Part III. Polymorphism of protein misfolded and aggregated species

31. Structural basis for the polymorphism of β-lactoglobulin amyloid-like fibrils

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Structural basis for amyloid polymorphism
Abstract

The whey protein β-lactoglobulin (β-lg) forms amyloid fibrils upon hydrolysis or partial unfolding. The morphology of these fibrils depends on the environmental conditions during formation, including pH, ionic strength, protein concentration and reaction time. Long and straight, as well as short, worm-like β-lg fibrils are observed. The molecular basis of this polymorphism remains poorly understood. In this chapter we review the relation between the fibril morphology and the peptide molecular structure of amyloids formed from β-lg. Fibril morphologies are typically measured by atomic force microscopy and electron microscopy, and the molecular structure is measured by a variety of spectroscopic techniques, such as Raman and FT-IR spectroscopy. Recent studies have begun to combine spectroscopy with imaging techniques to correlate fibril morphology with the underlying molecular structure. It was shown that straight and rigid fibrils have a substantially higher β-sheet content than worm-like fibrils. However, to elucidate in future the local molecular structure of peptides within fibrils, newly developed techniques such as tip-enhanced Raman spectroscopy, will be necessary.

Keywords
Amyloid, secondary structure, whey protein, FT-IR, circular dichroism, Raman, spectroscopy, Sum Frequency Generation, Atomic Force Microscopy, supramolecular polymers, self-assembly
1. Why beta-lactoglobulin?

β-Lactoglobulin (β-lg) is the main protein in whey and represents 0.2-0.4% (w/v) of skim milk. In the native state, β-lg has a predominantly β-sheet structure (Figure 1). Upon heating it is capable of self-assembling into a variety of supramolecular structures. The fibers in the fine-stranded gels that are formed upon incubation below the isoelectric point (pH=5.1) have been identified as amyloid fibrils. β-lg is of great importance to the dairy industry, similar to other whey proteins, and is particularly useful for controlling the texture of a variety of foods. At the same time, its tunable structuring capacity makes β-lg an interesting target for material science. Additionally, β-lg has become a major model protein to investigate the self-assembly mechanism of amyloid fibrils. Although the formation kinetics and morphology of β-lg amyloid fibrils have been extensively studied, the secondary structure of the fibrils has still not yet been fully elucidated. Here we review the knowledge that has been accumulated so far on the relation between the fibril morphology and the underlying molecular structure of β-lg amyloids by a variety of experimental techniques.

***Insert Figure 1***

2. Formation mechanisms of amyloid-like fibrils from beta-lactoglobulin

The generally accepted model for amyloid fibril formation comprises the assembly of oligomers into protofilaments, which can further aggregate into amyloid fibrils. Under normal environmental conditions, globular native proteins including β-lg in solution are in equilibrium with their partially unfolded state. This equilibrium can be displaced towards partially and completely unfolded states, if conditions such as temperature and pH are changed outside of the physiological range. Partially unfolded proteins can refold into cross-β-sheet secondary structure. This can induce a linear growth of oligomers, which are composed of hydrogen-bonded β-sheets, orthogonal to the peptide
backbone.(12) However, another mechanism of the formation of oligomers is via hydrolysis of β-lg into short peptide fragments. Akkermans et al. have shown by mass spectrometry that β-lg fibrils formed upon heating at pH=2 are composed of peptide fragments, and not of intact β-lg monomers.(13) The same mechanism has since been demonstrated for other proteins.(14) Which one of the two routes – partial unfolding or fragmentation – is followed, appears to depend on the conditions employed to promote fibrillation (Figure 2).(15)

***Insert Figure 2***

After the formation of oligomers, the so-called nucleation step, the elongation into protofilaments is a rapid process. Based on the observation of spontaneous alignment of protofilaments in atomic force microscopy (AFM) images, it has been suggested that their aggregation into mature fibrils is driven by short-range attractions.(16) These attractions are presumably of hydrophobic or Lennard-Jones type. Multi-stranded amyloid ribbons composed of 2 to 16 β-lg protofilaments have been observed, all showing a left-handed twist.(17) The maximal conversion of monomers into amyloid fibrils upon heating β-lg at pH=2 is between 50-90%, depending on protein concentration.(18)

3. Polymorphism of beta-lactoglobulin amyloids: rod-like, worm-like and straight fibrils

Upon partial unfolding or hydrolysis of β-lg monomers, different structural morphologies can appear, depending on experimental conditions. Since the fibrils have nanometric dimensions, they are usually examined either by AFM or by electron microscopy (EM). The amyloid fibrils that are formed are never monodisperse; length, diameter and persistence length vary among fibrils.(19, 20) Under certain conditions, also spherulites composed of a central region constructed from radially aligned amyloid fibrils are formed.(8, 21) Here we focus on fibrils and we discuss the parameters that affect the morphology and formation kinetics of β-lg amyloid fibrils.
1. Protein concentration

The critical aggregation concentration (CAC) of β-lg at pH=2 and 80°C was determined using Thioflavin T (ThT) fluorescence intensity measurements, and was found at 1.6 mg/mL (~87 µM).(22) The rate of aggregation increases with increasing β-lg concentration. Heating 5 mg/mL β-lg solutions at pH=2.5 and 80°C for 4 hrs did not show a loss of native-like β-lg, whereas at concentrations of 80 mg/mL, aggregation was very fast.(23) It was shown that between concentrations of 5 to 40 mg/mL, the concentration only affects the kinetics of fibril formation, while the structure of the fibrils was not influenced.(24) However, at higher concentrations (up to 75 mg/mL), a clear change in the fibril morphology and molecular structure was observed.(18) At 30 mg/mL β-lg, long, straight fibrils were formed which can be classified as semiflexible polymers (persistence length of same order as contour length). In contrast, the fibrils are much shorter and worm-like at 75 mg/mL (Figure 3a and b). Also, the conversion of monomers into fibrils shifted from ~50% at 30 mg/mL to ~90% at 75 mg/mL under the employed conditions.

***Insert Figure 3***

2. Solution pH

The most widely used protocol to form β-lg amyloid fibrils is incubation in acidified water (pH=2) for several hours at high temperature (80-90°C). It was shown that there is a small effect on the morphology of fibrils when the pH is varied between pH=1.6 and 2.4.(1) In all samples, the majority of the fibrils appeared semiflexible, up to 5 µm long and 5-10 nm in diameter. However, at pH=1.6, a small number of worm-like fibrils (similar to the ones shown in Figure 3b) were present, while at pH=2.4 some wavy fibrils with a period of ~64 nm were observed (Figure 3c). The persistence length of the fibrils varied between 2.1 and 3.1 µm. At higher pH values, between pH=3 to pH=7,
short, worm-like fibrils have been observed (Figure 3d). At pH=3.35, the contour length of these short wormlike amyloids was about 130 nm and the persistence length ~35 nm.

3. Solvent
The long, straight fibrils aggregated at low pH and high temperature are composed of peptides, which are formed due to acid hydrolysis. Another route to obtain fibrils is by enzymatic hydrolysis of β-lg at pH=8 and 37°C using the enzyme AspN endoproteinase, which cleaves the peptide bonds N-terminal to aspartic acid residues. When after cleavage the pH is lowered to pH=2 and the temperature to room temperature, long fibrils form. Alternatively, fibrils can be formed at room temperature by incubation in alcohol-water mixtures. Fibrils were shown to form in 50:50 water-alcohol solutions based on methanol, ethanol, propanol and 2,2,2-trifluoroethanol (TFE) at pH=2 and also at pH=7. TFE-induced fibrils appeared more worm-like than heat-induced fibrils, and showed a beaded structure under certain conditions (Figure 3 e-g). They were typically 150-500 nm in length and about 7 nm in diameter. The morphology of fibrils formed in methanol, ethanol and propanol depends on the pH: at pH=2 fibrils appear similar to those formed in water-TFE, while at pH=7 thinner filaments (5 nm) and bundles thereof are observed.

4. Ionic strength
The morphology of fibrils is highly dependent on the ionic strength of the solution. While at low monovalent salt concentrations long and straight fibrils are formed, at higher concentrations the fibrils become shorter and worm-like. Yet, fibrils obtained at low and high ionic strength do exhibit a similar diameter and periodic morphology with a periodicity between 22 and 28 nm. This suggests that the general molecular mechanism of fibril formation is independent of the ionic strength. However, the rate of aggregation increases with ionic strength, and the critical concentration decreases. Possibly, because at higher ionic strength electrostatic
repulsions are weaker, the critical size of a stable nucleus is smaller. Then the nucleation process is no longer necessarily the limiting growth step and the rate of aggregation is faster.

5. Shear forces
Agitation of the solution during fibril formation affects the kinetics of fibril formation as well as the final fibril morphology. Shear flow significantly enhances the rate of the formation of fibrils but only when it is applied during the nucleation process. No differences in kinetics for continuous shear (at a shear rate of 200 s⁻¹ in a Couette geometry) and short shear pulses of 30 s (at the same shear rate) were observed. However, the length distribution for fibrils formed after continuous shear showed a smaller variance than for fibrils formed upon pulsed shear. The total fibril length concentration increased for increasing shear rate up to a shear rate of 337 s⁻¹. When the shear rate was increased even more, the total fibril length decreased again. This could be related to breakage of fibrils by exposure to high shear rates. It has been suggested that breaking of filaments can induce secondary nucleation events, which play an important role in the kinetics of the proliferation of amyloid fibrils.

6. Reaction time
In the first hours of β-lg amyloid formation at pH=2 and without agitation, individual protofilaments of ~2.6 nm in diameter are formed. When the incubation time is increased from 16 to 96 hrs, an increased number of fibrils with a twisted ribbon shape are observed, and the average diameter increases to ~4 nm. This is consistent with the proposed mechanism where protofilaments align and aggregate into mature fibrils. No clear effect of reaction time on contour length was observed, but the persistence length decreased with incubation time, despite the increase in fibril diameter (Figure 4).

***Insert Figure 4***
4. Molecular structure of amyloid fibrils

X-ray diffraction studies have revealed that amyloid fibrils have a characteristic cross-β core structure, composed of a stack of β-strands. The β-strands are perpendicular to the fibril axis and form a continuous β-sheet along the fibrils axis that is stabilized by extensive hydrogen bonding (Figure 5). The sequence-specific side-chains influence the propensity to form fibrils. Below we discuss several techniques that are routinely used to study the secondary structure of β-lg amyloid fibrils and we compare the structure of morphologically different fibrils.

***Insert Figure 5***

1. Circular dichroism spectroscopy

Circular dichroism (CD) is defined as the unequal absorption of left- (L) and right- (R) handed circularly polarized light. Chiral molecules may absorb these two components to different extents, and may also have different indices of refraction for the two waves. CD spectroscopy is an excellent technique to determine the secondary structure of proteins and aggregates. Absorption in the region between 180 and 240 nm is due to the peptide bonds and provides information on the secondary structure. The spectra are usually analyzed using algorithms based on databases compiled for peptides for which the X-ray crystal structure is known. A drawback of CD spectroscopy, however, is the weak contribution of β-sheets, resulting in difficulties in quantifying secondary structure compositions for proteins with high β-sheet contents. Nevertheless, the secondary structure content of native β-lactoglobulin, oligomers and aggregates has been determined using CD. The aggregates, a mixture of amyloid fibrils and spherulites, were found to contain higher β-strand and lower α-helical structure contents than the oligomers, which were mainly dimers. Compared to the native protein, oligomers partially lost the helical and β-strand structure (Table 1). This is in agreement with the observation that dimers are linked by a disulfide bond, which requires at least unfolding of the protein’s main helix.
CD has also been used to study the effect of shear on the secondary structure of β-lg fibrils (Figure 6).(29) After heating, samples were sheared in variable (stirring) or controlled (Couette cell) shear flows. In variable shear, helical fibrils with proportions of 51% β-sheet structure and 32% random coil were formed. However, under controlled shear the fibrils showed a segmented or beaded morphology, a lower β-sheet (44%) and increased α-helix (37%) content. Also a lower mechanical strength was measured for these fibrils in AFM nanomechanical manipulation experiments, where the fibrils were stretched with an AFM tip. The pulling force magnitude, an indication of the energy required to stretch or unravel the fibrils, was ~20 pN for fibrils formed under controlled shear, while variable shear-induced fibrils showed force magnitudes of ~100 pN. Samples formed without shearing showed an even higher alpha-helix proportion (49%) compared to the ones formed with controlled shear, however, no fibrils were observed in AFM images. This can be explained by the fact that shear increases the rate of fibril formation, and that without shear only oligomers and no fibrils were formed. It has to be noted that the sheared samples showed a large variability in fibrillar and non-fibrillar structures, and it is unclear how this mixture contributes to spectroscopic measurements, which are intrinsically ensemble measurements.

2. FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectroscopy provides insight into the overall secondary structure of proteins. The first FT-IR experiments on proteins were demonstrated in the 1950s.(42) An advantage of infrared absorption spectroscopy is that proteins in a wide variety of environments can be studied: gasses, liquids, solids, crystals, membrane proteins and insoluble aggregates.(43) It is a technique that can be used to rapidly acquire spectra from small amounts (down to ~100 μg) of
The most widely used vibrational mode in studies of protein secondary structure is the amide I band, between 1600 and 1700 cm⁻¹. This vibrational mode originates mainly from the C=O stretching vibration of the amide group in the protein backbone, which is sensitive to the hydrogen bonding network of the local secondary structure. The amide I band overlaps spectrally with the O-H bending mode of water. To avoid strong water absorption, often high protein concentrations, dried samples or the attenuated total reflection (ATR) mode are employed. An established approach for quantitative analysis of FT-IR data is curve-fitting of the amide I band of a protein with its component bands. Each of the Lorentzian components correlates to a certain secondary structure element through its spectral position. The area of a component band is usually directly related to the amount of the respective secondary structure element. A drawback of FT-IR spectroscopy for analysis of secondary structure is the difficulty to separate between the contributions of unordered and α-helical structures in an aqueous environment.

FT-IR experiments have revealed that worm-like amyloids formed from β-lg in alcohol-water mixtures contain an increased β-sheet content compared to native β-lg. Dependent on the type of alcohol, both the β-sheet and α-helix intensity increase during fibril formation. For heat-induced fibrils formed at pH=2, which are longer and smoother in appearance than the worm-like fibrils, only the β-sheet intensity increased, while the helical intensity remained the same as for native β-lg (Figure 7). Because no quantitative analysis of the amide I band was performed, it was not possible to compare the different amyloid types with each other.

An alternative route to obtain worm-like fibrils is by disassembly and reassembly of multistranded fibrils formed at low pH and high temperature upon incubation in ethanol-water mixtures. The α-helical content of the worm-like fibrils is lower than that of the multistranded fibrils, while their persistence length is shorter. They are enriched in random coil and turn content and have a different peptide composition compared to the original multistranded fibrils.
Real-time ATR-FTIR experiments have shown that in the first stages of fibril formation from native β-lg, the β-sheet content increases, whereas it decreases again in the fibril elongation stage. The α-helical content first decreases and then increases slightly during elongation. Also, breaking up fibrillar proteins by high pressure microfluidization could be followed using ATR-FTIR. The inter-molecular β-sheet component decreased, which was attributed to breakage of the fibrils into short fibrillar structures. At the same time, the intensity of β-turns and α-helix increased.

***Insert Figure 7***

3. Raman spectroscopy

Raman spectroscopy provides information on the vibrational motions of molecules and has been widely used to study protein aggregation. It can be used to elucidate the structure of proteins in aqueous solutions, fibers, films, gels and crystals. The approach to determine a protein secondary structure composition is very similar to that in FT-IR. One advantage of Raman over FT-IR is that it does not suffer from overlapping water absorption; water vibrations are not Raman active. On the other hand, the process of Raman scattering is improbable. The use of high laser intensities, to increase the Raman signal, can damage protein samples. Sophisticated techniques like surface enhanced Raman scattering (SERS), where metal surfaces are used to enhance the Raman signal for proteins containing chromophores, can lead to a substantial increase of the signal-to-noise ratio.

The β-sheet development during the formation of amyloids can be followed in the amide I spectral region by Raman spectroscopy, as has been shown for α-synuclein. Raman spectroscopy on spherulites formed from insulin shows that regardless of whether aggregation is induced at the isoelectric point or away from it, and regardless of the ionic strength, protein aggregation favors the formation of beta-sheet structures. Raman spectroscopy has also been used to compare secondary structure of β-lg fibrils prepared at pH=2 in the presence of various alcohols. Significant differences in the band in the amide I region for the native protein compared to the
spectra of the fibrils were observed (Figure 8). The shift seems to correlate with the degree of aggregation which has occurred, and is interpreted as a significant change in the existing native β-sheet structure, a net increase in β-sheet, or perhaps both.(46) Upon heating of β-lg monomer at pH=2, the Raman peak positions shifted from 1238 to 1242 cm⁻¹, indicating that β-sheets are strongly hydrogen bonded.(54)

4. VSFG spectroscopy

Vibrational sum-frequency generation (VSFG) spectroscopy is a surface-specific vibrational spectroscopic technique, which was only recently used for the first time to study the secondary structure of amyloids at interfaces.(55, 56) VSFG spectroscopy was pioneered in the 1980s.(57) In the nonlinear optical process of VSFG, two pulsed lasers, one at visible and one at infrared (IR) frequency, interact with the molecules at an interface or on a surface. As a result, light at the sum frequency of the visible and IR beams can be generated. The process is resonantly enhanced when the IR frequency matches a surface vibrational mode frequency.(58-60) An advantage of VSFG is its surface-specificity, what makes it possible to look at formation of amyloid on membranes or interfaces. This may be interesting for applications like stabilization of foams by fibrils and in disease-related research. Besides, the same samples (e.g. on mica) can be used for VSFG and for AFM.

We recently used VSFG to resolve the difference in secondary structure between straight and worm-like β-lg amyloid fibrils.(18) The different fibril types were prepared by incubation of β-lg at pH=2 and 80°C at varying concentrations. Low concentrations resulted in straight fibrils, while at high concentrations worm-like fibrils were formed (Figure 3a and b). Figure 9a shows VSFG spectra of the amide I region. At 3.0% β-lg, a peak at 1625 cm⁻¹ is observed, indicating β-sheet structure. In contrast, the peak position shifts to higher wavenumbers for worm-like fibrils formed at higher concentrations. The fractional integrated intensity of peaks fitted to the VSFG data for bands at
~1625 cm\(^{-1}\) and ~1650 cm\(^{-1}\) gives an indication of the β-sheet and the α-helical/random intensities, respectively. In Figure 9b the fractions of the fitted peaks versus the concentration β-lg is plotted, clearly showing a decrease from nearly 100% to approximately 50% β-sheets. These results show a clear positive correlation between the β-sheet content (secondary structure) and the persistence length (morphology) of β-lg amyloid fibrils.

***Insert Figure 9***

5. TERS

An important drawback of spectroscopic techniques like FT-IR, Raman and VSFG spectroscopy is that they can only be used to measure average properties of a large ensemble of fibrils. Given that amyloid fibrils are notoriously polymorphic, there is a growing interest in the development of nanoscale techniques like tip-enhanced Raman spectroscopy (TERS), which can provide 1-10 nm spatial resolution in combination with structural sensitive spectroscopic information. First TERS spectra, of a C\(_{60}\) thin film, were published in 2000.(61) The technique offers depth and lateral spatial resolution in the nanometer range due to a strong field enhancement by a metalized tip of an AFM coupled to a Raman spectrometer.(62) Therefore it provides the opportunity to spatially map the secondary structure and amino acid residue composition along the surface of individual amyloid fibrils.(63, 64) It was shown using TERS that the surface of insulin fibrils is highly heterogeneous, containing β-sheet, α-helical and unordered structures. Certain amino acids on the surface, like phenylalanine and tyrosine, show a higher relative propensity for β-sheet areas than for α-helical regions and turns.(65) No TERS studies on β-lg amyloids have been reported yet, but it is a promising technique to investigate the structure and its relation to the morphology of single β-lg fibrils that vary in morphology. Moreover, TERS could be used to map how different secondary structure elements are distributed along the fibril.
Conclusions

The formation, morphology, mechanics and structure of amyloid fibrils are of interest to, and widely studied in, diverse research fields from health care to biophysics. However, it remains challenging to completely elucidate the molecular structure of amyloid fibrils and relate it to their morphology. Using CD, the polymorphism of fibrils formed under different levels of shear was studied. An increased \( \alpha \)-helical and decreased \( \beta \)-sheet content were observed for beaded, segmented fibrils, and this was related to a decreased mechanical strength. FT-IR experiments on the secondary structure of solvent-induced fibrils indicated higher \( \alpha \)-helical intensities for worm-like fibrils, compared to straight fibrils. On the other hand, it was shown that worm-like fibrils that form upon disassembly of multistranded fibrils have a decreased \( \alpha \)-helical intensity and an increased intensity for random structure and coils. A direct comparison between worm-like and straight fibrils was performed for fibrils formed at varying protein concentrations using VSFG. Long fibrils with near-100% \( \beta \)-sheet content had a 40-times higher persistence length than short, worm-like fibrils with \( \beta \)-sheet contents below 80%. In conclusion, several studies have shown that high \( \beta \)-sheet contents are related to more straight fibrils with increased mechanical strength, while \( \alpha \)-helical and unordered structures are more pronounced in worm-like fibrils. However, until now mainly bulk studies on \( \beta \)-lg amyloid fibrils were performed. An important complication that should be kept in mind is the polymorphism that is observed in every sample. Therefore, a high-resolution technique like TERS is very promising to elucidate the sub-structure of single fibrils and to relate this with its morphology.
Figure 1. Structure of bovine β-lactoglobulin from heteronuclear NMR spectroscopy. The monomeric native-state structure comprises an eight-stranded continuous anti-parallel β-barrel and one major α-helix. Reprinted with permission from Kuwata et al., PRS, 1999.(10)

Figure 2. Schematic representation of the mechanism of conversion of globular protein into amyloid fibrils. Reprinted with permission from Adamcik et al., Macromolecules, 2012.(12) Copyright 2012 American Chemical Society.

Figure 3. AFM height and TEM images of β-lg amyloid fibrils prepared under different conditions. (a) Incubation at pH=2 and 80°C in 16 hrs at 30 mg/mL β-lg, and (b) 75 mg/mL β-lg. Scalebars are 500 nm. Color bar shows height calibration. Reprinted with permission from vandenAkker et al., J. Am. Chem. Soc., 2011.(18) Copyright 2011 American Chemical Society. (c) Incubation at pH=2.2 and 80°C in 6 hrs at 10 mg/mL β-lg: fibril with wavy structure. Scalebar is 30 nm. Reprinted from Loveday et al., Int. Dairy J., 2010, with permission from Elsevier.(1) (d) Incubation at pH=3 and 80°C in 6 hrs at 30 mg/mL β-lg. Scalebar is 50 nm. Reprinted from Kavanagh et al., Int. J. Biol. Macromol., 2000, with permission from Elsevier.(25) (e) Incubation at pH=7 in 50% TFE-water mixtures at 40 mg/mL β-lg. Scalebar is 250 nm. Comparison of (f) heat-induced fibrils at pH=2 and 80°C and (g) solvent-induced fibrils, 50% TFE-water mixture. Scalebars are 100 nm. Reprinted with permission from Gosal et al., Langmuir, 2002.(3) Copyright 2002 American Chemical Society.

Figure 4. (a) A split fibril imaged using AFM in liquid and reconstructions of left-handed helical fibril formation from the twisting of a two-stranded ribbon (left) and a protofibril (right). Scale bar is 100 nm. (b) Diameter distribution of fibrils formed at 3.0% β-lg during incubation times of 16 and 96 h. Black lines are Gaussian fits. (c) Persistence length of fibrils formed at concentrations of 3.0 to 7.5%

Figure 5. Structural model for Aβ₁₋₄₀ protofilaments. (a) Ribbon representation viewed down the long axis of the protofilament. (b) Atomic representation with color coding to indicate residues with hydrophobic (green), polar (magenta), positively charged (blue) and negatively charged (red) side chains. Reprinted with permission from Tycko et al., Curr. Opin. Struct. Biol., 2004.(35)

Figure 6. CD spectra for native, heated, and heated and sheared β-lg. Samples were heated at 80°C for 12 hrs prior to controlled shear measurements. Variable sheared samples were stirred; controlled shear was performed using a Couette shear cell. The arrows indicate the wavelength of maximum negative ellipticity. Reprinted with permission from Dunstan et al, Soft Matter, 2009.(29)

Figure 7. Difference FT-IR spectra for β-lg fibrils formed (a) at pH = 2, 80°C for 24 hrs at a concentration of 40 mg/mL (broken line) and 100 mg/mL (solid line), (b) at pH = 2 in 50% w/w water-alcohol mixtures at β-lg concentration of 3.5% w/w in 40 days, and (c) at pH = 2 in 50% w/w water-alcohol mixtures at β-lg concentration of 7.0% w/w in 85 days. M: methanol, E: ethanol, P: propanol, T: TFE. Each spectrum is intensity normalized and the spectrum for native β-lg is subtracted from it. Reprinted with permission from Gosal et al., Biomacromolecules, 2004.(46) Copyright 2004 American Chemical Society.

Figure 8. Raman spectra of (a) native β-lg; β-lg incubated at 80°C for 14 hrs at a concentration of (b) 40 mg/mL and (c) 80 mg/mL; powdered aggregates from solutions of (d) 20 mg/mL (e) 35 mg/mL (f) 70 mg/mL in 50% v/v methanol; (g) 20 mg/mL (h) 35 mg/mL (i) 70 mg/mL in 50% v/v ethanol; (j) 35 mg/mL (k) 70 mg/mL in 50% v/v propan-2-ol; (l) 35 mg/mL (m) 70 mg/mL in 50% v/v TFE.
The 20 and 35 mg/mL alcohol-induced samples were incubated for 40 days, and 70 mg/mL alcohol-induced samples were incubated for 85 days. Showed are the region sensitive for $\alpha$-helical structure (left), the amide III (middle) and the amide I (right) spectral regions. Reprinted with permission from Gosal et al., Biomacromolecules, 2004.(46) Copyright 2004 American Chemical Society.

Figure 9. (a) Normalised VSFG spectra and (b) fractions of $\beta$-sheet and random/$\alpha$-helical intensity of fibrils formed at a concentration ranging from 30 to 75 mg/mL $\beta$-lg after an incubation time of 16 hrs at 80$^\circ$C and pH=2. Spectra and fractions are averages from three measurements. Scale bars in AFM images are 100 nm. Reprinted with permission from vandenAkker et al., J. Am. Chem. Soc., 2011.(18) Copyright 2011 American Chemical Society.
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