Gelation of Misfolded Proteins

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Summary: Insulin protein was exposed to mildly denaturing conditions (heat and low pH) to encourage the formation of beta-sheet rich amyloid fibrils. This resulted in an increase in viscosity of our protein samples and the morphology and thermodynamics of the resulting hydrogel were monitored using environmental scanning electron microscopy and micro differential scanning calorimetry respectively. It was found that the beta-sheet fibrils aggregated further to form macrofibrils, 2 μm in diameter and several microns in length. These long, flexible macrofibrils became entangled to form hydrogels with controllable mesh size: the higher the incubation temperature the higher the number of entanglements, and consequently the smaller the mesh size.

Keywords: amyloid fibril; fibrillar hydrogel; self-assembly

Introduction

Protein folding has become the focus of much attention world-wide because of its implication in a wide range of diseases including v-CJD and Alzeimers'.[1,2] The basic steps are being elucidated, with the recognition that probably all proteins are capable – under appropriate conditions – of mis-folding and subsequently self-assembling into the beta-sheet rich filaments that make up an amyloid fibril.[3] These fibrils consist of several protofilaments intertwined in a helical fashion to form a very stable, internally hydrogen -bonded fibril, which is typically nanometres in width but microns in length.[4] In laboratory experiments, it is common to encourage proteins to form fibrils by exposing the (correctly) folded protein to mildly denaturing conditions, such as low pH or elevated temperatures. As denaturation and self-assembly proceeds, the interaction of individual amyloid fibrils, which is considered to be entirely through non-covalent interactions, leads to a significant increase in viscosity, which is apparent immediately in the ampoule. Furthermore we have recently shown that individual fibrils further associate, presumably through secondary interactions, providing a hierarchy of supramolecular structures some of which are large enough to be easily visible in the optical microscope.[5] The mechanisms and thermodynamics of amyloid fibril self-assembly, and the morphology of the viscous solutions formed, still remain largely unclear.

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Such knowledge of the structure-property relationships could facilitate the design and implementation of novel biomaterials based on mis-folded proteins.

Bovine insulin is an ideal model to use to study the self-assembly of amyloid fibrils due to it's non-toxicity and its physiological importance, as it can form fibrils both in vivo and in vitro. Insulin is a small, helical protein consisting of two polypeptide chains, a 21 residue long A chain and a 30 residue long B chain, linked together by two disulphide bridges and in vitro it readily self-assembles following incubation in solution at pH 2.0 and temperatures above 30 °C. Under such conditions it forms viscous solutions that contain beta-sheet fibrils with an average diameter of ca. 10 nm. Here we focus on the formation of the hydrogel and the influence of environmental conditions, such as gelation temperature and protein concentration, on the kinetics of gelation and morphology of the resulting gel.

Materials and Methods

Samples. Bovine pancreatic insulin and all other chemicals were purchased from Sigma-Aldrich Company (Sigma-Aldrich co., Gillingham, UK) and used without purification. Solutions were made up by dissolving the protein powder in doubly distilled water and the pH adjusted with HCl to pH 2. Samples were incubated at temperatures ranging from 55 to 8-°C for 24 hours or until a gel had formed.

Environmental Scanning Electron Microscopy (ESEM). For all ESEM experiments reported here, we used a FEI Quanta 200 instrument with a Peltier stage and gaseous secondary electron detector (GSED) to produce an image. All samples were contained within a circular brass stub with a diameter of 1 cm and a depth of 0.5 cm. The temperature of the stub, and hence sample, was controlled using the Peltier cooling stage with a water/propanol coolant maintained at 283 K. The sample was placed on the stub, left for a few minutes to equilibrate, before initiating pump down of the chamber from ambient to a few torr. In all cases, the sample was cooled to 275 K (2 °C) and surrounded by water vapour at a pressure of ca. 4 torr. Working distances were typically 8-10 mm and images were collected using an accelerating voltage of 5 keV. Under such conditions signal to noise ratio was maximized, sample dehydration minimized, and detailed high-resolution images were obtained. Dimensions of the fibrillar network and mesh sizes were subsequently determined directly from the images using computer software built into the ESEM.

Micro Differential Scanning Calorimetry (μDSC). Highly sensitive DSC measurements (Seteram) were carried out by filling the sample cell with ca. 0.5 ml solution, while the

reference cell was filled with a matching buffer. All samples were equilibrated at 10 °C for 1 hour prior to any experiment. A scan rate of 0.5 °C min^{-1} was selected for all samples, and isothermal temperatures were varied to include: 55, 60, 65, 70, 75 and 80 °C.

Results and Discussion

The morphology of the viscous solutions (hydrogels) formed after exposing insulin solutions to heat was monitored using environmental scanning electron microscopy (ESEM). Typically gels formed after 4-6 hours, and if left for prolonged periods of time particulate structures, reminiscent of spherulites, begin to form and drop out of solution.[5] A typical micrograph of insulin hydrogel is given in Figure 1a where the network is composed of entangled macrofibrils and pores ranging in size from 4 to 10 μm. In all cases, the macrofibrils forming the networks are 1–2 *μm* in diameter and are of indefinite length. Slowly dehydrating the sample inside the ESEM chamber (Figure 1b) revealed that the macrofibrils are made up of what appear to be discs that are stacked face to face. These discs have a diameter equal to the length of one amyloid fibril, suggesting that they are composed of self-assembled beta-sheet fibrils, but the exact orientation of these fibrils in the discs is still not known.

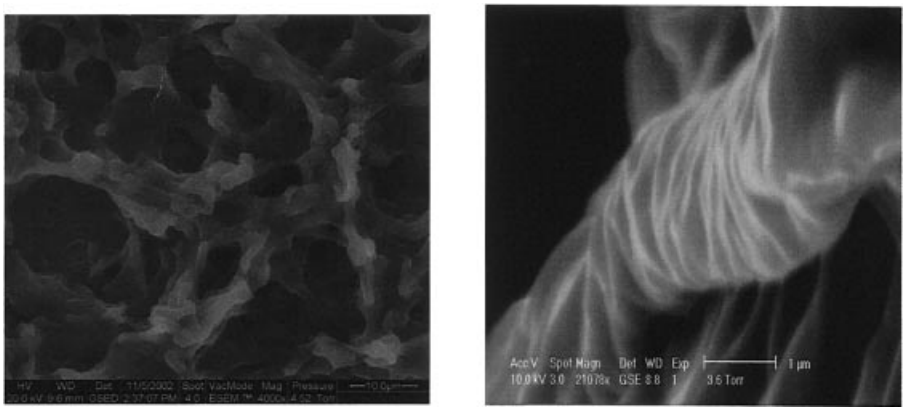

Figure 1. Typical ESEM micrographs of an 11 $mgml^{-1}$, pH 2, hydrogel sample obtained from aggregation of fibrillar insulin protein (6 hours, 75°C), where sample in a) is hydrated, but sample b) has been dried in order to obtain further structural detail. Scale bar represents a) 10 and b) 1μm.

Incubation temperature was found to have little effect on macrofibril formation or its dimensions, but temperature did affect the number of fibrillar entanglements and consequently mesh size: the higher incubation temperature, the greater the number of

entanglements and consequently the smaller the hydrogel mesh size (Figure 2). A similar decrease in mesh size was observed when the concentration of protein was increased. To investigate such differences further sensitive micro differential scanning calorimetry (μDSC) was employed to identify the denaturation temperature and to detect the onset, kinetics and enthalpy of gelation.

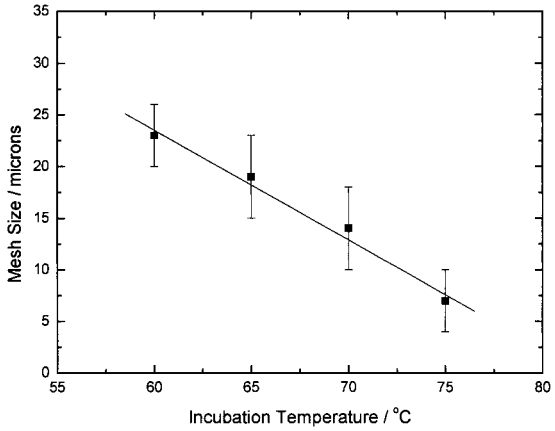

Figure 2. Average mesh size of the fibrillar network as a function of incubation temperature.

Figure 3 shows the DSC scan obtained for an 11 $mg.ml^{-1}$ insulin sample, before and after incubation, i.e. gelation. Such data are typical of all samples run here. During the initial heating ramp an endothermic peak with a minimum value at 57.5 ± 1 °C was observed due to denaturation of the protein. The heat capacity of this transition was always ca. 2 kJ mol^{-1}, which is typical for insulin.[6] The DSC scan of the sample after incubation showed no endothermic peak, implying that all protein had denatured and had been incorporated into the fibrillar network during the first heating ramp, and moreover that this gelation process is not reversible.

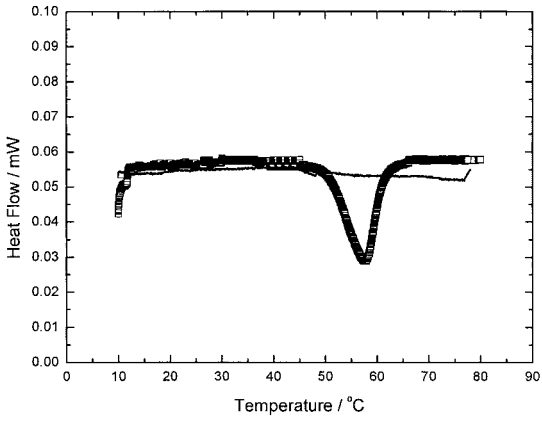

Figure 3. DSC scan of the endothermic denaturation peak of 11 $mg.ml^{-1}$, pH 2, insulin sample before (□) and after (¨) incubation. Average scan rate was 0.5 °Cmin^{-1}.

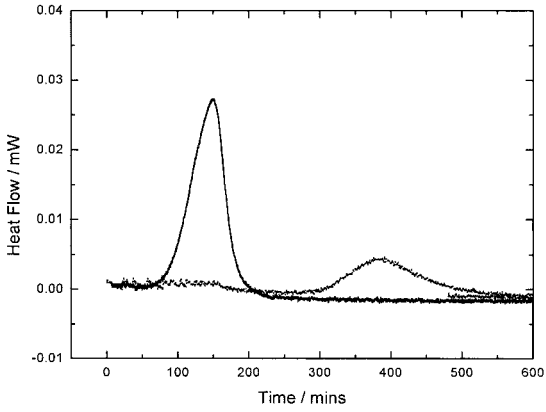

Figure 4. Isothermal calorimetry curve for 11 $mgml^{-1}$, pH 2 insulin sample incubated at 75 °C (—) and 60 °C (¨).

To determine the thermodynamics of protein gelation isothermal calorimetric scans at 6 temperatures were recorded for 6 hours. At 55 °C no peak was observed in the DSC trace, presumably due to the protein not having denatured and consequently not able to aggregate into beta-sheet rich fibrils. Alternatively gelation could have been too slow to be detected. At

60 °C a distinct, broad exothermic peak was observed (see Figure 4) after 314 ± 12 minutes. As the incubation temperature increased the exothermic peak began to appear more quickly and became narrower in shape (Figure 4), suggesting that protein aggregation, and consequently gelation, occurred more rapidly. Furthermore as the gelation rate increased, so too did the exothermic character (Table 1); for example gelation began 314 ± 12 minutes after the incubation time began for the sample at 60 °C with 65 ± 3 kJ mol^{-1} energy, but only after 68 ± 8 minutes and 131 ± 4 kJ mol^{-1} for the sample incubated at 75 °C. Such an increase in enthalpy of gelation suggests that the degree of gelation, i.e. number of entanglements, has also increased. This is confirmed from ESEM experiments where the number of macrofibril entanglements increased, and the average mesh size decreased with increasing incubation temperature (Figure 2). At 80 °C no well-defined exothermic peak was detected as the protein had already formed a gel before the isothermal temperature had been reached.

Table 1. Heat of gelation for 11 $mgml^{-1}$, pH 2 aggregated insulin fibrils incubated at different temperatures.

Temperature / °C	ΔH / kJ mol^{-1}	Time taken to reach maximum of peak / mins
55	-	No peak observed
60	65 ± 3	314 ± 12
65	79 ± 4	233 ± 10
70	114 ± 6	108 ± 5
75	131 ± 4	68 ± 8
80	Not able to determine	24 ± 10

Conclusions

The results of the present study show that under conditions that favour insulin denaturation the protein self-assembles to form beta-sheet rich fibrils. These consequently aggregate further into macrofibrils that become entangled and result in the formation of a fibrillar hydrogel. The rate and degree of macrofibril entanglements, and consequently hydrogel mesh size, can be manipulated by varying protein concentration and incubation temperature.

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