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*β_2 -Type Amyloid-Like Fibrils of Poly-L-Glutamic Acid
Convert into Long Highly Ordered Helices upon
Dissolution in Dimethyl Sulfoxide*

Sylwia Berbeć^a, Robert Dec^a, Dmitry Molodenskiy^b, Beata Wielgus-Kutrowska^c, Christian Johannessen^d, Agnieszka Hernik-Magoń^a, Fernando Tobias^e, Agnieszka Bzowska^c, Grzegorz Ścibisz^a, Timothy A. Keiderling^e, Dmitri Svergun^b, Wojciech Dzwolak^{a*}

^a Faculty of Chemistry, Biological and Chemical Research Centre, University of Warsaw, 1 Pasteur Str., 02-093 Warsaw, Poland.

^b European Molecular Biology Laboratory, Hamburg Outstation, c/o DESY, Hamburg, Germany.

^c Division of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw, Warsaw, Poland

^d Department of Chemistry, University of Antwerp, Belgium.

^e Department of Chemistry, University of Illinois at Chicago, Chicago, USA

* Corresponding author:

Phone: +48 22 552 6567; E-mail: wdzwolak@chem.uw.edu.pl

Abstract

Replacing water with dimethyl sulfoxide (DMSO) completely reshapes the free-energy landscapes of solvated proteins. In DMSO, a powerful hydrogen-bond (HB) acceptor, formation of HBs between backbone NH groups and solvent is favored over HBs involving protein's carbonyl groups. This entails a profound structural disruption of globular proteins and proteinaceous aggregates (e.g. amyloid fibrils) upon transfer to DMSO. Here, we investigate an unusual DMSO-induced conformational transition of β_2 -amyloid fibrils from poly-L-glutamic acid (PLGA). The infrared spectra of β_2 -PLGA dissolved in DMSO lack the typical features associated with disordered conformation that are observed when amyloid fibrils from other proteins are dispersed in DMSO. Instead, the frequency and unusual narrowness of the amide I band imply the presence of highly ordered helical structures which is supported by complementary methods including vibrational circular dichroism (VCD) and Raman optical activity (ROA). We argue that the conformation most consistent with the spectroscopic data is that of a PLGA chain essentially lacking non-helical segments such as bends that would provide DMSO acceptors with direct access to the backbone. A structural study of DMSO-dissolved β_2 -PLGA by synchrotron small-angle X-ray scattering (SAXS) reveals the presence of long uninterrupted helices lending direct support to this hypothesis. Our study highlights dramatic effects that solvation may have on conformational transitions of large polypeptide assemblies.

Introduction

Interactions with the surrounding environment (aqueous or otherwise) have a profound impact on the free-energy landscape and dynamics of solvated biopolymers, and are one of the key driving forces behind self-assembly of biomacromolecular components of the cell.¹ The fact that replacing water (in part, or entirely) with another solvent strongly affects thermodynamics of protein folding has been often utilized in biophysics, pharmacy and biotechnology.² As DMSO has found many applications e.g. as cryoprotector and trans-epidermal carrier³, or simply as a means to solubilize polypeptides, the effect of DMSO on protein stability has been attracting much interest.⁴⁻¹⁵ When small doses of DMSO are added to aqueous solutions of proteins, the solvent tends to stabilize the native state.¹⁶ Low concentrations of DMSO in water were shown to accelerate refolding of denatured lysozyme¹⁷ and increase enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase.¹⁸ The few reports on destabilizing effects of diluted DMSO mostly pertain to dissociation of oligomeric proteins (e.g.¹⁹⁻²⁰). On the other hand, at high concentrations, DMSO usually causes denaturation of proteins.^{6,9,16} Apart from perturbing water-water and water-protein interactions (e.g.²¹), one of the key mechanisms through which DMSO induces denaturation relies on its strong H-bond acceptor properties. Protein conformation in DMSO can be accessed using infrared (IR) spectroscopy. Typically, in IR spectra of DMSO-dissolved proteins, the amide I (amide I' for backbone-deuterated samples) vibrational band is observed at approximately 1662 cm⁻¹. This indicates that solvent molecules outcompete main chain carbonyls as H-bond acceptors leading to 'release' of free peptide C=O groups that absorb at this frequency.⁴ As a consequence, the protein becomes denatured. According to some studies, the DMSO-unfolded state is similar to that induced by conventional means (e.g. guanidine hydrochloride).¹⁷ However, others pointed to distinct scenarios of DMSO-induced denaturation, for example, in terms of higher selective vulnerability of native α -helices to

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2
3 DMSO vis-à-vis denaturation in concentrated urea.¹² For a number of proteins examined in
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5 detail, the DMSO-denatured state appears to be rather heterogeneous with a significant
6
7 presence of poly(L-proline)-II (PPII) conformation.^{10,15,22}
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10 DMSO has also proven to be an effective denaturant for amyloid fibrils, the linear
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12 aggregates of misfolded proteins whose occurrence *in vivo* hallmarks several degenerative
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14 maladies such as Alzheimer's, Parkinson's, and Huntington's Diseases, and diabetes type II.²³
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16 We have shown previously that with increasing DMSO concentration, superstructural
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18 arrangements of individual insulin amyloid fibrils are destabilized first, followed by a
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20 complete dissection of aggregates into disordered monomers spectrally indistinguishable from
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22 those obtained by dissolving native protein in pure solvent.^{22,24-26} The capacity of DMSO to
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24 act as the ultimate denaturant for amyloid fibrils has led to some very insightful works on
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26 misfolded proteins (e.g.²⁷). Meanwhile, as the self-assembly into amyloid fibrils is widely
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28 considered to be a generic property of proteins as polymers²⁸, a lot of basic research has been
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30 conducted on synthetic amyloidogenic peptides including polymerized amino acids.²⁹⁻³¹ In
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32 this respect, PLGA constitutes a particularly interesting case. Amyloid-like fibrils self-
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34 assembled from PLGA at low pH contain β -sheet structure with an exotic hydrogen bonding
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36 pattern involving bifurcated carbonyl acceptors (with main chain NH and side chain $-\text{COOH}$
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38 groups as hydrogen donors). This structure, termed β_2 , has a very characteristic infrared
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40 feature: the amide I' band is shifted below 1600 cm^{-1} .³²⁻³⁸ As the current understanding of the
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42 complex relationship between solvation phenomena and stability of misfolded protein
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44 aggregates remains unsatisfactory, following our previous works we investigated DMSO
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46 effects on stability of β_2 -fibrils of PLGA. In the current study, a variety of spectroscopic
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48 methods including VCD³⁹⁻⁴² and ROA⁴³ which have proven instrumental in illuminating
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50 conformational states of proteins in solution, were employed along with a structural technique,
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52 synchrotron small-angle X-ray scattering (SAXS).
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Materials and Methods

Samples

PLGA (as sodium salt) was purchased from Sigma-Aldrich, USA (cat. No. P4761). Nominal molecular weight (MW) of the PLGA lot (# 096K5103V) used in this study was 15–50 kDa. However, as this value proved inconsistent with the SAXS data obtained in the course of this work, we have re-evaluated MW of the PLGA lot using analytical ultracentrifugation (AUC), see Supporting Information: Fig. S1 and Table S1. According to the AUC-based assay, the actual MW of PLGA was closer to 10 kDa (11.2–11.7 kDa – see Supporting Information for details) which is in reasonable agreement with the SAXS data. All other non-deuterated chemicals, e.g. anhydrous DMSO, pure isopropanol, BioUltra (‘for molecular biology’ grade) 2,2,2-trifluoroethanol (TFE) were purchased from Sigma-Aldrich. Deuterated compounds (D_2O , DCl , d^6 -DMSO) were purchased from ARMAR Chemicals, Switzerland. As bending vibrations of H_2O molecules overlap the diagnostically useful amide I band of PLGA, D_2O was used instead, except for Raman/ROA measurements where H_2O does not pose a similar problem. For most spectral measurements (unless specifically indicated otherwise), deuterated PLGA samples were dissolved in pure DMSO or mixed D_2O -DMSO solutions which were prepared using non-deuterated DMSO. However, as aliphatic C-H hydrogens do not exchange with D_2O under ambient conditions, this does not result in back D/H-exchange of PLGA. In the case of TFE- D_2O mixed solvent used for FT-IR and VCD measurements, non-deuterated TFE was used. Although TFE molecule does contain a single exchangeable hydrogen atom, the actual fraction of exchangeable protons introduced to the sample along with TFE is very low (due to the relatively high molecular weight of TFE) and is not expected to significantly affect spectral position of the amide I' band. For example, a freely exchanging diluted PLGA in 25 v/v % TFE (H) in D_2O is expected to have only 1 in 30 of exchangeable hydrogen atoms unsubstituted for deuterium (which is negligible from the

standpoint of amide I' band characteristics). In the case of Raman/ROA measurements, deuterated DMSO (d^6 -DMSO) was used in order to access the potentially interesting spectral range corresponding to CH_2 deformations within the PLGA side chains.

Unless indicated otherwise, the typical preparative routine of β_2 -aggregates of PLGA consisted in dissolving the commercial sodium salt of PLGA in D_2O (or H_2O) at 10 mg/ml concentration followed by acidification with diluted DCl (HCl) to pD (pH) approx.~ 4.1 (uncorrected for isotopic effects in the case of D_2O -dissolved samples), as described earlier.³²⁻³³ Thus prepared liquid samples were incubated at 60 °C for 24 hours which resulted in formation and precipitation of β_2 -PLGA. For DMSO-titration experiments (0% - 90% range) reported in Fig. 2, β_2 -PLGA was pelleted with a centrifuge and subsequently re-suspended/dissolved in mixtures of DMSO and D_2O , pD 4.1 at the desired final concentrations of DMSO while maintaining a constant PLGA concentration of 10 mg/ml. PLGA samples in "100% DMSO" were obtained by dissolving freeze-dried β_2 -PLGA in anhydrous DMSO (or d^6 -DMSO for VCD and ROA measurements) at various concentrations (typically 10 mg/ml for FT-IR measurements but considerably higher for VCD and ROA, as specified). The initial stages of preparation of β_2 -PLGA take place when the polypeptide is fully dissolved in water first in random coil then (after acidification) in α -helical conformation. When preparation of β_2 -PLGA is carried out in D_2O , these conditions are conducive to full and practically instantaneous H/D-exchange of backbone NH and Glu side chain $-COOH$ groups. Hence β_2 -PLGA precipitated and freeze-dried afterward is labelled as 'fully deuterated'. Samples of PLGA in the random coil (RC) conformations for FT-IR (Fig. 1) and AUC (Supporting Information) measurements were prepared by dissolving commercial sodium salt of PLGA in pure D_2O (FT-IR) or NaCl solution in H_2O (AUC, as specified) at neutral pD/pH. Unless stated otherwise aqueous α -helical PLGA (e.g. Fig. 1) was obtained by

dissolving PLGA sodium salt in 25 v/v %, or 40 v/v % solutions of TFE in D₂O at 10 mg/ml concentration and adjusting pD with diluted DCl to 4.1.

The isopropanol-precipitated helical PLGA (PLGA-iso) was obtained by rapid mixing a 600 μ L portion of 10 mg/ml solution of β_2 -PLGA in anhydrous DMSO (see above) with a 1000 μ L volume of cooled isopropanol resulting in precipitation of polypeptide. The pellet was centrifuged and washed several times with excess volumes of cooled isopropanol to remove traces of DMSO. Subsequently, the polypeptide was re-suspended in isopropanol at desired concentrations for far-UV circular dichroism (CD) measurements (liquid sample). Alternatively, PLGA-iso suspension in isopropanol was spread over a CaF₂ window and dried to collect Fourier-transform infrared (FT-IR) and far-UV CD spectra of films, or, after further dilution with isopropanol was applied onto mica surface for AFM analysis.

FT-IR Spectroscopy

The spectra were acquired using a CaF₂ transmission cell and 0.05 mm Teflon spacer on Nicolet iS50 FT-IR spectrometer (Thermo, USA) equipped with a DTGS detector. Typically, FT-IR spectra were obtained by co-adding 32 interferograms of nominal resolution of 2 cm⁻¹. During measurements, the spectrometer's sample chamber was continuously purged with dry air while the temperature in the cell (25 °C, or as specified) was controlled through dedicated Peltier system. From each sample's spectrum the corresponding buffer and water vapor spectra were subtracted. Baseline correction, calculation of Savitzky-Golay second derivative spectra and peak-fitting procedure were performed using GRAMS software (Thermo). All further experimental details were the same as specified earlier.³⁸

Far-UV CD Spectroscopy

For the acquisition of far-UV CD spectra fresh 0.025 mg/ml suspension of PLGA-iso in excess of pure isopropanol was prepared. Reference samples of PLGA (also at 0.025 mg/ml concentration) were prepared in D₂O, pD 4.1, or in 25 v/v % TFE in D₂O, pD 4.1. Liquid samples were placed in a 1 cm quartz cuvette. Measurements were carried out at 25 °C by accumulation of 5 independent spectra on Jasco J-815 S spectropolarimeter (Jasco, Japan) equipped with a Peltier module. PLGA-iso films for far-UV CD were prepared by drying droplets of diluted suspension of PLGA in isopropanol on a CaF₂ window. The films selected for CD measurements did not exhibit significant optical anisotropy (in terms of birefringence or linear dichroism).

VCD Spectroscopy

Samples for VCD measurements were obtained by dissolving freeze-dried deuterated β_2 -PLGA in d⁶-DMSO at approx. 37 mg/ml concentration. The reference sample of helical PLGA was obtained by dissolving sodium salt of PLGA at approx. the same concentration in 25 v/v % TFE in D₂O and subsequent adjustment of pD to 4.3 using diluted DCl. At this pD value the overall helical content in aqueous PLGA remains high while the aggregation and precipitation of polypeptide is decelerated (VCD requires both high concentration of PLGA and relatively long acquisition times). Spectra were measured with 12 cm⁻¹ spectral resolution and accumulated as the average of 4 scans using a homemade dispersive VCD instrument previously described in the literature⁴⁴ for samples in CaF₂ cells with 50 μ m Teflon spacer. Baselines were partially corrected by subtraction of identically collected spectra of appropriate solvents. In parallel to VCD measurements, control infrared spectra (at 4 cm⁻¹ resolution) were collected on the same samples with Bruker Vertex 80 spectrometer with a DTGS detector.

ROA Spectroscopy

PLGA samples for ROA / Raman measurements were typically of 45 mg/ml concentration (in d^6 -DMSO or 25 v/v % TFE in D_2O , pD 4.5). The spectra were collected at ambient temperature on a ChiralRAMAN spectrometer (BioTools, Inc.), which employs the scattered circular polarization measurement strategy in backscattering. Total acquisition time of a single ROA spectrum was 12-13 hrs. The ROA difference spectra are presented as circular intensity differences ($I_R - I_L$) and the parent Raman spectra as circular intensity sums ($I_R + I_L$), with I_R and I_L denoting the Raman-scattered intensities with right- and left-circular polarization states, respectively. Samples were pipetted into quartz microfluorescence cells and the measurements were carried out under the following conditions: laser excitation 532 nm; laser power measured at the sample ~ 200 mW; spectral resolution ~ 7 cm^{-1} . Pure solvent spectra were subtracted from the parent Raman spectra and all spectra were subsequently smoothed using a second level Savitzky-Golay filter.

AFM microscopy

Samples of isopropanol-precipitated PLGA (washed with excess of isopropanol several times) or of β_2 -PLGA aggregates were diluted approximately 100 times with excess of isopropanol or acidified H_2O (pH 4.1), respectively. A small droplet of 8 μl of either PLGA sample was deposited onto freshly cleaved mica and left to dry for 24 hours. AFM tapping-mode measurements were carried out using Nanoscope III atomic force microscope (Veeco, USA) and TAP300-A1 sensors with cantilever resonance frequency of 300 kHz (BudgetSensors, Bulgaria).

SAXS

The SAXS data were collected at the P12 beamline⁴⁵ of the EMBL at the PETRA III storage ring (DESY, Hamburg) on PLGA dissolved in DMSO at solute concentrations 10, 20, and 30 mg/ml. The samples and buffer (pure DMSO) underwent continuous sample flow during SAXS measurements at the temperature 20 °C consisting of 40 × 50 ms exposures, for a total exposure time of 2 s. The data were recorded on a Pilatus 2M detector at the wavelength 1.24 Å, covering the momentum transfer range $0.025 < s < 4.8 \text{ nm}^{-1}$ ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). The experimental data was processed using standard procedures⁴⁶⁻⁴⁷, the radius of gyration (R_g) and the particle distance distribution function ($P(r)$) were calculated with Autorg⁴⁸ and Datgnom⁴⁸, respectively. The molecular mass (MM) of the solute was calculated by comparison of the forward scattering $I(0)$ of the sample with that of the scattering from a reference bovine serum albumin solution and also from the excluded solute volume calculated with Datporod.⁴⁸ The shape of the solute was calculated ab initio with DAMMIF⁴⁹ (twenty models were generated and averaged by Damaver⁵⁰). The scattering from the atomistic models was calculated and compared with the experimental data by CRY SOL.⁵¹

Results and Discussion

Transmission FT-IR spectra of various conformational states of fully deuterated PLGA are shown in Figure 1. At neutral and alkaline pD, as is the case of aqueous solution of PLGA sodium salt, the polypeptide is in the random coil (RC) conformation with the corresponding broad amide I' band at 1646 cm^{-1} and another large band at 1563 cm^{-1} assigned to antisymmetric stretches of side chain ionized carboxyl groups. Acidification neutralizes Glu side chains (reflected by the rise of $-\text{COOD}$ (ν) peak above 1700 cm^{-1} and simultaneous disappearance of the 1563 cm^{-1} band) allowing the PLGA backbone to acquire an α -helical conformation, wherein the amide I' band becomes slightly narrower and shifts to 1643 cm^{-1} . As helical PLGA in concentrated aqueous solutions is metastable, TFE was added to stabilize this conformation. Without such measures incubation of aqueous α -helical PLGA, especially at low pH and elevated temperatures, results in rapid self-assembly and precipitation of β_2 -fibrils (Supporting Information Fig. S2). The unique infrared characteristics of β_2 -fibrils consists in the splitting of the amide I' band into two sharp components: the major shifted to 1595 cm^{-1} and the minor at $\sim 1637\text{ cm}^{-1}$ (black line).^{32-34,52} This unusual amide I' band redshift has been attributed, in part, to the occurrence of three-center hydrogen bonds within β_2 -fibrils.³²⁻³³ Namely, a single bifurcated carbonyl acceptor (of the peptide bond) binds two hydrogens: one of peptide NH (ND) group, another of Glu side chain's $-\text{COOH}$ ($-\text{COOD}$) group. Combined, these two hydrogen bonds significantly decrease electron density on the carbonyl and hence the frequency of C=O stretch which accounts for the largest contribution to the amide I band. The involvement of side chains in this bonding pattern is reflected by the changes in the contour of the $-\text{COOD}$ stretching band which in the spectra of β_2 -fibrils splits into two peaks at 1731 and 1718 cm^{-1} . The three conformational states of PLGA retain the key infrared features when isolated as solids (see: Supporting Information Fig. S3).

For an initial assessment of the influence of DMSO on stability of β_2 -fibrils, measurements of optical density were carried out (Figure 2). Light-scattering aqueous suspensions of fibrils become gradually translucent with the increasing concentration of the solvent. Eventually, freeze-dried β_2 -PLGA dissolves easily when transferred to pure DMSO producing completely clear solution (see the inset in Figure 2A). However, a decrease in optical density of β_2 -PLGA suspensions is noticeable for DMSO concentrations well below 20 v/v % while the titration trajectories are clearly biphasic: most rapid changes take place between 10 and 20, and above 50 v/v % of DMSO. The corresponding solvent-subtracted transmission FT-IR spectra are shown in panels B and C of Figure 2. Interestingly, for DMSO concentration up to 60 v/v %, virtually no identifiable spectral changes in β_2 -fibrils are observed. Only at 70 v/v % of DMSO the β_2 -structure reveals first signs of breaking down: decrease in intensity of the 1596 cm^{-1} band with a new band at around 1643 cm^{-1} starting to overlie the 1637 cm^{-1} component. The spectra collected for PLGA fibrils dissolved in 80, 90, and 95 v/v % DMSO in acidified D_2O distinctly point to complete dissection of β_2 -structure. Surprisingly, the frequency of the amide I' band ($1643\text{-}1644\text{ cm}^{-1}$) is entirely different from that observed for insulin amyloid fibrils at this DMSO concentration range ($1661\text{-}1663\text{ cm}^{-1}$ – see²⁵⁻²⁶); even though the non-cooperativity of decreasing optical density and FT-IR-detected β -sheet content is similar. For freeze-dried β_2 -fibrils dissolved in 100 % DMSO, the amide I' band shifts to 1652 cm^{-1} and becomes considerably narrower which clearly contrasts with the aforementioned typical denaturation scenario observed for most proteins including amyloid fibrils^{4,22} (see also Supporting Information Fig. S4). The transition in the amide I' band frequency range is accompanied by spectral changes in -COOD band that, at 1715 cm^{-1} , is no longer split. The amide II band visible at 1550 cm^{-1} in Figure 2C arises from partial D/H-back-exchange taking place during the freeze-drying of deuterated β_2 -PLGA.

The frequency and narrow bandwidth of the amide I' band prove that β_2 -fibrils transferred to DMSO convert into a structural state entirely distinct from the disordered coil found in DMSO-denatured amyloid fibrils from globular proteins^{22,25-27}, and is reminiscent of helical conformation. The DMSO-induced conformation of PLGA is very stable over time and resists mild heating (also Supporting Information Fig. S5). In Figure 3, the solvent-subtracted spectrum of DMSO-dissolved PLGA (A) is juxtaposed with the typical spectrum of α -helical PLGA in 25 v/v % TFE in D₂O, pD 4.1 (B), i.e. under conditions promoting highest helical content in the polypeptide. The spectra were subjected to peak-fitting with Lorentzian components.⁵³⁻⁵⁴ Parameters of fitted Lorentzian components (see Supporting Information, Table S2) clearly indicate that in DMSO, PLGA acquires a more homogenous conformation. This is reflected both in terms of full width at half maximum (FWHM) of individual components, their numbers and distribution of intensities. For both cases corresponding second-derivative spectra were calculated as well (Figure 3C). Again, within the amide I' band frequency range, the single well-defined peak at 1652 cm⁻¹ for DMSO-dissolved polypeptide contrasts with three peaks at 1653, 1643, and 1627 cm⁻¹ for the sample dissolved in TFE/D₂O mixture. The –COOD band is well-resolved only in the former case.

While the data presented so far is consistent with a rather homogenous, and putatively helical conformation being formed upon dissolving of β_2 -fibrils in DMSO, FT-IR spectroscopy alone is not a sufficient tool to verify whether this conformation is indeed α -helical or not. The observed frequency of the main amide I' component falls within the range ascribed to helices. On the other hand, the blue shift vis-à-vis frequencies observed for helices in aqueous environment (1654 vs. 1641 cm⁻¹) could be rationalized in terms of solvent effects recognized to affect amide I band.⁵⁵⁻⁵⁸ In order to gain an additional conformational insight into the state of DMSO-dissolved PLGA, we have employed two chiroptical methods: VCD and ROA.²²

A comparison of VCD spectra collected for β_2 -filbrils dissolved in DMSO and regular helical PLGA in 25 v/v % TFE/D₂O, pD 4.1 is presented in Figure 4A (the top panel shows control FT-IR spectra re-collected for the same samples). The VCD spectral signature of the DMSO-dissolved sample is dominated by a positive couplet consisting of a single narrow negative peak at 1659 cm⁻¹ (to the high frequency side of the absorbance maximum) with a low-intensity positive band at ca. 1643 cm⁻¹. This pattern is typical of α -helices⁴¹ and is very different from VCD spectra of β_2 -filbrils consisting of two minor negative bands at 1632 / 1605 cm⁻¹ and a single major positive band at 1598 cm⁻¹ (e.g.³³), but it is also distinct from the negative couplet (positive at 1685 cm⁻¹ and negative at 1655 cm⁻¹) typical of disordered proteins and peptide structures having significant PPII conformation⁵⁹ as observed, for example, for insulin amyloid dissolved in DMSO.²² In fact, such a VCD spectrum is consistent with a right-handed helical, most likely α -helical conformation.⁶⁰⁻⁶² Again, in comparison with the PLGA sample dissolved in acidified TFE/D₂O system, the DMSO-induced state appears to be more structurally homogenous. The three-lobed VCD band for the TFE dissolved PLGA is typical of N-deuterated helices, but the relatively high intensity of the 1624 cm⁻¹ band suggests the sample retains some disorder. It should be noted that while the VCD signal intensity scaled to absorption values ($\Delta A/A$) is larger ($\sim 5 \times 10^{-5}$) for the DMSO-based sample, as expected for a highly helical conformation, it does not reach the extreme values that have been observed for helical poly(γ -benzyl-L-glutamate) in chloroform, which were of the order of 2.5×10^{-4} .⁶³

Paralleling the FT-IR/VCD data, Raman and ROA spectra were also collected for DMSO- and TFE/D₂O-dissolved PLGA samples (Figure 4B). In unison with infrared absorption and VCD results, Raman peaks including amide I band and C-H bending modes (1250-1350 cm⁻¹) reveal again significantly reduced bandwidths for the DMSO-dissolved sample. In the diagnostically useful range of the ROA spectra, a marked narrowing and

simultaneous blue-shift of amide I band to 1668 cm^{-1} is observed, the former indicating a decrease in conformational flexibility. More importantly, its couplet (- / +) structure is an important indicator of order in form of α -helices⁶⁴⁻⁶⁵, as disordered peptides are characterized by a broad, all positive amide I band. Furthermore, in both helical forms of PLGA, a strong positive band at 1346 cm^{-1} (in a couplet with unusual negative band at $\sim 1300\text{ cm}^{-1}$ for the DMSO-dissolved peptide) is observed. This band has recently been shown to be the most stable ROA marker band for α -helices⁶⁶, which, together with the other spectroscopic evidence, strongly points towards a highly ordered, α -helical conformation.

Due to very strong UV-absorption by DMSO, electronic circular dichroism (ECD) of samples dissolved in this solvent is practically unmeasurable below 260 nm. However, in an attempt to use ECD as a probe of the DMSO-induced conformation of PLGA, we have established an experimental protocol of a rapid isolation of PLGA from DMSO aimed at avoiding or minimizing conformational disruptions of polypeptide as checked by infrared absorption. The approach (described in Materials and Methods section) consisted in an abrupt mixing of DMSO-dissolved PLGA with an excess of cooled isopropanol which competitively replaces PLGA's -COOH groups as hydrogen donors to DMSO. The instantaneously formed precipitate was washed several times with isopropanol to remove traces of DMSO. The red FT-IR spectrum in Figure 5A corresponds to a film of thus obtained 'PLGA-iso', and is overlaid with a control solution spectrum of PLGA in DMSO taken before the addition of isopropanol. Both spectra are very similar in respect to frequency and FWHH of the amide I band although the precipitate appears to be slightly 'contaminated' with other, possibly less ordered, conformations (vide the overlapping shoulder at lower wavenumbers). The -COOH band becomes broad, and decreases in intensity which, at least in part, could be attributed to changes in interactions with the surroundings. In precipitated solvent-depleted state of PLGA, regardless of the backbone conformation, clumped Glu side chains interact with each other

through hydrogen bonds in a number of ways depending on local geometries. Averaging of these contributions to the spectra leads to broadening of vibrational bands of involved chemical groups. However, the overall similarity of the main amide I bands suggests that the procedure is effective in preserving the secondary structure of PLGA in DMSO. This has become a starting point for measuring far-UV CD spectra shown in Figure 5B. The spectra were collected for PLGA-iso both as a suspension in isopropanol and as a dry film deposited onto an UV-transparent substrate. In either case, there are prominent pairs of negative peaks at ca. 209 and 225 nm (suspension) or 208 and 223 nm (film). In contrast to the typical spectra of α -helical structures (e.g. of PLGA dissolved in the acidified TFE/D₂O mixture) where intensity ratio of the bands at 208 and 222 nm is approximately 1.0, for PLGA-iso the long wavelength band is relatively ‘enhanced’ by 25 % (for film), or 80 % (suspension). While similar spectra have been assigned to canonical type-I turns⁶⁷ such conformation is problematic as a dominant secondary structure of long polypeptide chains and even harder to reconcile with the previously discussed spectroscopic data. It should be also stressed that for 3_{10} -helix the opposite type of spectral deformation is expected (the short wavelength band becomes more intensive⁶⁸⁻⁶⁹). Given the fact that once-precipitated PLGA-iso chains remain strongly agglomerated either as dry films or as suspensions in isopropanol, the lack of molecular-level dispersion of PLGA could explain the observed deformation of the far-UV CD spectra. Firstly, strong light-scattering of short UV on insoluble particles is a known factor distorting CD spectra. Secondly, tight lateral agglomeration of PLGA-iso could, in principle, enable lateral couplings of electronic transition moments in amide chromophores that would lead to deformation of CD signals. According to the auxiliary AFM-based analysis of PLGA-iso shown in Figure 5C, the morphology of precipitates, while clearly different from β_2 -fibrils does reveal elongated worm-like features which could arise from such tightly self-associated helices. We have also examined the conformational stability of PLGA-iso upon

exposure to water using infrared spectroscopy (see Fig. S6). The helical structure remained remarkably stable, possibly due to the fact that it remained in a strongly self-associated and poorly dispersed state. A prolonged room-temperature incubation of PLGA-iso suspension in acidified (pH 4.1) water triggered noticeable partial conversion to β_2 -form (Fig. S6 in Supporting Information).

The picture emerging from the data depicts an unusual scenario of DMSO-induced disassembly of β_2 -fibrils of PLGA which apparently leads to highly ordered α -helices in which backbone NH groups do not interact directly (via hydrogen bonds) with the solvent. The hypothetical conformational homogeneity of PLGA in DMSO entails that these α -helices must have very high persistence lengths with few (if any) bends and loops such that the entire PLGA molecule would likely have a rod-like shape. To obtain a direct information about the overall shape of PLGA in DMSO, SAXS was employed, and the experimental data is presented in Figure 6.

The zero-angle intensity (inset in Figure 6) and the calculated radius of gyration R_g of the solute decrease with concentration pointing to repulsive interactions between PLGA molecules. The data extrapolated to infinite dilution was used to evaluate the MW of the solute (see Methods) yielding an estimate of $MW = 7 \pm 1$ kDa. This suggests, similarly to above AUC results, that the PLGA molecules in DMSO have a somewhat lower MW than that indicated by Sigma-Aldrich for the given lot. The particle radius of gyration is $R_g = 1.8 \pm 0.1$ nm and the distance distribution $p(r)$ computed from the experimental data (Figure 7) reveals the maximum size of $D_{max} = 7.5 \pm 0.5$ nm. The $p(r)$ function displays a skewed profile typical for rod-like particles, and the cross-section of the rod determined by the position of the maximum in the $p(r)$ is about 1 nm. The *ab initio* low resolution shape of PLGA restored by DAMMIF yields a good fit to the experimental data with discrepancy $\chi^2 = 0.68$ (Figure 8) and reveals a rod-like structure with repeating nodes along the long axis

(Figure 9B), which can be interpreted as turns of a helix. We further utilized DARA Web server⁷⁰ to find the structural neighbors in the Protein Data Bank⁷¹ yielding the scattering patterns similar to that observed from PLGA. The best hits found by DARA and yielding good agreement to the experimental data were short helical peptides with MW around 5 kDa and maximum size about 7 nm (the overall best fitting model, human vimentin coil 1A fragment with PDB code 1GK7, is displayed in Figure 9C, and its fit with discrepancy $\chi^2 = 0.69$ in Figure 8). To obtain further evidence, tentative models of PLGA in the form of α -helical fragments of different length were constructed and screened against the experimental data. Thirty fits were calculated for α -helical models containing from thirty to sixty PLGA molecules (inset in Figure 9); the best fit with $\chi^2 = 0.64$ in Figure 8 corresponds to a helix containing 42 monomers (11.6 turns, MW = 5.7 kDa) displayed in Figure 9C. Taken together, all SAXS-derived parameters, model-free analysis and also structural modelling provide a consistent picture of PLGA in DMSO adopting a rod-like shape, highly likely formed by α -helical folds (Figure 9). This result further corroborates the spectral data providing a strong indication that in DMSO, β_2 -fibrils of PLGA convert into structurally homogenous and practically uninterrupted (and therefore rod-like) α -helices.

The destabilizing influence of high DMSO concentrations on globular proteins and amyloid aggregates has been known for many years. The underlying physicochemical mechanisms may be complex, but at least those arising from the solvent's potential to disrupt networks of hydrogen bonds stabilizing protein secondary structures seem intuitive. Yet, the outcome of DMSO-induced structural transitions in various proteins and peptides is often perplexing. For example, while in globular proteins with various α/β content, DMSO appears to target selectively helical conformation leaving β -sheets mostly unaffected^{13,72}, β -sheets stacked within amyloid fibrils are promptly converted into PPII conformation (e.g.²⁵). Elsewhere, DMSO was shown to stabilize the 3₁₀-helical conformation in certain peptides.⁷³

According to infrared study by Mirtič and Grdadolnik, poly-L-lysine in DMSO is in helical conformation.⁷⁴ All these examples highlight the fact that the conformational fate of a polypeptide chain in DMSO is sequence-dependent and cannot be predicted based on the backbone-DMSO-solvation paradigm. Our study presents evidence that the DMSO-induced conformational transition in amyloid-like β_2 -fibrils self-assembled from PLGA ultimately leads to long practically uninterrupted α -helices. In light of the α -helix-disrupting ‘reputation’ of the solvent, the fact that PLGA forms longer helices in DMSO than it does in aqueous environment is puzzling. Length of an α -helix as a structural segment of the native state of a globular protein is determined both by local amino acid sequence and interactions with surroundings. An interplay of these two groups of factors dictates at which point (i.e. amino acid residue) the helix transitions into a turn. Statistically, the demands of dynamics and size of globular proteins are best satisfied with relatively short α -helices with very few of them consisting of more than 30 amino acid residues.⁷⁵ In this respect, a helical homopolypeptide in chemically uniform environment (solvent) constitutes a very interesting model to study intrinsic factors determining helical length such as the aggregate dipole moment of an α -helix.⁷⁶ As oriented polar molecules of the surrounding solvent are expected to interact with this macrodipole⁷⁷ a tilted layer of DMSO molecules bound to Glu –COOH groups could, in principle, provide an additional stabilization to the helix resulting in its elongation. This reasoning remains however purely speculative at this point.

Conclusions

In summary, we have shown that DMSO-induced destabilization of β_2 -amyloid like fibrils derived from poly-L-glutamic acid leads to formation of a stable α -helical conformation instead of the PPII conformation, which is the case for other globular proteins and amyloid fibrils obtained from them. The application of several chiroptical methods, as well as

synchrotron SAXS allowed us to determine the exceptional persistence length of DMSO-stabilized helices: in the absence of turns the whole PLGA becomes a rod-like molecule. Our study highlights a profound impact of solvation on polypeptide folding. Further studies are needed to provide detailed mechanistic explanation of the observed phenomenon.

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Supporting Information

Additional AUC and a FT-IR data. This information is available free of charge via the Internet at <http://pubs.acs.org>

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FIGURE 1. Transmission FT-IR spectra of PLGA in various conformational states: α -helical PLGA (formed in the presence of 25 or 40 v/v % TFE in D₂O, pD 4.1: red and blue lines, respectively); β_2 -form in D₂O, pD 4.1 (black line); random coil (sodium salt of PLGA dissolved in D₂O without pD adjustment – green line). Traces of β_2 -form already forming in the helical sample are marked with an asterisk.

FIGURE 2. Gradual dissolution of β_2 -fibrils of PLGA in the presence of increasing concentration of DMSO (v/v) in D₂O, pD 4.1 probed by optical density (A, absorbance units) at three different wavelengths and FT-IR spectroscopy (B-C). Fresh samples were incubated at 25 °C and gently agitated at 250 rpm for 24 hours prior to the measurements. Despite liquid samples becoming translucent already in the presence of 30-50 v/v % DMSO the corresponding FT-IR spectra remain intact (B). Only with DMSO concentration above 70 v/v % pronounced spectral changes take place (C). The “100 %” spectrum was obtained by dissolving freeze-dried β_2 -fibrils in anhydrous DMSO.

FIGURE 3. Peak-fitting of solvent-subtracted FT-IR spectra of PLGA in DMSO (A) and in 25 % TFE (v/v) in D₂O, pD 4.1 (B) with Lorentzian components, as indicated. The corresponding values of FWHM (full width at half maximum) are given in brackets. (C) Second derivative spectra of PLGA in DMSO (blue) and in 25 % TFE (v/v) in D₂O, pD 4.1 (red).

FIGURE 4. Chiroptical characterization of PLGA dissolved in DMSO vs. the typical helical form of PLGA in 25% TFE-aqueous (v/v) acidified solution. For the acquisition of VCD/IR spectra (A) non-deuterated DMSO and deuterated TFE/water (D₂O, pD 4.1) were used, whereas for ROA/Raman measurements PLGA was fully protonated and dissolved either in 25% TFE in H₂O, pH 4.1, or in fully deuterated DMSO (used in order to exhibit the amide III band region).

FIGURE 5. Properties of PLGA-iso: the rapidly precipitating form of PLGA upon mixing of solution of (β_2 -form) PLGA in DMSO with excess of isopropanol. (A) Transmission FT-IR spectra of dry film of PLGA-iso deposited on CaF₂ window overlaid with spectrum of solvent-subtracted PLGA in DMSO. (B) Far-UV CD spectra of suspension of PLGA-iso in isopropanol overlaid with spectra of typical α -helical PLGA in acidified aqueous solution in the presence and absence of TFE (left) and CD spectrum of dry film of PLGA-iso. (C) Amplitude AFM images of dry PLGA-iso (left) and β_2 -PLGA fibrils (right).

FIGURE.6. SAXS curves of PLGA in DMSO with sample concentrations $c = 30$ (black), 20 (red) and 10 (green) mg/ml. Concentration dependence shows a linear character (shown in inset), which allows to apply extrapolation on zero concentration

FIGURE.7. Normalized pair-distance distribution function $P(r)$ for the PLGA sample.

FIGURE 8. Experimental data extrapolated to infinite dilution (black markers) and corresponding fitted curves obtained according to: [i] manually constructed atomistic model (red line), $\chi^2=0.64$ (the goodness of fit as a function of model length is displayed in the inset); [ii] DAMMIF shape model (green line), $\chi^2=0.68$; [iii] closest structural neighbor from the PDB (orange line), $\chi^2=0.69$.

FIGURE 9. Models of PLGA in DMSO: A) best model manually built from PLGA monomers; B) ab initio shape restored by DAMMIF; C) the closest structural neighbor in the PDB as found by DARA.

TOC Graphic