Protein aggregation: more than just fibrils

Mark R.H. Krebs1, Kristin R. Domike2 and Athene M. Donald3
Cavendish Laboratory, University of Cambridge, J.J. Thomson Avenue, Cambridge CB3 0HE, U.K.

Abstract
The aggregation of misfolded proteins into amyloid fibrils, and the importance of this step for various diseases, is well known. However, it is becoming apparent that the fibril is not the only structure that aggregating proteins of widely different types may adopt. Around the isoelectric point, when the net charge is essentially zero, rather monodisperse and quasi-amorphous nanoscale particles form. These particles are found to contain limited runs of β-sheet structure, but their overall organization is random. These nanoparticles have the potential to be useful for such applications as the slow release of drugs. The amyloid fibrils form away from the isoelectric point, but over certain ranges of, e.g., pH, the fibrils themselves do not exist freely, but form suprafibrillar aggregates termed spherulites. These consist of fibrils radiating from a central nucleus, and form by new species attaching to the ends of growing fibrils, rather than by the aggregation of pre-existing fibrils. Under the polarizing light microscope, they exhibit a Maltese cross shape due to their symmetry. The rate of aggregation is determined by factors involving (at least) protein size, concentration, presence of salt and charge. The occurrence of spherulites, which have been found in vivo as well as in vitro, appears to be generic, although the factors which determine the equilibrium between free fibril and spherulite are not as yet clear.

Introduction
That partially or completely misfolded proteins can aggregate to form amyloid fibrils is well established and it is partly accepted that this is a generic property of proteins under the appropriate conditions (see, e.g., [1]). Because of the association of amyloids with a variety of neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease, amyloid fibrils are perhaps the most studied of the forms in which protein aggregation occurs, but they are not the only structure that misfolded proteins can adopt.

In the present paper, we consider two further forms of aggregate which appear to be common in proteins and may be universal. We will concentrate on their appearance in β-lactoglobulin and, to a lesser extent, insulin. The first aggregate consists of particulates of a rather uniform size distribution, which form around the isoelectric point of the protein. We have recently shown that these aggregates, which have been extensively studied for the case of the milk protein β-lactoglobulin because of their use in texturing foods, occur in a variety of different and structurally unrelated proteins [2]. Given their easy manufacture and uniform controllable size, we explored whether these particles offer scope for biocompatible delivery vehicles, for instance by loading with appropriate drugs.

The second aggregate structure is that which we have termed a spherulite [3], because of its similarity under the polarizing microscope to the semi-crystalline structures seen in synthetic polymers such as polyethylene. Protein spherulitic structures have been seen not only in vitro, but also in plaques from animal brains with diseases equivalent to Alzheimer’s disease and the new variant of Creutzfeldt–Jakob disease. The spherical structures consist of radiating fibrils from a central core, and appear to form via the symmetric growth of fibrils from a nucleus, whose origin is as yet unclear.

Types of protein aggregate
Particulate structures
Particulate structures have been found in a variety of structurally unrelated proteins [2]. In the present paper, we focus on β-lactoglobulin, the most extensively studied protein in this context in the literature. The particles form after thermal denaturation, and the average size of particles can be modified by changing the thermal history (time, temperature and heating rate all affect the size [4]). Large particles, visible by confocal microscopy, were created by heating solutions of β-lactoglobulin to 80°C at a heating rate of 1°C min⁻¹; ESEM (environmental scanning electron microscopy) [5,6] showed the particles to be relatively monodisperse, with radii of 1230 ± 425 nm [4]. To model the particles’ functionalization potential, we modified the particles to allow FRET (fluorescence resonance energy transfer) experiments, since for it to occur, the donor and acceptor molecules need to be within a few nanometres of each other, implying that interaction is possible [7]. The particles were covalently modified with FITC [8]. The particles can easily be visualized by confocal microscopy (Figure 1A) and appear as spherical objects with uniform
Confocal microscopy and fluorescence spectroscopy of labelled particulates

The amounts released vary between the different molecules varied between the dye and the drug molecules. Tetracycline and quinine are released the fastest, reaching maximum after the same time, but much less protein was released: 9.3 ± 0.3 μM. The rhodamine-infused particles released 114 ± 9 μM protein after ~100 h. Final particulate size confirms these observations. The average starting radius, 445 ± 75 nm [2,4,9], decreased to 430 ± 85 nm (quinine), 380 ± 70 nm (tetracycline) and 410 ± 75 nm (rhodamine) after incubation in PBS (phosphate buffered saline) for 9 days. Although these differences are within the range of statistical errors, the decreases correspond closely to the amount of protein released from the particulates, suggesting they may be significant. Incubating 30× diluted particulates at 37°C in PBS for 2 weeks resulted in similar size decreases (Figure 2D). Again, the differences are within the range of statistical errors, but the trends are identical with those observed before, with tetracycline-infused particles decreasing the most in size, followed by Rhodamine 6G-infused and quinine-infused particles, suggesting that the effect may be small, but real.

Given that molecules can apparently easily diffuse into the particulates, we studied the possibility of incorporation and subsequent release of active ingredients. Smaller particles with an average radius of 445 ± 75 nm were formed by rapidly heating (100°C-min⁻¹) solutions of lactoglobulin. Particulates were ‘loaded’ with Rhodamine 6G, quinine or tetracycline. The latter two are used here as model drug compounds. Over a short period (lasting days), the dye and drugs diffused out of the particulates, as shown by UV–visible light absorbance of the supernatant, although there was also some rapid release of dye and protein. The kinetics of release varied between the dye and the drug molecules. Tetracycline and quinine are released the fastest, reaching maximum cumulative release after ~75 h, whereas Rhodamine 6G is released the slowest, reaching a maximum only after ~100 h. The amounts released vary between the different molecules as well: the cumulative release of tetracycline is 1409 ± 6 μM; that of quinine is 175 ± 1 μM and that of Rhodamine 6G is 205 ± 5 μM. It is not clear why there is such a substantial variability in release, but it is likely that there are significant differences in the manner in which the different molecules interact with the protein. Given that the protein is neither in a native state nor completely unfolded when in the particles [9], it is difficult to quantify this experimentally.

After correcting for dye or drug absorbance at 280 nm, it was observed that protein was released from the particulates as well, suggesting the particulates dissolve slowly upon dilution (Figure 2). Similar to the release of dye and drug molecules, particulate dissolution is different between the three samples. Again, the tetracycline-loaded particles released the most protein the fastest, reaching a maximum cumulative release of 848 ± 5 μM after ~75 h. The quinine-loaded particles reached their protein release maximum after the same time, but much less protein was released: 9.3 ± 0.3 μM. The rhodamine-infused particles released 114 ± 9 μM protein after ~100 h. Final particulate size confirms these observations. The average starting radius, 445 ± 75 nm [2,4,9], decreased to 430 ± 85 nm (quinine), 380 ± 70 nm (tetracycline) and 410 ± 75 nm (rhodamine) after incubation in PBS (phosphate buffered saline) for 9 days. Although these differences are within the range of statistical errors, the decreases correspond closely to the amount of protein released from the particulates, suggesting they may be significant. Incubating 30× diluted particulates at 37°C in PBS for 2 weeks resulted in similar size decreases (Figure 2D). Again, the differences are within the range of statistical errors, but the trends are identical with those observed before, with tetracycline-infused particles decreasing the most in size, followed by Rhodamine 6G-infused and quinine-infused particles, suggesting that the effect may be small, but real.

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Spherulites

Spherulites have been observed in β-lactoglobulin, initially by Sagis et al. [10], and also by inference in proteins associated with disease [11–15]; insulin has also been studied in depth. The growth of spherulites has been followed by time-lapse microscopy, permitting modelling of the process kinetics [16,17]. Whereas we have found β-lactoglobulin only forms spherulites slowly and infrequently, insulin forms them readily and with faster kinetics [16]. Nevertheless, modelling their growth has shown very similar overall behaviour, even to the extent of having comparable thermal activation energies [16]. The questions of what causes spherulites to nucleate, or indeed to shift the balance between free amyloid fibrils and radially growing fibrils from a central core, remain unanswered.

Figure 3 shows the typical structure of such spherulites in both insulin and β-lactoglobulin. Spherulites have been shown to consist of radially oriented fibrils, as demonstrated by the colours of the quadrants in Figure 3, which shows that the alignment of the ‘fast’ direction of a 551-nm waveplate is radial, implying that the fibrils are aligned in this direction. It has also been demonstrated, at least for the parallel case of insulin [17,18], that spherulites form simultaneously with the growth of the amyloid fibrils, rather than assemble from pre-formed fibrils.

Ubiquity of the β-sheet in protein aggregation

It is well known that amyloid fibrils comprise a high percentage of cross-β structure [19] and they are frequently identified by their propensity to take up so-called characteristic dyes such as thioflavin T. The extent to which this is a unique identifier has been discussed previously [20]. Recently, it has been shown that the particulate structures in β-lactoglobulin also contain some level of β-sheet [9]. SAXS (small-angle X-ray scattering) experiments revealed linear structures at the molecular level. Uptake of thioflavin T by the particles suggested that these structures were correlated with the presence of β-sheet [9]. Thus we have a new paradigm for
thinking about protein aggregation. Once the native protein structure is lost, in general in the types of experiments described in the present paper via heat, there seems a strong tendency for aggregation involving β-sheets to occur. However, how extensive such sheets are, and what long-range structures may or may not form, as opposed to short runs of sheets, is where the particular conditions of aggregation come into play.

For conditions around the isoelectric point, the net charge on the protein is low, and there will be only small patches of electrostatic attraction. Thus the large-scale structure is amorphous, but locally some β-sheets will form. In the presence of salt, remaining electrostatic interactions are screened so that the propensity to form β-sheet is reduced further, demonstrating the importance of the electrostatic interactions [9]. Moving away from the isoelectric point, the charge on the chains increases. Initially, both particles and amyloid fibrils can coexist, but at increasing net charge, the amyloid fibril dominates. These fibrils may exist freely in solution, or they may form on a central nucleus and grow outwards to give the spherulite morphology. The factors controlling the position of this equilibrium between free and aggregated fibrils are not yet clear. However, charge has a role to play, since in the presence of salt, the morphology is significantly different: the spherulites tend to grow larger and have a central core which seems to consist of collapsed fibrils [16]. Thus the intrinsic stiffness of the fibrils appears to be charge/pH-dependent.

It therefore appears that the β-sheet is a fundamental and presumably stable state of aggregation for different proteins. Different aggregation conditions govern the way in which local runs of β-sheet come together into larger-scale structures. It is also clear that the amyloid fibril is only one form of aggregation. The implications for bionanotechnology of being able to control and manipulate the aggregates, as manifest by the model experiments on drug release described in the present paper, are many and varied. This is a rich field.

Towards generalizing protein aggregation

Although there is a general level of acceptance that amyloid fibrils are an apparently universal form of protein aggregate, if the correct conditions for misfolding or unfolding are found, there are other ubiquitous forms of protein aggregation that also seem to have associated universality. In the present paper, we discuss particulate structures that form around the protein’s isoelectric point, and spherulite structures that can coexist with amyloid fibrils when there is substantial charge on the protein chain, i.e. away from the isoelectric point. In both of these cases, as well as the well-known amyloid fibril structure, there is a high proportion of β-sheet present, demonstrating the stability and ubiquity of this motif. For the particulate structures, only short runs of β-sheet occur: in the presence of salt, screening the low levels of charge which may be present in this regime, the aggregation is presumed to be even more random and so these runs are particularly short. With or without salt, the overall morphology is amorphous, but both SAXS and thioflavin T data nevertheless indicate the presence of the β-sheet motif. Away from the isoelectric point, some conditions favour spherulite formation rather than simply free amyloid fibrils. The spherulites appear to consist of fibrils which grow radially outwards from some central nucleus, whose origin as yet remains unclear. A significant proportion of β-sheet is also found in the spherulites.

Spherulitic structures have been found previously in diseased tissue, as well as in vitro. Particulate structures have not been found associated with any diseases. On the contrary,
we believe they may have promise as nanoencapsulants and candidate particles for drug release. Their potential in this role has been demonstrated here using drug uptake and release experiments.

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**References**