit amyloid fibrils for a variety of uses, including the formation of "nanowires",[9,10] attachment of GFP, enzymes, and cytochromes[11,12] and the formation of 3D scaffolds for cell culture.[13] We believe that considering, in addition to the biochemical detail of proteins, the physicochemical properties of proteins and their aggregates, such as their ability to form liquid crystal phases, should provide powerful new opportunities for the development of functional, biological materials that self-structure on a nanoscale level.

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Supporting Information Available: Experimental details for fibril formation, demonstration of the presence of amyloid fibrils in the liquid crystal states, EM evidence for the breaking of fibrils upon freeze−thawing and the effect of pH on the liquid crystal phases. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Dobson, C. M. *Nature* **2003**, *426*, 884−890.
(2) Donald, A. M.; Windle, A. H.; Hanna, S. *Liquid Crystalline Polymers*, 2nd ed.; Cambridge University Press: Cambridge, U.K., 2006; p 604.
(3) Krebs, M. R. H.; Bromley, E. H. C.; Rogers, S. S.; Donald, A. M. *Biophys. J.* **2005**, *88*, 2013−2021.
(4) Krebs, M. R. H.; MacPhee, C. E.; Miller, A. F.; Dunlop, I.; Dobson, C. M.; Donald, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 14420−14424.
(5) Krebs, M. R. H.; Wilkins, D. K.; Chung, E. W.; Pitkeathly, M. C.; Chamberlain, A. K.; Zurdo, J.; Robinson, C. V.; Dobson, C. M. *J. Mol. Biol.* **2000**, *300*, 541−549.
(6) Aggeli, A.; Bell, M.; Carrick, L. M.; Fishwick, C. W. G.; Harding, R.; Mawer, P. J.; Radford, S. E.; Strong, A. E.; Boden, N. *J. Am. Chem. Soc.* **2003**, *125*, 9619−9628.
(7) Tang, J. X.; Kang, H.; Jia, J. *Langmuir* **2005**, *21*, 2789−2795.
(8) Jin, L.-W.; Claborn, K. A.; Kurimoto, M.; Geday, M. A.; Maezawa, I.; Sohraby, F.; Estrada, M.; Kaminsky, W.; Kahr, B. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 15294−15298.
(9) Reches, M.; Gazit, E. *Science* **2003**, *300*, 626−627.
(10) Scheibel, T.; Parthasarathy, R.; Sawicki, G.; Liu, X.-M.; Jaeger, H.; Lindquist, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4527−4532.
(11) Baldwin, A. J.; Bader, R.; Christodoulou, J.; MacPhee, C. E.; Dobson, C. M.; Barker, P. D. *J. Am. Chem. Soc.* **2006**, *128*, 2162−2163.
(12) Baxa, U.; Speransky, V.; Steven, A. C.; Wickner, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5253−5260.
(13) Zhang, S. *Nat. Biotechnol.* **2003**, *21*, 1171−1178.

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