pH-Driven Polymorphism of Insulin Amyloid-Like Fibrils

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Abstract

Prions are infective proteins, which can self-assemble into different strain conformations, leading to different disease phenotypes. An increasing number of studies suggest that prion-like self-propagation may be a common feature of amyloid-like structures. Thus it is important to unravel every possible factor leading to the formation of different amyloid strains. Here we report on the formation of two types of insulin amyloid-like fibrils with distinct infrared spectroscopic features grown under slightly different pH conditions. Similar to prion strains, both insulin fibril types are able to self-propagate their conformational template under conditions favoring spontaneous formation of different type fibrils. The low-pH-induced insulin amyloid strain is structurally very similar to previously reported strains formed either in the presence of 20% ethanol, or by modification of the amino acid sequence of insulin. A deeper analysis of literature data in the context of our current findings suggests a shift of the monomer-dimer equilibrium of insulin as a possible factor controlling the formation of different strains.

Introduction

Amyloid-like structures are associated with a number of pathological conditions including neurodegenerative diseases, such as Alzheimer’s and Parkinson’s, and infectious prion diseases, also a number of nonneuropathic systemic amyloidoses, and even type II diabetes [1]. In some cases amyloid-like folds can play a positive role as well: they have a structural function in spider silk and biofilm formation in bacteria, and a regulatory function in fungi or hormone storage in humans [2]. Experiments in vitro revealed even more amyloid-forming proteins and peptides, including proteins with no link to in vivo amyloids, such as polyaminoacids (e.g., polyllysine, polythreonine and polyglutamic acid) [3], and short oligopeptides [4–6]. Finally, even an amyloid-like self-assembly of phenylalanine was recently reported [7]. All these findings support the idea that amyloid-like folds may be a generic property of all polypeptides, while the propensity of fibril formation would depend on the sequence of the polypeptide and on the environmental conditions (i.e., temperature, pressure, solution milieu, interaction with lipid interfaces, pH) [1].
Prions stand out among other amyloid-forming proteins as the only proteinaceous infectious pathogens [8]. Identical amino acid sequences of prion protein can adopt distinct pathogenic conformations, referred to as prion strains [9,10]. Different strains lead to distinct incubation periods and patterns of neuropathology in prion diseases [10]. Similar conformational variations were detected in other amyloid-forming proteins both in vitro [11–22] and in vivo [23–25]. With growing evidence of the involvement of prion-like mechanisms in the progression of other amyloid-related diseases [23–33], it is indispensable to understand all the factors determining formation of different amyloid strains.

The new variant Creutzfeldt-Jakob disease (vCJD) is thought to be caused by a bovine spongiform encephalopathy (BSE) strain [34]. In this case, the determining factor for the formation of distinct prion strains is cross-species infection. Similar to prions, formation of distinct amyloid strains for two slightly different insulin forms was recently reported [19,35]. When protein sequences are identical, the environment plays the key role in straining of amyloid-like fibrils. The presence of co-solvents [11,14,15,20], different temperatures [36–38], different concentrations of denaturants [38,39] and salts [21], or different ways of agitation [12,40] may lead to distinct amyloid fibril strains. Here we report on the formation of distinct insulin amyloid strains at slightly different pH values.

As diagnostic tool, Fourier-transform infrared (FTIR) spectroscopy has been used, which has proven to be an important method for the characterization of secondary structural changes of prion and amyloid strains [11,19,41], supplemented by atomic force microscopy (AFM) measurements of the topology of amyloid fibrils and thioflavin T (ThT) fluorescence for recording the fibrillation kinetics.

Results and Discussion

In our recent work on potential inhibitors of insulin amyloid-like fibrillation, we followed the aggregation of insulin at pH 2 in the presence of 5% residual dimethylsulfoxide (DMSO) [42]. To test if the presence of a small amount of DMSO affects the fibrillation process, we compared the FTIR spectra of insulin amyloid-like fibrils spontaneously formed in D2O in the presence (Fig 1A) and absence (Fig 1B) of 5% DMSO. To reveal possible changes upon using D2O instead of H2O, as required for the better quality FTIR measurements, and for looking into subtle pH changes on the fibrillation propensity of insulin, fibrils were prepared in heavy water samples at two pH/C3 values (where pH/C3 is the pH-meter readout uncorrected for isotopic effects, see Methods section), pH/C3 1.6 to mimic similar concentrations of H+ and D+, and pH/C3 2 to reach the same ionization state of the protein in the two solvents. The FTIR spectra look similar in the presence and absence of DMSO, but a rather small difference in pH/C3 leads to significant differences in amide I’ band contours (Fig 1A and 1B). Spectra of fibrils prepared at pH/C3 2 exhibit maxima in the amide I’ region at ~1628 cm⁻¹ (with the main minimum of the second derivative at 1628 cm⁻¹ and a weaker one at 1615 cm⁻¹), while spectra of fibrils grown at pH/C3 1.6 exhibit maxima in the amide I’ region at ~1621–22 cm⁻¹ (with the main minimum of the second derivative at 1619 cm⁻¹ and a weaker one at 1631 cm⁻¹), pointing toward predominantly beta-sheet structures but with a significantly different hydrogen-bonding patterns. A small band outside of the amide I’ region at ~1728 cm⁻¹ is present only in the spectra of fibrils grown at pH/C3 1.6 and can be attributed to deuterated carboxyl groups [19]. Very similar spectral characteristics were recently described as a hallmark of two different insulin amyloid strains [19,35].

Fibrils grown at pH/C3 1.6 in the presence of 5% DMSO are usually 2–4 nm in diameter and exhibit both a curved and straight morphology (Fig 1C), while fibrils grown at pH/C3 2 both in the presence (Fig 1D) and absence (Fig 1E) of DMSO are thicker (4–16 nm) and usually
The structure of the fibrils at pH 1.6 in the absence of DMSO (Fig 1E) looks similar to the case at pH 2, suggesting no clear morphological differences between strains.

Both types of fibril seeds induce aggregation of insulin at either pH* and 37°C (Fig 2). Seeds grown at pH* 1.6 fibrillate insulin at similar rates under both pH* conditions and faster than seeds grown at pH* 2. The latter seeds elongate faster at solution conditions of the same pH*.

As clearly visible, the fluorescence intensity of Thioflavin T (ThT), which marks formation of fibrillar amyloid states, is seed-dependent: pH* 1.6-seed-induced aggregates result in an about double ThT intensity when compared to pH* 2-seed-induced aggregates (Fig 2A). The light absorbance data at 600 nm—as measure of formation of larger insulin aggregates due to light scattering—show the reverse effect (Fig 2B). The pH* 2-type fibrils induce aggregates which strongly absorb visible light (600 nm), the absorbance being ~25% lower in the case of seeding in the pH* 1.6 environment. pH* 1.6-type fibrils induce weakly absorbing aggregates (about 5 times lower than pH* 2-type fibrils); however, the absorbance is strongly increased in the pH* 2 solution.

The FTIR spectra of the seeded fibrils clearly demonstrate the superiority of the seed template versus the pH*-environment in controlling the fibrillar structure (Fig 3). The spectra of pH* 2-seed-induced aggregates grown at pH* 2 and pH* 1.6 look identical. In case of the pH* 1.6-seeded aggregates, the spectral signature is similar for both solution conditions; however, in pH* 2, the intensity of the band at 1631 cm⁻¹ is increased. These data confirm the ability

**Fig 1. Polymorphism of insulin amyloid-like fibrils formed at different pH* values.** FTIR absorption spectra (second derivative spectra in the inset) of fibrils grown in the presence (A), and absence (B) of 5% DMSO (spectra were repeated using different FTIR instruments in different labs, see S1 Fig). AFM images of fibrils prepared in the presence of DMSO at pH* 1.6 (C), and pH* 2 (D) or in absence of DMSO at pH* 1.6 (E), and pH* 2 (F). Fibril height measurements are shown in S2 Fig.

doi:10.1371/journal.pone.0136602.g001
of both types of insulin fibrils to self-propagate their conformational template in spite of unfavorable environmental factors (here different pH conditions), suggesting the existence of two different insulin amyloid strains.

Surprisingly, the FTIR spectra of insulin amyloid-like fibrils spontaneously formed in H2O at pH 1.6 and pH 2 look almost identical (Fig 4). Both spectra exhibit maxima in the amide I/I' region at ~1628 cm⁻¹ (with the main minimum of the second derivative at 1628 cm⁻¹ and a weaker one at 1641 cm⁻¹), and a small band outside of the amide I/I' region at ~1730 cm⁻¹. A different spectrum was obtained using fibrils spontaneously formed in H2O at a slightly higher pH, at pH 2.4: it also exhibits a maximum in the amide I/I' region at ~1628 cm⁻¹, but the second derivative profile is different – two similar sized bands, at 1625 cm⁻¹ and 1636 cm⁻¹, respectively. As the amyloid-like fibrils are highly protected from hydrogen/deuterium exchange, most of the amide hydrogens stay unchanged despite resuspension of the aggregates in D2O. It reflects in the blue-shift of the spectra compared to insulin fibrils, prepared in D2O.

Different types of insulin fibrils were first mentioned more than 60 years ago [43], however no structural or cross-seeding data were presented. In more recent studies, formation of different strains were reported in the presence and absence of 20% ethanol (at pH 1.5–1.8) [11,14,15], and using slightly different insulin forms (bovine insulin (BI) and recombinant Lys⁶³¹-Arg⁶³² human insulin analog (KR)) at pH 1.9 [19,35]. Spectral characteristics of the latter strains are very similar to our data. The spectrum of the fibrils formed at pH 2 is similar to the spectrum of the BI strain, and the spectrum of the fibrils formed at pH 1.6 reminds us of the one of the KR strain. So the effect of two additional positively charged amino acids on the fibrillar structure is similar to the effect of ΔpH by -0.4 units. The change in net charge of the protein due to such ΔpH is minor, and taking into account that in normal water at pH 1.6 and pH 2 insulin aggregates into the same strain, we may conclude that ionization state of the protein is not the factor inducing formation of different strains. So what is the factor?

A possible answer to that question can be found by analyzing recent studies, which, at first sight, seem to contradict our findings [44–46]. In these works, no differences in the FTIR spectra of insulin fibrils formed at different pH values in the range between 1.3 and 3.1 are reported, however, a marked change of the vibrational circular dichroism (VCD) spectra are seen.

Fig 2. Kinetics of seed-induced aggregation of insulin; followed by ThT fluorescence intensity (A) as maker of fibril formation, and light absorbance at 600 nm (B). Measurements were repeated using 3 batch preparations showing similar results.

doi:10.1371/journal.pone.0136602.g002
between pH 2.1 and 2.4, which is explained by a different supramolecular chirality [44,45]. Furthermore, it was shown that the chirality can be converted by incubation of preformed fibrils at different pH, thus excluding the possibility of different strains [46]. The reported FTIR spectra lack a detailed description, however, the shape of the amide I band looks very similar to the amide I' band of the pH*2 fibrils [44–46]. A closer inspection reveals one major experimental difference, which can affect the mechanism of insulin fibrillation. The concentration of insulin used in the aforementioned studies was 60 mg/mL (compared to 1 mM (~5.8 mg/mL) in our study), which means a strong shift towards a higher oligomeric state of insulin in solution, as even at much lower concentrations insulin tends to oligomerize [47–49]. Hence, the factor which determines the formation of different strains could be due to a shift in the monomer-oligomer equilibrium.

The spectral features of the insulin amyloid strain formed in the presence of 20% ethanol [11,14,15] are similar to those of the pH*1.6 and KR strain [19,35]. In all three cases the second derivative FTIR spectra in the amide I' region exhibit strong minima at 1619–1620 cm⁻¹, and a weaker one at 1630–31 cm⁻¹, plus a small band outside of amide I' region at 1728–30 cm⁻¹. Hence, it should be concluded that the same amyloid strain is present in all three cases. It is known that the presence of 20% ethanol strongly increases the dissociation of insulin dimers...
[50], leading to predominantly monomeric insulin at moderate concentrations [14,51]. The C-terminal part of the B-chain of insulin is involved in the formation of intramolecular antiparallel β-sheet that binds together native insulin dimers [19]. Thus there is a high probability that two additional charged amino acids would lead to dissociation of dimers in case of KR insulin. There is no data on the monomer-dimer equilibrium of insulin at pH 1.6, but the fact that different strains can be formed not only with increasing pH, but also with increased concentration of insulin, suggests a shift of the equilibrium to the monomeric state. We may hence hypothesize that the major factor which determines formation of different strains is a shift of the equilibrium between insulin monomers and dimers (oligomers) (Fig 5). If the equilibrium
is shifted towards dimers (or higher oligomers), insulin aggregation would result in the pH*2-like strain, and in case that the equilibrium is shifted towards monomers, growth of the pH*1.6-like strain is fostered.

To further test the hypothesis we carried out several additional experiments. First we checked if an increased insulin concentration would explain the differences observed between the pH*1.6 and pH*2 data. As seen in Fig 6A, and Table 1, the spectrum of 10 mM insulin aggregates, prepared in the pH*1.6 environment, is slightly different from the other spectra. The blue shift of the amide I’ maximum, when compared to the spectra of the pH*1.6 strain, and the absence of the band around 1728 cm⁻¹ suggests that the increased protein

Table 1. Summary of FTIR band positions of insulin amyloid-like fibrils.

| Insulin aggregation conditions | Amide I’ band (2ⁿᵈ derivative), cm⁻¹ | Additional bands, cm⁻¹ |
|-------------------------------|-------------------------------------|------------------------|
|                               | Beta-sheets                         | Turns/loops            | Carboxyl groups⁵       |
| 1 mM insulin, pH*1.6          | 1619/1631⁴                          | 1665                   | 1728                   |
| 1 mM insulin, pH*2            | 1628/1615                           | 1665                   | absent                 |
| 1 mM insulin, pH*1.6, 5% DMSO | 1619/1631                           | 1664                   | 1728                   |
| 1 mM insulin, pH*2, 5% DMSO   | 1628/1615                           | 1665                   | absent                 |
| 10 mM insulin, pH*1.6         | 1623                                | 1662                   | absent                 |
| 1 mM insulin, pH*2, 20% ethanol | 1620/1631                           | 1663                   | 1728                   |
| 1 mM insulin, pH*2, 20% DMSO  | 1619/1629                           | 1661                   | 1731                   |
| 1 mM insulin, pH 1.6          | 1628/1641                           | 1672/1661              | 1729                   |
| 1 mM insulin, pH 2            | 1628/1641                           | 1672/1661              | 1729                   |
| 1 mM insulin, pH 2.4          | 1625/1636                           | 1673/1661              | absent                 |

⁴All FTIR measurements were repeated at least three times showing similar results.
⁵Band assigned to carboxyl groups according to Surmacz-Chwedoruk et al [19]

doi:10.1371/journal.pone.0136602.g006
concentration leads to formation of different strain. Nevertheless, the spectrum is also different from the pH 2 strain, so this data does not add much to strengthen our hypothesis. It is worth to mention that at high insulin concentration insulin aggregates form gel-like substance (which is not the case at lower insulin concentrations). It may point to the different aggregation mechanism thus explaining difference in FTIR spectra.

We also repeated previously described data on insulin aggregation in the presence of ethanol and examined the effect of higher DMSO concentrations. As seen in Fig 6B, the presence of 20% of both organic cosolvents during insulin aggregation in the pH 2 environment leads to formation of aggregates exhibiting pH 1.6-like IR spectra. This confirms that ethanol and, to a lower extent DMSO shifts the equilibrium towards formation of pH 1.6-like insulin amyloid strains.

Finally, we used dynamic light scattering (DLS) to determine the size distribution of insulin under the various solution conditions. The data reveal that average size of insulin, dissolved in pH 1.6 is lower than in pH 2 (Fig 7). The measured diameter of insulin in pH 1.6 is 3.4±0.7 nm, which is bigger than monomer, but smaller than dimer, while in pH 2, the diameter is 4.0±0.6, which is a little bigger than insulin dimer. Owing to the polydispersity of the sample, the method does not allow the exact estimation of the monomer and oligomer content, however the shift of the equilibrium towards dimeric/oligomeric species at higher pH is unarguable, hence supporting our hypothesis.

Taken together, our data indicates different factors inducing polymorphism of insulin amyloid-like fibrils. However it seems that all the presented cases can be reduced to the formation
of two amyloid strains and possibly explained by the differences in the equilibrium between insulin monomers and dimers (oligomers).

**Materials and Methods**

**Preparation of insulin fibrils**

Recombinant human insulin was purchased from Sigma Aldrich (91077C). Insulin amyloid-like fibrils were prepared as described previously [52]. Briefly, fresh 1 mM insulin solution (in 100 mM phosphate buffer (PB), at different pH (in H2O) and pH\textsuperscript{D2O} (in D2O) values was incubated at 60°C for 24 hours with 300 rpm agitation (using a MHR 23 thermomixer, Ditabis, Germany). The secondary structures and morphological signatures of the aggregates obtained were tested using FTIR spectroscopy and AFM.

Seeds were prepared as described previously [52]. Briefly, 1 mL of fibrils were sonicated for 10 minutes using a Bandelin Sonopuls 3100 ultrasonic homogenizer equipped with a MS73 tip (using 50% of the power, cycles of 30 s/30 s sonication/rest, total energy applied to the sample per cycle, 0.56 kJ). The sample was kept on ice during the sonication procedure. Right after the treatment, one part of the fibrils was mixed with 9 parts of the fresh 1 mM insulin solution in the appropriate buffer and incubated at 37°C for 24 hours without agitation. The secondary structures of the aggregates obtained were tested using FTIR spectroscopy.

**Elongation kinetics**

To follow the seeding kinetics, samples were prepared as described above, with addition of 50 μM ThT. Right after the mixing the fresh insulin with seeds, samples were divided into 200 μL aliquots, in 96-well plates. The plates were sealed using clear polyolefin sealing tape. The aggregation kinetics was followed at constant 37°C temperature using a Biotek Synergy H4 plate reader without agitation. ThT fluorescence intensity upon fibril formation was observed using 440 nm excitation and 482 nm emission with simultaneous measurement of absorbance at 600 nm.

**Infrared spectroscopy**

To avoid overlapping of protein amide I and water bands, D2O is used as solvent in FTIR measurements. At equal concentrations of D\textsuperscript{+} and H\textsuperscript{+}, respectively, the pH-meter reading with a glass electrode is 0.4 pH units lower in D2O than in H2O [53]. However, isotopes affect the pK\textsubscript{a} of protein ionizable groups, and for solutions of globular proteins the ΔpK\textsubscript{a} was found to be 0.4 pH units in the acidic range, thus the isotope effect on the glass electrode and the ionization constant cancel each other, so that an identical pH-meter reading (in the acidic range) refers to an identical ionization state of the biopolymer in D2O and H2O solutions [54]. To prepare samples for the FTIR measurements, insulin fibrils prepared in H2O were separated from water by centrifugation (30 min., 15000 g), and resuspended in D2O, the procedure was repeated three times. All samples were sonicated for 1 minute using a Bandelin Sonopuls 3100 ultrasonic homogenizer equipped with a MS73 tip. The FTIR spectra were recorded using a Nicolet 5700 spectrometer from Thermo Scientific equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector, and using Bruker Alpha spectrometer equipped with deuterium triglycine sulfate (DTGS) detector. For all measurements, CaF\textsubscript{2} transmission windows and 0.05 mm Mylar spacers or 0.05 and 0.1 mm Teflon spacers (with Bruker instrument) were used. Spectra were recorded at room temperature. For each spectrum, 256 interferograms of 2 cm\textsuperscript{-1} resolution were co-added. A corresponding buffer spectrum was subtracted from each sample spectrum. All the spectra were baseline-corrected and normalized to the...
same area of amide I/I’ band (1700–1580 cm⁻¹) before further data processing. All data processing was performed using GRAMS software.

Dynamic light scattering
For DLS experiments, freshly prepared insulin solutions at different buffers were filtered using 0.22 μm syringe filter. The size measurements were performed using Zetasizer μV (Malvern instruments) with low-volume quartz batch cuvette at 60°C.

Atomic force microscopy
For AFM experiments, 1 mM insulin was diluted 100 times with deionized water, 30 μL of the sample were deposited on freshly cleaved mica and left to adsorb for 1 min, the sample was rinsed with 1 mL of water and dried gently using airflow. AFM images were recorded in the Tapping-in-Air mode at a drive frequency of approximately 300 kHz, using a MultiModee SPM microscope equipped with a NanoScope IIIa controller. PointProbe NCHR aluminium-coated silicon tips from Nanosensors were used as a probe.

Supporting Information
S1 Fig. Example of the repeatability of FTIR spectra. Red and blue spectra were collected using Thermo Nicolet instrument in TU Dortmund University, black and green—using Bruker Alpha instrument in Vilnius University.
(PDF)
S2 Fig. Fibril height measurements.
(PDF)

Author Contributions
Conceived and designed the experiments: VS. Performed the experiments: TS DD AB MG VS. Analyzed the data: TS DD VS. Contributed reagents/materials/analysis tools: RW VS. Wrote the paper: RW VS.

References
1. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. Annu Rev Biochem. 2006; 75: 333–366. doi:10.1146/annurev.biochem.75.101304.123901 PMID: 16756495
2. Pham CL, Kwan a H, Sunde M. Functional amyloid: widespread in Nature, diverse in purpose. Essays Biochem. 2014; 56: 207–219. doi:10.1042/BSE0560207 PMID: 25131597
3. Fändrich M, Dobson CM. The behaviour of polyamino acids reveals an inverse side chain effect in amyloid structure formation. EMBO J. 2002; 21: 5682–5690. doi:10.1093/emboj/cdf573 PMID: 12411486
4. Azriel R, Gazit E. Analysis of the minimal amyloid-forming fragment of the islet amyloid polypeptide. An experimental support for the key role of the phenylalanine residue in amyloid formation. J Biol Chem. 2001; 276: 34156–34161. doi:10.1074/jbc.M102883200 PMID: 11445568
5. Sawaya MR, Sambashivan S, Nelson R, Ivanova MI, Sievers S a, Apostol MI, et al. Atomic structures of amyloid cross-beta spines reveal varied steric zippers. Nature. 2007; 447: 453–457. doi:10.1038/ nature05695 PMID:17468747
6. Tamamis P, Adler-Abramovich L, Reches M, Marshall K, Sikorski P, Serpell L, et al. Self-assembly of phenylalanine oligopeptides: Insights from experiments and simulations. Biophys J. Biophysical Society; 2009; 96: 5020–5029. doi:10.1016/j.bpj.2009.03.026
7. Adler-Abramovich L, Vaks L, Carny O, Trudler D, Magno A, Caflisch A, et al. Phenylalanine assembly into toxic fibrils suggests amyloid etiology in phenylketonuria. Nat Chem Biol. Nature Publishing Group; 2012; 8: 701–706. doi:10.1038/nchembio.1002 PMID: 22706200
8. Prusiner SB. Prions. Proc Natl Acad Sci U S A. 1998; 95: 13363–13383. doi:VL—95 PMID: 9811807
9. Safar J, Willie H, Itri V, Groth D, Serban H, Torchia M, et al. Eight prion strains have PrP(Sc) molecules with different conformations. Nat Med. 1998; 4: 1157–1165. doi: 10.1038/2654 PMID: 9771749

10. Collinge J, Clarke AR. A general model of prion strains and their pathogenicity. Science. 2007; 318: 930–936. doi: 10.1126/science.1138718 PMID: 17991853

11. Dzwolak W, Smirnovas V, Jansen R, Winter R. Insulin forms amyloid in a strain-dependent manner: an FT-IR spectroscopic study. Protein Sci. 2004; 13: 1927–1932. doi: 10.1110/ps.03607204 PMID: 15169954

12. Petkova AT, Leapman RD, Guo Z, Yau W-M, Mattson MP, Tycko R. Self-propagating, molecular-level polymorphism in Alzheimer’s beta-amyloid fibrils. Science. 2005; 307: 262–265. doi: 10.1126/science.1105850 PMID: 15653506

13. Heise H, Hoyer W, Becker S, Andronesi OC, Riedel D, Baldus M. Molecular-level secondary structure, polymorphism, and dynamics of full-length alpha-synuclein fibrils studied by solid-state NMR. Proc Natl Acad Sci U S A. 2005; 102: 15871–15876. doi: 10.1073/pnas.0506109102 PMID: 16247008

14. Dzwolak W, Gruzielianek S, Smirnovas V, Ravindra R, Nicolini C, Jansen R, et al. Ethanol-perturbed amyloidogenic self-assembly of insulin: Looking for origins of amyloid strains. Biochemistry. 2005; 44: 8948–8958. doi: 10.1021/bi050281l PMID: 15966720

15. Dzwolak W, Jansen R, Smirnovas V, Loksztajn A, Porowski S, Winter R. Template-controlled conformations of insulin fibrillar self-assembly reflect history of solvation of the amyloid nuclei. Phys Chem Chem Phys. 2005; 7: 1349–1351. doi: 10.1039/b502255p PMID: 19787953

16. Paravastu AK, Leapman RD, Yau W-M, Tycko R. Molecular structural basis for polymorphism in Alzheimer’s beta-amyloid fibrils. Proc Natl Acad Sci U S A. 2008; 105: 18339–18354. doi: 10.1073/pnas.0806270105 PMID: 19155323

17. Debelouchina GT, Platt GW, Bayro MJ, Radford SE, Griffin RG. Magic angle spinning NMR analysis of beta-2-microglobulin amyloid fibrils in two distinct morphologies. J Am Chem Soc. 2010; 132: 10414–10423. doi: 10.1021/ja102775u PMID: 20662519

18. Dinkel PD, Siddiqua A, Huynh H, Shah M, Margittai M. Variations in filament conformation dictate seeding barrier between three- and four-repeat tau. Biochemistry. 2011; 50: 4330–4336. doi: 10.1021/bi2004685 PMID: 21510682

19. Surmacz-Chwedoruk W, Nieznańska H, Wójcił S, Dzwolak W. Cross-seeding of fibrils from two types of insulin induces new amyloid strains. Biochemistry. 2012; 51: 9460–9469. doi: 10.1021/bi301144d PMID: 23127165

20. Chatani E, Yagi H, Naiki H, Goto Y. Polymorphism of beta-2-microglobulin amyloid fibrils manifested by ultrasonication-enhanced fibril formation in trifluoroethanol. J Biol Chem. 2012; 287: 22827–22837. doi: 10.1074/jbc.M112.393415 PMID: 22853256

21. Bouisset L, Pieri L, Ruiz-Arlandis G, Gath J, Jensen PH, Habenstein B, et al. Structural and functional characterization of two alpha-synuclein fibrils. Nat Commun. 2013; 4: 2575. doi: 10.1038/ncomms3575 PMID: 24108358

22. Tycko R. Physical and structural basis for polymorphism in amyloid fibrils. Protein Sci. 2014; 23: 1528–39. doi: 10.1002/pro.2544 PMID: 25179159

23. Aguzzi A. Neurodegeneration: Alzheimer’s disease under strain. Nature. 2014; 512: 32–4. doi: 10.1038/512023a PMID: 25100477

24. Stöhr J, Condello C, Watts JC, Bloch L, Oehler A, Nick M, et al. Distinct synthetic Aβ prion strains producing different amyloid deposits in bigenic mice. Proc Natl Acad Sci U S A. 2014; 111: 2–7. doi: 10.1073/pnas.1319594110 PMID: 140896111

25. Watts JC, Condello C, Stöhr J, Oehler A, Lee J, DeArmond SJ, et al. Serial propagation of distinct strains of Aβ prions from Alzheimer’s disease patients. Proc Natl Acad Sci. 2014; 111: 10323–10328. doi: 10.1073/pnas.140900111 PMID: 24982139

26. Westmark GT, Westmark P. Prion-like aggregates: Infectious agents in human disease. Trends Mol Med. Elsevier Ltd; 2010; 16: 501–507. doi: 10.1016/j.molmed.2010.08.004 PMID: 20870462

27. Brundin P, Melki R, Kopito R. Prion-like transmission of protein aggregates in neurodegenerative diseases. Nat Rev Mol Cell Biol. Nature Publishing Group; 2010; 11: 301–307. doi: 10.1038/nrm2873 PMID: 20308987

28. Frost B, Diamond MI. Prion-like mechanisms in neurodegenerative diseases. Nat Rev Neurosci. Nature Publishing Group; 2010; 11: 155–159. doi: 10.1038/nrm2786 PMID: 20029438

29. Angot E, Steiner J a., Hansen C, Li JY, Brundin P. Are synucleinopathies prion-like disorders? Lancet Neurol. Elsevier Ltd; 2010; 9: 1128–1138. doi: 10.1016/S1474-4422(10)70213-1 PMID: 20848907

30. Prusiner SB. A Unifying Role for Prions in Neurodegenerative Diseases. Science (80: -). 2012; 336: 1511–1513. doi: 10.1126/science.1222951
31. Eisele YS. From soluble Abeta to progressive Abeta aggregation: Could prion-like templated misfolding play a role? Brain Pathol. 2013; 23: 333–341. doi: 10.1111/bpa.12049 PMID: 23587139

32. Masuda-Suzukake M, Nonaka T, Hosokawa M, Oikawa T, Ariai T, Akiyama H, et al. Prion-like spreading of pathological α-synuclein in brain. Brain. 2013; 136: 1128–1138. doi: 10.1093/brain/awi307 PMID: 23466394

33. Goedert M, Falcon B, Clavaguera F, Tolnay M. Prion-like Mechanisms in the Pathogenesis of Tauopathies and Synucleinopathies. Curr Neurol Neurosci Rep. 2014; 14: 495. doi: 10.1007/s11910-014-0495-z PMID: 25218483

34. Hill a F, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, et al. The same prion strain causes vCJD and BSE. Nature. 1997; 389: 448–450, 526. doi: 10.1036/S0887-7963(98)80051-5 PMID: 9333232

35. Surmacz-Chwedoruk W, Babenko V, Dzwolak W. Master and Slave Relationship Between Two Types of Self-Propagating Insulin Amyloid Fibrils. J Phys Chem B. 2014; 118: 13582–13589. doi: 10.1021/jp510980b PMID: 25373010

36. Tanaka M, Chien P, Naber N, Cooke R, Weissman JS. Conformational variations in an infectious protein determine prion strain differences. Nature. 2004; 428: 323–328. doi: 10.1038/nature02392 PMID: 15029196

37. Tanaka M, Collins SR, Toyama BH, Weissman JS. The physical basis of how prion conformations determine strain phenotypes. Nature. 2006; 442: 585–589. doi: 10.1038/nature04922 PMID: 16810177

38. Colby DW, Giles K, Legname G, Wilie H, Baskakov I V., DeArmond SJ, et al. Design and construction of diverse mammalian prion strains. Proc Natl Acad Sci U S A. 2009; 106: 20417–20422. doi: 10.1073/pnas.0910350106 PMID: 19915150

39. Cobb NJ, Apostol MI, Chen S, Smirnovas V, Surewicz WK. Conformational stability of mammalian prion protein amyloid fibrils is dictated by a packing polymorphism within the core region. J Biol Chem. 2014; 289: 2643–2650. doi: 10.1074/jbc.M113.520718 PMID: 24338015

40. Ostapchenko VG, Sawaya MR, Makarava N, Savtchenko R, Nilsson KPR, Eisenberg D, et al. Two amyloid states of the prion protein display significantly different folding patterns. J Mol Biol. Elsevier Ltd; 2010; 400: 908–921. doi: 10.1016/j.jmb.2010.05.051 PMID: 20553730

41. Caughey B, Raymond GJ, Bessen R a. Strain-dependent differences in beta-sheet conformations of abnormal prion protein. J Biol Chem. 1999; 273: 32230–32235. doi: 10.1074/jbc.273.48.32230

42. Malisauskas R, Botyriute A, Cannon JG, Smirnovas V. Flavone Derivatives as Inhibitors of Insulin Amyloid-Like Fibril Formation. PLoS One. 2015; 10: e0121231. doi:10.1371/journal.pone.0121231 PMID: 25799281

43. Waugh DF, Wilhelmson DF, Commerford SL, Sackler ML. Studies of the nucleation and growth reactions of selected types of insulin fibrils. J Am Chem Soc. 1952; 75: 2592–2600. doi: 10.1021/ja1107a013

44. Kurouski D, Lombardi R a, Dukor RK, Lednev IK, Nafie L a. Direct observation and pH control of reversed supramolecular chirality of insulin fibrils by vibrational circular dichroism. Chem Commun. 2010; 46: 7154–7156. doi: 10.1039/c0cc02423f

45. Kurouski D, Dukor RK, Lu X, Nafie L a, Lednev IK. Normal and reversed supramolecular chirality of insulin fibrils probed by vibrational circular dichroism at the protofilament level of fibril structure. Biophys J. Elsevier; 2012; 103: 522–531. doi: 10.1016/j.bpj.2012.04.042 PMID: 22947868

46. Kurouski D, Dukor RK, Lu X, Nafie L a, Lednev IK. Spontaneous inter-conversion of insulin fibril chirality. Chem Commun. 2012; 48: 2837. doi: 10.1039/c2cc16895b

47. Bryant C, Spencer DB, Miller a, Bakaysa DL, McCune KS, Maple SR, et al. Acid stabilization of insulin. Biochemistry. 1993; 32: 8075–8082. doi: 10.1021/Bi00083a004 PMID: 8394123

48. Nielsen L, Frokjaer S, Brange J, Uversky VN, Fink a, L. Probing the mechanism of insulin fibril formation with insulin mutants. Biochemistry. 2001; 40: 8397–8409. doi: 10.1021/bi0105983 PMID: 11444987

49. Nettleton EJ, Tito P, Sunde M, Bouchard M, Dobson CM, Robinson C V. Characterization of the oligomeric states of insulin in self-assembly and amyloid fibril formation by mass spectrometry. Biophys J. Elsevier; 2000; 79: 1053–1065. doi: 10.1016/S0006-3495(00)76359-4 PMID: 10920035

50. Kim SK, Ha T, Schermann J-P. Biomolecular structures: from isolated molecules to the cell crowded medium. Phys Chem Chem Phys. 2010; 12: 3334–3335. doi: 10.1039/c004156b PMID: 20336242

51. Grudzielanek S, Jansen R, Winter R. Solvational tuning of the unfolding, aggregation and amyloidogenesis of insulin. J Mol Biol. 2005; 351: 879–894. doi: 10.1016/j.jmb.2005.06.046 PMID: 16051271

52. Mitko K, Botyriute A, Smirnovas V. Amyloid-Like Fibril Elongation Follows Michaelis-Menten Kinetics. Baskakov I V., editor. PLoS One. 2013; 8: e66884. doi: 10.1371/journal.pone.0066884 PMID: 23874721
53. Glasoe PK, Long FA. Use of glass electrodes to measure acidities in deuterium oxide. J Phys Chem. 1960; 64: 188–190. doi: 10.1021/j100830a521

54. Bundi A, Wüthrich K. 1H-nmr parameters of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-L-Ala-OH. Biopolymers. 1979; 18: 285–297. doi: 10.1002/bip.1979.360180206