Morphology and Persistence Length of Amyloid Fibrils are Correlated to Peptide Molecular Structure

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Materials and Methods

Bovine β-lactoglobulin (genetic variants A and B, Sigma, L0130) was dissolved in HCl solution (pH 2). To remove traces of electrolytes, the solution was extensively dialyzed (Slide-a-Lyzer, MWCO 10kDa, Thermo) against an HCl solution (pH 2). Insoluble protein was removed by filtration (0.1 μm filter, Millipore). The final protein concentration was determined using UV spectrophotometry (Perkin Elmer, Lambda 35 UV/VIS Spectrometer) at a wavelength of 278 nm based on an extinction coefficient[1] of 16.8 $mM^{-1}cm^{-1}$. To obtain fibrils of the desired morphology, a range of conditions was tested. The β-lactoglobulin stock solution was diluted with HCl solution (pH 2) to a concentration between 3.0 and 7.5% (w/w). Samples in an eppendorf tube were heated in an oven at 80°C during a period varying between 2 and 96 hours, followed by quenching in ice water. Fibrils were separated from non-aggregated material by centrifuging the solution over centrifugal filters (MWCO 100kDa, Millipore) at 1000 *g* for 30 min.[2] The retentate containing the fibrils was diluted with HCl (pH 2) and centrifuged two additional times over the centrifugal filters to remove residual non-aggregated material. To quantify the extent of protein

conversion into fibrils, the total amount of protein in the filtrate was quantified by determining the protein concentration with UV spectrophotometry at 278 nm and weighing the filtrate fraction (Figure 1). Atomic force microscopy (AFM) on the filtrate confirmed that fibrils were absent in this fraction. Conversion data are averages over at least three independent experiments with standard deviations. The filtered fibril suspension was centrifuged for 5 min at 2000 *g* to remove fibril aggregates. To determine the final protein concentration, fibril solutions were mixed with an equal volume of formic acid to solubilize the aggregated protein. The protein contents of these solutions were measured with the Bio-Rad protein assay (Bradford, Bio-Rad) using monomeric β-lg in 50% formic acid as the standard.[3]

The morphology, dimensions and persistence length of the fibrils were examined using tapping mode AFM with a Dimension 3100 Scanning Probe Microscope (Veeco). For AFM in air, fibril suspensions were diluted in HCl solution (pH 2) to concentrations of ~0.01%. A 20 µl aliquot was put on freshly cleaved mica, incubated for 5 min, washed with HCl solution (pH 2), and dried in air. For imaging in air, silicon cantilevers (force constant 5 N/m) were used. Images were flattened using Nanoscope 6.14 software and the diameter of fibrils was measured from their maximum height. For every condition, we averaged over at least n=110 fibrils. To investigate the effect of drying the fibrils, we also performed AFM experiments on fibrils in solution. For liquid AFM, fibril suspensions were diluted to ~0.1%, a drop was put on freshly cleaved mica, and measurements were performed directly at room temperature using silicon nitride cantilevers (force constant 0.24-0.35 N/m). The mean diameter of long, straight fibrils in liquid after 16 hrs incubation was 2.3 nm (n = 118), while for dried fibrils this was 2.6 nm (n=114). This indicates that the effect of drying on fibril diameter is small.

The contour length (C) of the fibrils and the end-to-end distance (E) between the fibril ends were measured with the Simple Neurite Tracer in Fiji[4] ($n \geq 155$). The persistence length, *P*, is

given by $\langle E \rangle_{2D} = 4PC\,(1\text{-}2P/C\,(1\text{-}e^{-C/2P}))$, assuming that the fibrils interact weakly with the mica surface and can relax to a two-dimensional equilibrium conformation.[5-7] This assumption is valid when the fibrils have no cross-overs,[6,8] as is clearly the case for all our samples. We verified that there was only one fibril population per sample by calculating the distribution of η, which is defined[7] as the difference between C and E, normalized by C: $\eta = (C - E) / C$. As a consistency check, we also calculated the persistence length based on the average cosine of the angle Θ between tangent vectors of the fibrils[6]: $\langle \cos(\Theta_C)_{2D} \rangle = e^{C/(2P)}$. The persistence lengths determined from $\langle E \rangle$ and $\langle \cos(\Theta c) \rangle$ were in good agreement; therefore only values determined from the $\langle E \rangle$ data are shown (SI Table 1).

For vibrational sum-frequency generation (VSFG) spectroscopy, a drop of undiluted fibril suspension was put on freshly cleaved mica, incubated for 5 min, washed with HCl (pH 2) and dried in air. The VSFG setup has been described in detail elsewhere.[9] Briefly, a visible beam (VIS) (800 nm, 20-30 μJ/pulse, spectral bandwidth of 25 cm^{-1}) is overlapped at the sample position with an infrared (IR) beam (2-3 μJ/pulse, 150 fs broadband) which is centered at 1640 cm^{-1} and has a spectral bandwidth of 150 cm^{-1}. Both beams are focused down to a ~100 μm beam waist. The incident angles of the VIS and IR beams are 35° and 40°, respectively, both defined relative to the surface normal. The SFG light generated by the sample is detected with a monochromator connected to a charge-coupled device camera. All spectra were collected under unpolarized SFG, *s*-polarized VIS, and *p*-polarized IR conditions and integrated over 6 min. We have repeatedly observed the absence of a psp signal for both β-lg fibrils and human Islet Amyloid Polypeptide (hIAPP) fibrils; therefore we have not used a polarizer in the detection path (SI Figure 2). The spectra were normalized to a reference signal from a z-cut quartz plate. Spectral analysis included background subtraction and division by the spectrum of the quartz

reference. The SFG spectra for the three locations of one sample were averaged, and subsequently fitted using an equation that is commonly used for fitting of SFG spectra.[10] Fitting was performed using a one- or two-component Lorentzian model and included maximum entropy method (MEM) analysis to verify the fit.[10] The fit yields values for the amplitude and phase of the non-resonant susceptibility, and for the amplitude, wavenumber and line width of each component, as shown in SI Table 2. Next, the amplitude divided by the full width at half height (FWHH) is calculated. This value is proportional to the amount of secondary structure, when assuming that the IR and Raman dipole do not depend on the secondary structure and that orientation effects are cancelled out by the random orientation of the fibrils on the mica. The value of Amp/FWHH has been normalized and plotted against concentration. The errors on these values are estimated to be ±0.15, based on the variation in fit results when different fit assumptions are made.

By comparing SFG spectra from fibrils on mica that were dried or hydrated by D_2O, we confirmed that the structure of wet and dry β-lg fibrils prepared under our conditions is very similar in line with recent studies[11,12].

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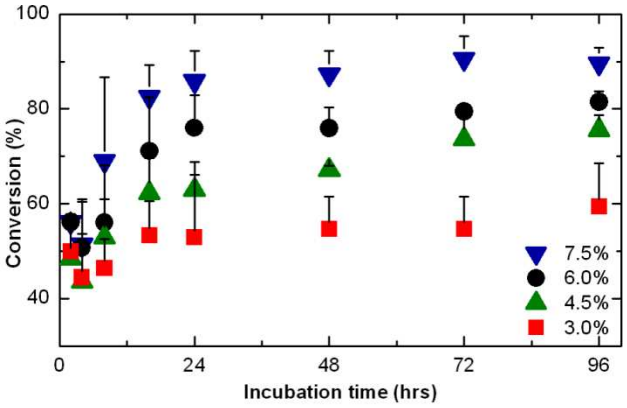
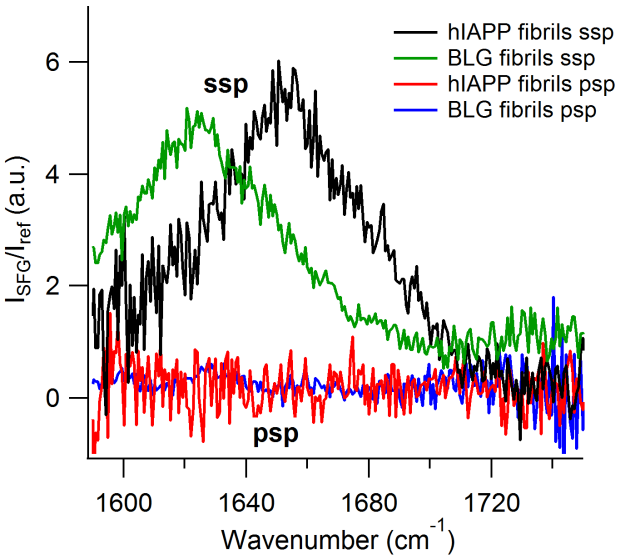


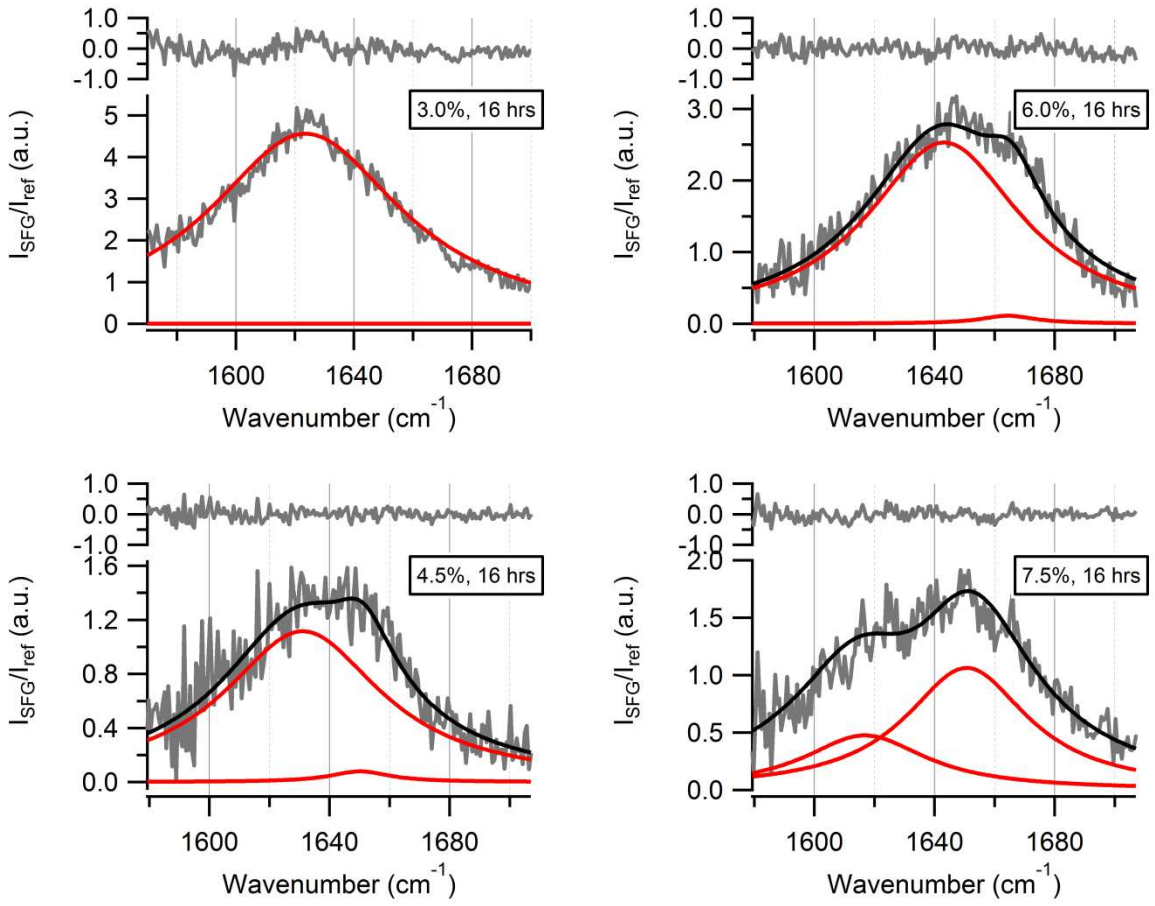
SI Figure 1. Conversion of monomer into aggregates for concentrations of 3.0 to 7.5% β-lg over an incubation time varying from 2 to 96 hrs.

SI Table 1. Average contour length, diameter and persistence length of fibrils formed under different conditions.

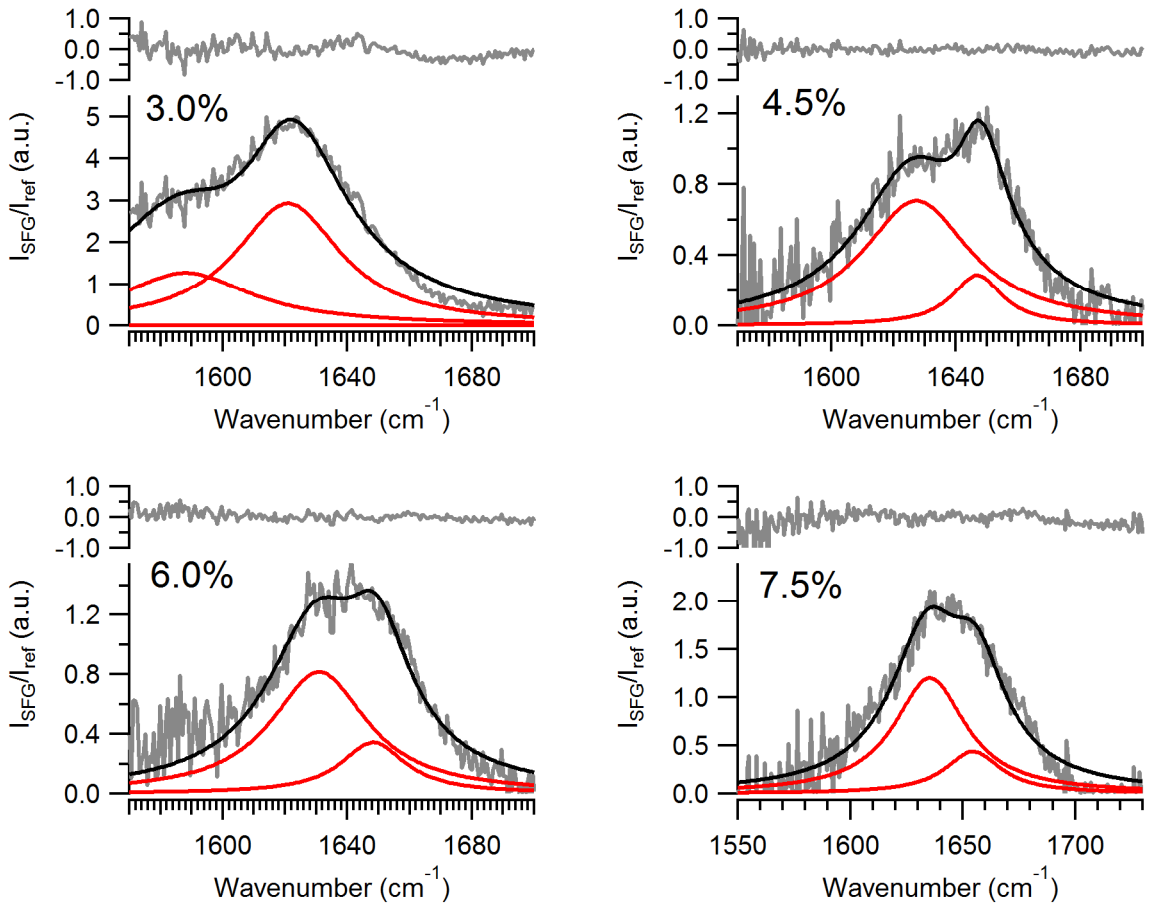
Conc. (%)	**Time (hrs)**	**Contour length (μm)**	**Diameter (nm)**	**Persistence length (nm)**
3.0	16	1.0 ± 0.79	2.6 ± 1.0	3818 ± 164
	96	0.92 ± 0.65	4.1 ± 0.9	1668 ± 46
4.5	16	0.86 ± 0.77	2.8 ± 1.1	2418 ± 149
	96	0.19 ± 0.13	5.2 ± 2.1	886 ± 18
6.0	16	0.41 ± 0.39	5.1 ± 1.0	237 ± 21
	96	0.14 ± 0.05	3.3 ± 1.0	139 ± 10
7.5	16	0.20 ± 0.07	2.9 ± 0.6	91.9 ± 7
	96	0.13 ± 0.04	6.1 ± 1.3	80.6 ± 5



SI Figure 2. SFG spectra of β-lg fibrils and hIAPP fibrils show that the SFG signal is absent for the psp polarization, whereas the ssp polarization combination results in a significant SFG signal.



SI Figure 3. Fitting of the VSFG spectra of β-lg fibrils prepared from solutions containing 3.0 to 7.5% monomers over a time span of 16 hrs. The spectra are the average of the three spectra measured at three locations of the same sample. The lower panels show data (grey line), the fit (black line), and the components of the fits (red lines). The residuals are shown in the top panels.

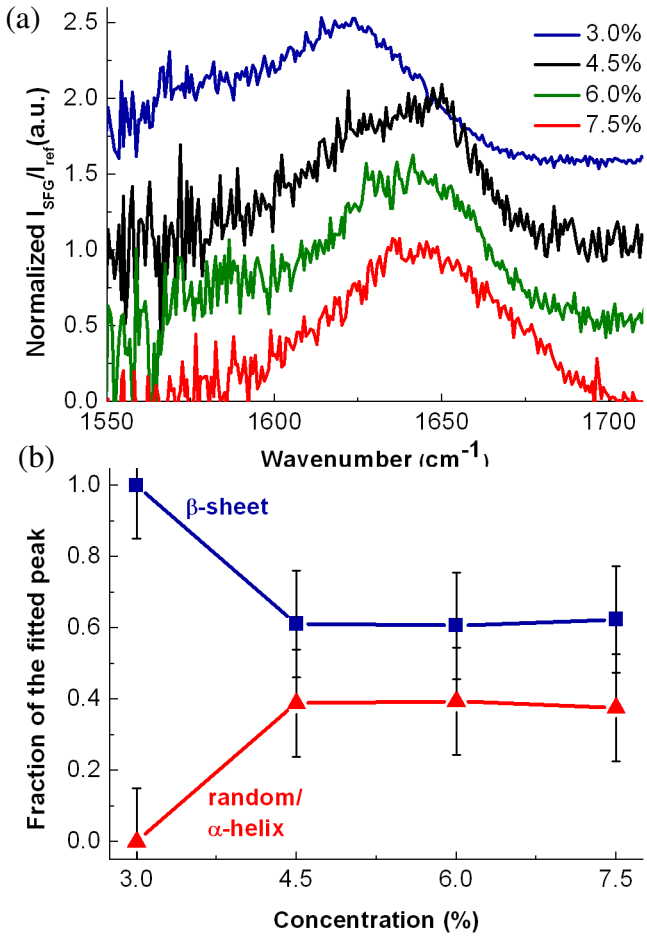


SI Figure 4. Fitting of the VSFG spectra of β-lg fibrils prepared from solutions containing 3.0 to 7.5% monomers over a time span of 96 hrs. The spectra are the average of the three spectra measured at three locations of the same sample. The lower panels show data (grey line), the fit (black line), and the components of the fits (red lines). The residuals are shown in the top panels.

SI Table 2. Fit parameters and standard deviations for fits of VSFG spectra.

Conc. (%)*,**	Incubation time (hrs)	Ampl. 1 (a.u.)	St.dev. (a.u.)	Freq. 1 (cm^{-1})	St.dev. (cm^{-1})	FWHH 1 (cm^{-1})	St.dev. (cm^{-1})	Ampl. 2 (a.u.)	St.dev. (a.u.)	Freq. 2 (cm^{-1})	St.dev. (cm^{-1})	FWHH 2 (cm^{-1})	St.dev. (cm^{-1})
3.0	16	80	1	1626	1	71	1	-	-	-	-	-	-
3.0	96	36	6	1621	1	42	3	-	-	-	-	-	-
4.5	16	39	5	1635	2	64	4	3	3	1656	2	28	12
4.5	96	18	3	1627	2	42	4	5	2	1647	1	20	3
6.0	16	32	7	1631	3	57	5	19	6	1654	1	39	4
6.0	96	17	4	1631	2	38	4	7	3	1648	1	26	4
7.5	16	15	6	1620	2	52	9	30	5	1650	2	57	4
7.5	96	21	6	1635	2	39	5	10	6	1655	2	31	7

For all fits, the non-resonant amplitude and phase were 0 and -1.6, respectively. The values for the standard deviation result from the fits in Igor. *For the concentration of 3% (16 hrs) a single component fitted the data based on visual inspection of the residuals. **For the concentration of 3% (96 hrs) an additional component at 1588 cm^{-1} was required, which is tentatively assigned to the contribution of side chains.



SI Figure 5. (a) Normalized VSFG spectra and (b) fraction of β-sheet and random/ α-helix content of fibrils formed at concentrations of 3.0 to 7.5% in 96 hrs. Spectra and fractions are averages from three measurements. Spectra have been normalized and are plotted with a offset of 0.5 a.u. offset along along the y-axis.