Exploring the occurrence of thioflavin-T-positive insulin amyloid aggregation intermediates

Mantas Ziaunys Corresp., 1, Andrius Sakalauskas 1, Kamile Mikalauskaite 1, Vytautas Smirnovas 1

1 Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius, Lithuania

Corresponding Author: Mantas Ziaunys
Email address: mantas.ziaunys@gmc.vu.lt

The aggregation of proteins is considered to be the main cause of several neurodegenerative diseases. Despite much progress in amyloid research, the process of fibrillization is still not fully understood, which is one of the main reasons why there are still very few effective treatments available. When the aggregation of insulin, a model amyloidogenic protein, is tracked using thioflavin-T (ThT), an amyloid specific dye, there is an anomalous occurrence of double-sigmoidal aggregation kinetics. Such an event is likely related to the formation of ThT-positive intermediates, which may affect the outcome of both aggregation kinetic data, as well as final fibril structure. In this work we explore insulin fibrillization under conditions, where both normal and double-sigmoidal kinetics are observed and show that, despite their dye-binding properties and random occurrence, the ThT-positive intermediates do not significantly alter the overall aggregation process.
**Exploring the occurrence of thioflavin-T-positive insulin amyloid aggregation intermediates**

Mantas Ziaunys*, Andrius Sakalauskas, Kamile Mikalauskaite, Vytautas Smirnovas

Institute of Biotechnology, Life Sciences Center, Vilnius University, Vilnius, Lithuania

*Corresponding Author:
Mantas Ziaunys
Saulėtekio al. 7, Vilnius, LT-10257, Lithuania
Email address: mantas.ziaunys@gmc.vu.lt

**Abstract**

The aggregation of proteins is considered to be the main cause of several neurodegenerative diseases. Despite much progress in amyloid research, the process of fibrillization is still not fully understood, which is one of the main reasons why there are still very few effective treatments available. When the aggregation of insulin, a model amyloidogenic protein, is tracked using thioflavin-T (ThT), an amyloid specific dye, there is an anomalous occurrence of double-sigmoidal aggregation kinetics. Such an event is likely related to the formation of ThT-positive intermediates, which may affect the outcome of both aggregation kinetic data, as well as final fibril structure. In this work we explore insulin fibrillization under conditions, where both normal and double-sigmoidal kinetics are observed and show that, despite their dye-binding properties and random occurrence, the ThT-positive intermediates do not significantly alter the overall aggregation process.

**Introduction**

Protein aggregation into amyloid fibrils is linked to multiple neurodegenerative disorders, such as Alzheimer’s, Parkinson’s or prion diseases (Knowles, Vendrauscolo & Dobson, 2014; Chiti & Dobson, 2017), affecting millions of people worldwide (Isik, 2010). Such protein assembly into insoluble aggregates is still not fully understood, despite the significant effort put into figuring
out both the mechanism of aggregation (Meisl et al., 2016; Castello et al., 2017; Biza et al., 2017; Giorgetti et al., 2018; Linse, 2019), as well as the resulting fibril structural aspects (Makin & Serpell, 2005; Fitzpatrick et al., 2013). As a consequence, there are still very few disease modifying drugs available (Mehta et al., 2017; Cummings et al., 2019; Maurer et al., 2018; Park et al., 2020).

The process of amyloid fibril formation consists of multiple microscopic events. The first one being nucleation, a process during which proteins lose their native structure and form a primary aggregation center (Chatani & Yamamoto, 2018). This structure then passes several growth phases, such as elongation (Gurry & Stultz, 2014), assembly into protofibrils (Dolui et al., 2018) and subsequent maturation into fully formed fibrils (Ma et al., 2013; Sidhu et al., 2017). The resulting aggregates are then capable of acting as catalysts for surface-mediated secondary nucleation (Törnquist et al., 2018), as well as fragmenting into smaller fibrils (Nicoud et al., 2015), thus creating new aggregation centers. Different proteins have also been shown to form specific oligomeric species prior to further aggregation processes (Nettleton et al., 2000; Chiti & Dobson, 2006; Danzer et al., 2007; Selivanova & Galzitskaya, 2012; Sengupta, Nilson & Kayed, 2016). Such a large number of possible steps involved in the aggregation process significantly complicates matters and requires extensive research in order to understand and prevent the progress of amyloid-related diseases.

In order to study aggregation reactions in vitro, insulin is often used as a model amyloidogenic protein (Brange et al., 1997). Despite its main application as a treatment for diabetes, insulin is capable of forming amyloid fibrils under acidic or neutral pH at an elevated temperature (Nielsen et al., 2001). This, coupled with its availability, has made it a widely used protein to study both the mechanisms of amyloid formation (Ahmad et al., 2003; Podestá et al., 2006; Malik & Roy, 2011), as well as possible inhibitory compounds (Wang, Dong & Sun, 2012; Malisauskas et al., 2015; Zheng & Lazo, 2018). Even though a large number of experiments have been conducted with insulin under various conditions, new information regarding its fibrillation continues to arise, such as new possible aggregation mechanisms or structural polymorphisms (Sakalauskas, Ziaunys & Smirnovas, 2019; Ratha et al., 2020). A factor that requires further attention is the seemingly random appearance of double-sigmoidal aggregation kinetics when examining the fibrillization of insulin with a fluorescent probe – thioflavin-T (ThT). This phenomenon was examined by Smirnovas et al. (Smirnovas & Winter, 2008), Grudzielanek et al. (Grudzielanek, Smirnovas & Winter, 2006) and Fodera et al. (Foderà et al., 2009), where it was shown that the first increase in the double-sigmoidal curve is likely related to the formation of oligomeric intermediate species capable of binding ThT and this event was consistently reproducible only under certain environmental conditions.

ThT is a benzothiazole dye that binds to the beta-sheet grooves of amyloid fibrils and attains a locked conformation (Robbins et al., 2012). This causes a red shift of its excitation and emission wavelengths, as well as a significant increase in fluorescence quantum yield (Gade Malmos et al., 2017; Xue et al., 2017). The fluorescence intensity, binding affinity and maximum
excitation/emission wavelengths are highly dependent on the conformation of fibrils and there
are even reports of multiple types of binding modes on the same type of aggregate (Sidhu et al.,
2018; Ziaunys, Sneideris & Smirnovas, 2020). Despite being widely used as a probe to track
amyloid formation, the dye’s fluorescence is not exclusively tied to such fibrillar aggregates, as it
has been shown to increase in fluorescence upon binding or being trapped in non-amyloid
structures (Singh et al., 2010; Sulatskaya et al., 2018). This, in turn, does not rule out the
possibility of the double-sigmoidal kinetics being the result of structures that are not amyloid in
nature.

In our research we observed that when human recombinant insulin is aggregated at pH 2.4, there
exist both regular sigmoidal fibrilization kinetic curves, as well as double-sigmoidal ones under
the same aggregation conditions. Unlike in the previously reported cases (Grudzielanek,
Smirnovas & Winter, 2006; Smirnovas & Winter, 2008; Foderà et al., 2009), this occurrence
appears to be random even when the same batch of protein is used. The double-sigmoidal curves
also possess a different ThT fluorescence intensity at the end of the reaction, which begs the
question whether the formation of these anomalous ThT-positive intermediates could yield
differently structured fibrils. Small variations in insulin aggregation conditions, such as pH value
or protein concentration (Sneideris et al., 2015; Sakalauskas, Ziaunys & Smirnovas, 2019) can
cause the formation of distinct conformation aggregates. Since both regular and double-
sigmoidal aggregation types exist under the same conditions, this creates an opportunity to
explore any possible differences during the whole fibrillization process. In this work we examine
a large sample size of insulin aggregation kinetic curves, isolate the double-sigmoidal kinetic
samples from regular ones and determine whether there are secondary structure, morphology,
ThT binding and seeding propensity differences between them. In addition, both regular and
double-sigmoidal aggregation reactions are tracked by scanning ThT fluorescence excitation-
emission matrices in order to determine if there are aggregate structural differences from distinct
dye binding, such as specific maximum excitation and emission wavelengths or bound-ThT
fluorescence intensity (Groenning et al., 2007; Ziaunys & Smirnovas, 2019b; Ziaunys,
Sakalauskas & Smirnovas, 2020).

95 Materials and Methods

96 Insulin aggregation

97 Human recombinant insulin powder (Sigma-Aldrich cat. No. 91077C) was dissolved in a 100
mM sodium phosphate buffer (pH 2.4) containing 100 mM NaCl (reaction buffer). ThT (Sigma-
Aldrich cat. No. T3516) was dissolved in H₂O to a final concentration of ~12 mM and mixed for
10 min using vigorous agitation, after which the dye solution was filtered through a 0.22 µm
pore syringe filter. An aliquot of the ThT stock solution was diluted 200 times and the exact dye
concentration was determined by measuring the solution’s absorbance at 412 nm (ε₄₁₂ = 23 250
M⁻¹cm⁻¹). The ThT stock solution was then diluted to a final concentration of 10 mM. The
protein solution was then combined with the reaction buffer and a 10 mM ThTsolution to a final
protein and ThT concentration of 100 µM (insulin $\varepsilon_{280}=6335$ M$^{-1}$ cm$^{-1}$, MW=5808 Da) and
distributed into 200 µL test tubes (20 µL final volume). These conditions result in both types of
aggregation, with a random appearance of double-sigmoidal kinetic curves.

The aggregation kinetics were tracked as previously described (Milto, Michailova & Smirnovas,
2014). In short, sample ThT fluorescence intensity was monitored using a Qiagen Rotorgene Q
real-time analyzer at a constant 60°C temperature with measurements taken every minute. A total
of one thousand samples were measured in batches of 36. After the aggregation reaction, the
samples were stored at 4°C.

For seeded aggregation, fibril samples were sonicated for 10 min using a Bandelin Sonopuls
ultrasonic homogenizer with a MS73 tip (40% power, with 30 s sonication / 30 s rest intervals).
Then insulin, ThT and fibril solutions were combined to a final protein concentration of 100 µM,
ThT concentration of 100 µM and 1% or $10^{-5}$% fibrils (% of total protein mass in solution). The
reaction was monitored as in the non-seeded aggregation experiment.

**Kinetic data analysis**

A first-order derivative was calculated for each sample’s kinetic data, using a 40-point averaging
range. The maximum value of the derivative curve corresponds to the rate of aggregation, while
its position – to the time at which the rate is highest ($t_r$). Samples which had one clear peak in the
first-order derivative were regarded as normal, while ones which were composed of two peaks
(regular, high-rate peak and a small, low-rate peak preceding it) - as double-sigmoidal. Data
processing was done using Origin 2018 software.

**Fluorescence measurements**

Each sample was diluted 5 times to 100 µL with the reaction buffer containing 100 µM of ThT
and their fluorescence intensity was measured using a Varian Cary Eclipse Fluorescence
Spectrophotometer with 440 nm excitation (slit width – 5 nm) and 480 nm emission (slit width –
5 nm) wavelengths. For each case, three measurements were taken and averaged.

**Atomic force microscopy**

The samples were separated into two groups based on their aggregation kinetic profiles and
mixed to result in a homogenous solution. 30 µL aliquots were deposited on freshly cleaved
mica, incubated for 1 min, washed with 1 mL of MilliQ water and dried under airflow. In the
case of intermediate aggregates during the double-sigmoidal aggregation, the real-time analyzer
was stopped when the aggregation curve reached the first minor plateau. Then the samples were
quickly removed and placed on freshly cleaved mica as mentioned earlier. For each condition,
three 10 x 10 µm AFM images were recorded as previously described (Sneideris et al., 2019)
using a Dimension Icon (Bruker) atomic force microscope, operating in tapping mode with a
silicon cantilever Tap300AI-G (40 N m$^{-1}$, Budget Sensors). High resolution (1024 x 1024 pixels)
images were flattened and analyzed using Gwyddion 2.5.5 and SPIP 6.7.8. Each fibril’s height
was determined by tracing lines perpendicular to the fibril’s axis. Height statistical analysis was conducted by taking into consideration all three repeats for each condition, i.e. a similar number of aggregates were examined in every image.

**Fourier-transform infrared spectroscopy**

The two sample groups were centrifuged at 10’000 g for 30 min and resuspended in 1 mL of D$_2$O. The centrifugation and resuspension step was repeated 3 times and the final resuspension volume was 0.25 mL. Before measurements, both samples were sonicated using a Bandelin Sonopuls ultrasonic homogenizer with a MS72 tip (20% power and constant sonication for 30 s). Sonication helps to break fibril clumps, which leads to less scattering effects and better-quality FTIR spectra. During sample preparation and measurement, H-D exchange is insignificant, as in the case of insulin fibrils it is very slow (Dzwolak, Loksztejn & Smirnovas, 2006). The spectra were recorded as previously described (Sneideris et al., 2019). In short, the concentrated fibril samples were scanned in near-vacuum conditions (~2 mbar) at room temperature using a Vertex 80v (Bruker) IR spectrometer. 256 interferograms were averaged for each spectrum. A D$_2$O spectrum was subtracted and the resulting spectra were normalized to the same area of amide I/I’ band (1700-1595 cm$^{-1}$). Data processing was performed using GRAMS software.

**ThT fluorescence excitation-emission matrices**

The aggregation solution was prepared as described in the insulin aggregation section to a final volume of 3 mL. The solution was then placed in a 10 mm pathlength cuvette, sealed with a plug cap to prevent evaporation and incubated at 60°C without agitation. EEMs were scanned every 5 min using a Varian Cary Eclipse fluorescence spectrophotometer using an excitation range from 440 to 465 nm and emission range from 475 to 500 nm (excitation and emission slit widths – 5 nm, wavelength step – 1 nm, scan rate – 600 points/min). Due to the analysis being conducted on a transitioning system, the EEM size was optimized to be as minimal as possible to lower the impact of an intensity drift, which results from different concentrations of aggregates present at the start and finish of each scan cycle. This was done by first acquiring a larger EEM, which encompassed the maximum ThT fluorescence zone, then it was narrowed down as much as possible to reduce scan time.

Each EEM was corrected for the inner filter effect caused by 100 µM of ThT as described previously (Ziaunys & Smirnovas, 2019b). In short, the correction was made by using the absorbance spectra of 100 µM non-bound ThT, as it is extremely difficult to account for absorbance changes throughout the entire reaction resulting from ThT becoming bound to fibrils and because the majority of ThT remains non-bound even when all insulin is aggregated (Ziaunys & Smirnovas, 2019b). The EEM “center of mass” was then calculated for the entire EEM after the inner filter correction. This was done to prevent signal noise, caused by light scattering, from affecting the maximum intensity position.
**Results**

**Aggregation kinetics**

A large number (n=1,000) of low volume insulin samples were aggregated under the exact same conditions and their kinetics were tracked by monitoring changes in ThT fluorescence intensity. Analysis of all the data revealed that a majority of samples experienced normal, sigmoidal spontaneous aggregation kinetics, with one rate maximum seen in the first order derivative (Figure 1A, C). A fraction of samples displayed double-sigmoidal kinetics, with two peaks in the first order derivative curve (Figure 1B, D). The first increase in fluorescence intensity of the double-sigmoidal aggregation kinetics occurred roughly 100 minutes before the second increase and its rate was, on average, nearly 10-fold lower.

A total of 55 samples possessed such unusual aggregation kinetics, constituting a probability of such an occurrence being at least 5.5% under the tested conditions. 77 samples had an intermediate kinetic profile (very small first peak or a large overlap between both peaks), which could not be accurately attributed to either type of aggregation. The normal and double-sigmoidal samples were then separated for further analysis.

In order to determine if there are any links between the rate of aggregation, the time at which this rate is highest and the resulting fluorescence intensity of fibrils, all three factor dependencies were examined. We can see that all three parameters are mostly independent from one another (Figure 2A-C). The time at which the aggregation rate is highest, does not influence the rate at which fibril elongation occurs, neither does it change the final fluorescence intensity of the formed fibrils. When we compare these three factors between the normal and double-sigmoidal samples (Figure 2D-I), it appears that the double-sigmoidal fibrillization has a slightly lower time at which the maximum aggregation rate is reached (Figure 2D, G) and it has no effect on the rate itself (Figure 2E, H). There is, however, a considerable difference in the final fluorescence intensity distribution (Figure 2F, I). The distribution maximum is more than 10% lower when the aggregation kinetics are double-sigmoidal, suggesting that there are either off-pathway aggregates or a fraction of fibrils possess a different ThT binding mode. Despite this distinction in average fluorescence intensity, a large portion of all three data sets overlap with one another due to a large spread caused by the stochastic nature of non-seeded insulin aggregation (Foderà et al., 2008).

**Fibril structure and seeding properties**

The fibril samples were examined using atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR) and used as seeds to examine their rate of self-replication. In the
AFM images acquired during the first increase in signal intensity during double-sigmoidal kinetics, we observe small, round oligomeric aggregate species (with most having a height of 1 to 2 nm) and short protofibrils (0.1 to 0.5 µm in length) (Figure 3A, Figure S1). When compared to a sample obtained before an increase in dye fluorescence is observed (Figure S1), there are far more aggregates present in the case of ThT-positive intermediates. The ThT-negative samples also contain a higher number of 0.5 – 1.5 nm height assemblies and very few elongated structures (Figure S2A-C). When the aggregation reactions are concluded, the fibrils are considerably longer and have a greater height, however, there do not seem to be any major differences between both cases, neither visually nor by their height distribution (Figure 3B-D, Figure S1). Due to formation of large aggregate clumps, the fibrils were sonicated to better examine any possible differences in their height. While the height distribution average values are similar, there is a wider spread in the case of the sonicated double-sigmoidal sample (Figure S3), likely caused by the existence of several smaller fibrils or amorphous aggregates. The FTIR second derivative spectra (Figure 3E) and seeding kinetics (Figure 3F) are also nearly identical for both cases, indicating that the double-sigmoidal aggregation does not have a significant influence on the final fibril secondary structure or self-replication properties.

ThT-positive intermediates

The normal and double-sigmoidal aggregation reactions were examined by scanning excitation-emission matrices of ThT fluorescence during aggregation. The kinetic curves were plotted as the maximum EEM signal intensity over time. When examining the kinetics of double-sigmoidal aggregation, we see that the lag phase is followed by a slow increase in ThT fluorescence intensity, then a sudden jump in intensity (marked as *), which then quickly returns to a low value and is continued by the second growth phase (Figure 4A). Such a jump is not visible in the normal aggregation data (Figure 4A), nor in any of the previous experiments, where samples were only scanned once a minute. This indicates that it may only be visible for a very short time during the EEM scan. When the excitation and emission wavelengths of the EEM “center of mass” are calculated (Figure 4B, C), we see that there are significant changes in both of these parameters during the first, anomalous increase in ThT intensity, as compared to relatively minor variations in the normal aggregation. The excitation wavelength shifts from 457 nm to 453 nm and then rises back to 454 nm, while the emission intensity shifts between 489 nm and 487 nm and gradually reaches 488 nm. Both wavelengths reach a constant value at roughly the same time after the sudden increase in ThT fluorescence intensity, marking the end of the anomalous phase.

If we examine the top fluorescence intensity value distributions in the EEMs before and after the sudden signal jump (Figure 4D), there are both significant shifts in the top value positions during the anomalous phase, as well as single, high intensity lines. Such lines can be caused by either a large particle floating past the optical path during a scan or by sudden association and dissociation of a ThT-positive aggregate. After the high intensity jump, once the signal returns to
normal, these lines are no longer present in any of the EEMs (as seen after 290 minutes (Figure 4D)) and they become nearly identical. Such an occurrence has been observed multiple times throughout the study and it ranged from being mild (Figure S4) to very extreme (Figure S5).

Discussion

The large sample size of aggregation reactions shows that under these conditions, the occurrence of such anomalous, double-sigmoidal fibrilization kinetics is both relatively rare (5.5%) and seemingly random. This is unlike the previously reported cases, where a certain set of conditions caused all of the kinetic curves to be double-sigmoidal (Grudzielanek, Smirnovas & Winter, 2006; Smirnovas & Winter, 2008; Foderà et al., 2009). The conditions used in this work allowed to examine how such peculiar kinetics affected the overall aggregation reaction, as both types of fibrillizations were observed. When compared to normal aggregation, this anomalous event occurs roughly 100 minutes before the second increase, however it does not influence the time at which maximum aggregation rate is reached nor the rate of aggregation and the resulting fibrils have an identical morphology, secondary structure and seeding properties. It is possible that it generates a small population of small, amorphous structures, as hinted by the fibril height range, AFM images and the lower ThT fluorescence intensity distribution. The fact that this anomaly does not influence the overall kinetic parameters (apart from lag time) or fibril structure is a positive aspect, considering that these values are often used to determine the effectiveness of anti-amyloid compounds. However, the random formation of different intermediate species does raise concerns. If these structures can appear in any reaction solution and there is minimal connection between their existence and the overall aggregation process, then there is essentially no way of controlling this event.

Judging from the ThT fluorescence EEMs, it appears that during the anomalous aggregation phase, there exists the formation of ThT-positive intermediates or structures that are capable of trapping and conformationally “locking” the dye molecules, unlike during a normal fibrilization process. AFM images acquired during this phase show small and round aggregates, as well as short protofibril species, with an average height that is much lower than observed for fully formed fibrils. Their ability to bind ThT in a different mode (as identified by the higher excitation and emission wavelengths) suggests that they possess a structure that is distinct from both normal intermediate species, as well as the fully formed fibrils. The differences in these parameters could also be an indicator that there is no actual surface-dye interaction, but rather an entrapment of ThT in the oligomeric structure. The lower ThT fluorescence intensity distribution at the end of the reaction and AFM images also hint at a possibility that some of these structures remain in solution and do not become incorporated into the amyloid structure of normal fibrils.

A possible explanation for the ThT-binding ability of these intermediates may stem from the high intensity lines seen in the ThT fluorescence EEMs. When the sample is continuously scanned to generate an EEM, such lines can only result from either a larger particle floating past
the optical path or by a quick association and dissociation of a ThT positive aggregate.
Considering that during fibrilization the concentration of large aggregates increases, an event
which results in enhanced light scattering and signal noise, we would expect to observe an
increasing amount of such high intensity lines, however, this is not the case. Once the first phase
of the double-sigmoidal kinetics is concluded, they are no longer seen, which means that they are
likely not the result of light scattering from larger aggregate particles. This leaves the hypothesis
that ThT-positive intermediates quickly associate and dissociate during this time period. If these
intermediates are capable of trapping ThT molecules within their structure, they may not even
require a similarity to amyloids, as it has been shown that ThT immobilization can cause an
increase in fluorescence intensity (Hutter et al., 2011; Ziaunys & Smirnovas, 2019a). This would
also explain the significantly different excitation and emission wavelengths, as these parameters
depend highly on the dye’s binding mode.

While these ThT-positive intermediates do not have any major effect on the final fibril structure
and most kinetic parameters, they could become an issue when testing potential anti-amyloid
compounds. If an inhibitor targets the process of primary nucleation and is specific towards a
certain type of intermediate structure, the formation of a different type of aggregate, which
incorporates ThT but is not amyloid-like, may not be affected at all and result in an increase in
ThT fluorescence intensity. This would then lead to a false interpretation on the effectiveness of
the tested compound and negatively affect the drug screening process.

Conclusions

The occurrence of double-sigmoidal kinetics during insulin amyloid aggregation does not
influence the final fibril structure or morphology, nor does it change the rate of the main
reaction. However, it does result in a lower ThT fluorescence intensity and may be related to the
formation of a different type of aggregates. The random variability observed during intermediate
oligomer formation may also have a negative impact during potential anti-amyloid drug
screenings and lead to false interpretations.
313 References

314 Ahmad A, Millett IS, Doniach S, Uversky VN, Fink AL. 2003. Partially Folded Intermediates in
315 Insulin Fibrillation. Biochemistry 42:11404–11416. DOI: 10.1021/bi034868o.

316 Biza K V., Nastou KC, Tsiolaki PL, Mastrokalou C V., Hamodrakas SJ, Iconomidou VA. 2017.
317 The amyloid interactome: Exploring protein aggregation. PLOS ONE 12:e0173163. DOI:
318 10.1371/journal.pone.0173163.

319 Brange J, Andersen L, Laursen ED, Meyn G, Rasmussen E. 1997. Toward Understanding Insulin
320 Fibrillation. Journal of Pharmaceutical Sciences 86:517–525. DOI: 10.1021/js960297s.

321 Castello F, Paredes JM, Ruedas-Rama MJ, Martin M, Roldan M, Casares S, Orte A. 2017. Two-
322 Step Amyloid Aggregation: Sequential Lag Phase Intermediates. Scientific Reports 7:40065.
323 DOI: 10.1038/srep40065.

324 Chatani E, Yamamoto N. 2018. Recent progress on understanding the mechanisms of amyloid
325 nucleation. Biophysical Reviews 10:527–534. DOI: 10.1007/s12551-017-0353-8.

326 Chiti F, Dobson CM. 2006. Protein Misfolding, Functional Amyloid, and Human Disease. Annual
327 Review of Biochemistry 75:333–366. DOI: 10.1146/annurev.biochem.75.101304.123901.

328 Chiti F, Dobson CM. 2017. Protein Misfolding, Amyloid Formation, and Human Disease: A
329 Summary of Progress Over the Last Decade. Annual Review of Biochemistry 86:27–68. DOI:
330 10.1146/annurev-biochem-061516-045115.

331 Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. 2019. Alzheimer’s disease drug development
332 pipeline: 2019. Alzheimer’s & Dementia: Translational Research & Clinical Interventions
333 5:272–293. DOI: 10.1016/j.trci.2019.05.008.

334 Danzer KM, Haasen D, Karow AR, Moussaud S, Habeck M, Giese A, Kretzschmar H, Hengerer
335 B, Kostka M. 2007. Different Species of α-Synuclein Oligomers Induce Calcium Influx and
336 Seeding. Journal of Neuroscience 27:9220–9232. DOI: 10.1523/JNEUROSCI.2617-
337 07.2007.

338 Dolui S, Roy A, Pal U, Saha A, Maoi NC. 2018. Structural Insight of Amyloidogenic Intermediates
339 of Human Insulin. ACS Omega 3:2452–2462. DOI: 10.1021/acsomega.7b01776.

340 Dzwolak W, Loksztejn A, Smirnovas V. 2006. New Insights into the Self-Assembly of Insulin
341 Amyloid Fibrils: An H–D Exchange FT-IR Study. Biochemistry 45:8143–8151. DOI:
342 10.1021/bi060341a.

343 Fitzpatrick AWP, Debelouchina GT, Bayro MJ, Clare DK, Caporini MA, Bajaj VS, Jaroniec CP,
344 Wang L, Ladizhansky V, Muller SA, MacPhee CE, Waudby CA, Mott HR, De Simone A,
345 Knowles TPJ, Saibil HR, Vendruscolo M, Orlova E V., Griffin RG, Dobson CM. 2013.
346 Atomic structure and hierarchical assembly of a cross-β amyloid fibril. Proceedings of the
347 National Academy of Sciences 110:5468–5473. DOI: 10.1073/pnas.1219476110.

348 Foderà V, Cataldo S, Librizzi F, Pignataro B, Spiccia P, Leone M. 2009. Self-Organization
Pathways and Spatial Heterogeneity in Insulin Amyloid Fibril Formation. *The Journal of Physical Chemistry B* 113:10830–10837. DOI: 10.1021/jp810972y.

Foderà V, Librizzi F, Groenning M, Van De Weert M, Leone M. 2008. Secondary nucleation and accessible surface in insulin amyloid fibril formation. *Journal of Physical Chemistry B* 112:3853–3858. DOI: 10.1021/jp710131u.

Gade Malmos K, Blancas-Mejia LM, Weber B, Buchner J, Ramirez-Alvarado M, Naiki H, Otzen D. 2017. ThT 101: a primer on the use of thioflavin T to investigate amyloid formation. *Amyloid* 24:1–16. DOI: 10.1080/13506129.2017.1304905.

Giorgetti S, Greco C, Tortora P, Aprile F. 2018. Targeting Amyloid Aggregation: An Overview of Strategies and Mechanisms. *International Journal of Molecular Sciences* 19:2677. DOI: 10.3390/ijms19092677.

Groenning M, Norrman M, Flink JM, van de Weert M, Bukrinsky JT, Schluckebier G, Frokjaer S. 2007. Binding mode of Thioflavin T in insulin amyloid fibrils. *Journal of Structural Biology* 159:483–497. DOI: 10.1016/j.jsb.2007.06.004.

Grudzielanek S, Smirnovas V, Winter R. 2006. Solvation-assisted Pressure Tuning of Insulin Fibrillation: From Novel Aggregation Pathways to Biotechnological Applications. *Journal of Molecular Biology* 356:497–509. DOI: 10.1016/j.jmb.2005.11.075.

Gurry T, Stultz CM. 2014. Mechanism of Amyloid-β Fibril Elongation. *Biochemistry* 53:6981–6991. DOI: 10.1021/bi500695g.

Hutter T, Amdursky N, Gepshtein R, Elliott SR, Huppert D. 2011. Study of Thioflavin-T Immobilized in Porous Silicon and the Effect of Different Organic Vapors on the Fluorescence Lifetime. *Langmuir* 27:7587–7594. DOI: 10.1021/la200875k.

Isik AT. 2010. Late onset Alzheimer’s disease in older people. *Clinical Interventions in Aging* 5:307. DOI: 10.2147/CIA.S11718.

Knowles TPJ, Vendruscolo M, Dobson CM. 2014. The amyloid state and its association with protein misfolding diseases. *Nature Reviews Molecular Cell Biology* 15:384–396. DOI: 10.1038/nrm3810.

Linse S. 2019. Mechanism of amyloid protein aggregation and the role of inhibitors. *Pure and Applied Chemistry* 91:211–229. DOI: 10.1515/pac-2018-1017.

Ma J, Komatsu H, Kim YS, Liu L, Hochstrasser RM, Axelsen PH. 2013. Intrinsic Structural Heterogeneity and Long-Term Maturation of Amyloid β Peptide Fibrils. *ACS Chemical Neuroscience* 4:1236–1243. DOI: 10.1021.cn400092v.

Makin OS, Serpell LC. 2005. Structures for amyloid fibrils. *FEBS Journal* 272:5950–5961. DOI: 10.1111/j.1742-4658.2005.05025.x.

Malik R, Roy I. 2011. Probing the mechanism of insulin aggregation during agitation. *International Journal of Pharmaceutics* 413:73–80. DOI: 10.1016/j.ijpharm.2011.04.024.
Malisauskas R, Botyriute A, Cannon JG, Smirnovas V. 2015. Flavone Derivatives as Inhibitors of Insulin Amyloid-Like Fibril Formation. *PLOS ONE* 10:e0121231. DOI: 10.1371/journal.pone.0121231.

Maurer MS, Schwartz JH, Gundapaneni B, Elliott PM, Merlini G, Waddington-Cruz M, Kristen A V., Grogan M, Witteles R, Damy T, Drachman BM, Shah SJ, Hanna M, Judge DP, Barsdorf AI, Huber P, Patterson TA, Riley S, Schumacher J, Stewart M, Sultan MB, Rapezzi C. 2018. Tafamidis Treatment for Patients with Transthyretin Amyloid Cardiomyopathy. *New England Journal of Medicine* 379:1007–1016. DOI: 10.1056/NEJMoa1805689.

Mehta D, Jackson R, Paul G, Shi J, Sabbagh M. 2017. Why do trials for Alzheimer’s disease drugs keep failing? A discontinued drug perspective for 2010-2015. *Expert Opinion on Investigational Drugs* 26:735–739. DOI: 10.1080/13543784.2017.1323868.

Meisl G, Kirkegaard JB, Arosio P, Michaels TCT, Vendruscolo M, Dobson CM, Linse S, Knowles TPJ. 2016. Molecular mechanisms of protein aggregation from global fitting of kinetic models. *Nature Protocols* 11:252–272. DOI: 10.1038/nprot.2016.010.

Milto K, Michailova K, Smirnovas V. 2014. Elongation of Mouse Prion Protein Amyloid-Like Fibrils: Effect of Temperature and Denaturant Concentration. *PLoS ONE* 9:e94469. DOI: 10.1371/journal.pone.0094469.

Nettleton EJ, Tito P, Sunde M, Bouchard M, Dobson CM, Robinson C V. 2000. Characterization of the Oligomeric States of Insulin in Self-Assembly and Amyloid Fibril Formation by Mass Spectrometry. *Biophysical Journal* 79:1053–1065. DOI: 10.1016/S0006-3495(00)76359-4.

Nicoud L, Lazzari S, Balderas Barragán D, Morbidelli M. 2015. Fragmentation of Amyloid Fibrils Occurs in Preferential Positions Depending on the Environmental Conditions. *The Journal of Physical Chemistry B* 119:4644–4652. DOI: 10.1021/acs.jpcb.5b01160.

Nielsen L, Khurana R, Coats A, Frokjaer S, Brange J, Vyas S, Uversky VN, Fink AL. 2001. Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. *Biochemistry* 40:6036–6046. DOI: 10.1021/bi002555c.

Park J, Egolum U, Parker S, Andrews E, Ombengi D, Ling H. 2020. Tafamidis: A First-in-Class Transthyretin Stabilizer for Transthyretin Amyloid Cardiomyopathy. Annals of Pharmacotherapy 54:470–477. DOI: 10.1177/1060028019888489.

Podestà A, Tiana G, Milani P, Manno M. 2006. Early Events in Insulin Fibrillization Studied by Time-Lapse Atomic Force Microscopy. *Biophysical Journal* 90:589–597. DOI: 10.1529/biophysj.105.068833.

Ratha BN, Kar RK, Bednarikova Z, Gazova Z, Kotler SA, Raha S, De S, Maiti NC, Bhunia A. 2020. Molecular Details of a Salt Bridge and Its Role in Insulin Fibrillation by NMR and Raman Spectroscopic Analysis. *The Journal of Physical Chemistry B* 124:1125–1136. DOI: 10.1021/acs.jpcb.9b10349.

Robbins KJ, Liu G, Selman V, Lazo ND. 2012. Conformational analysis of thioflavin T bound to the surface of amyloid fibrils. *Langmuir* 28:16490–16495. DOI: 10.1021/la303677t.
Sakalauskas A, Ziaunys M, Smirnovas V. 2019. Concentration-dependent polymorphism of insulin amyloid fibrils. *PeerJ* 7:e8208. DOI: 10.7717/peerj.8208.

Selivanova OM, Galzitskaya O V. 2012. Structural polymorphism and possible pathways of amyloid fibril formation on the example of insulin protein. *Biochemistry (Moscow)* 77:1237–1247. DOI: 10.1134/S0006297912110028.

Sengupta U, Nilson AN, Kayed R. 2016. The Role of Amyloid-β Oligomers in Toxicity, Propagation, and Immunotherapy. *EBioMedicine* 6:42–49. DOI: 10.1016/j.ebiom.2016.03.035.

Sidhu A, Segers-Nolten I, Raussens V, Claessens MMAE, Subramaniam V. 2017. Distinct Mechanisms Determine α-Synuclein Fibril Morphology during Growth and Maturation. *ACS Chemical Neuroscience* 8:538–547. DOI: 10.1021/acschemneuro.6b00287.

Sidhu A, Vaneyck J, Blum C, Segers-Nolten I, Subramaniam V. 2018. Polymorph-specific distribution of binding sites determines thioflavin-T fluorescence intensity in α-synuclein fibrils. *Amyloid* 25:189–196. DOI: 10.1080/13506129.2018.1517736.

Singh PK, Kumbhakar M, Pal H, Nath S. 2010. Viscosity Effect on the Ultrafast Bond Twisting Dynamics in an Amyloid Fibril Sensor: Thioflavin-T. *The Journal of Physical Chemistry B* 114:5920–5927. DOI: 10.1021/jp100371s.

Smirnovas V, Winter R. 2008. Revealing Different Aggregation Pathways of Amyloidogenic Proteins by Ultrasound Velocimetry. *Biophysical Journal* 94:3241–3246. DOI: 10.1529/biophysj.107.123133.

Sneideris T, Darguzis D, Botyriute A, Grigaliunas M, Winter R, Smirnovas V. 2015. pH-Driven Polymorphism of Insulin Amyloid-Like Fibrils. *PLOS ONE* 10:e0136602. DOI: 10.1371/journal.pone.0136602.

Sneideris T, Sakalauskas A, Sternke-Hoffmann R, Peduzzo A, Ziaunys M, Buell AK, Smirnovas V. 2019. The Environment Is a Key Factor in Determining the Anti-Amyloid Efficacy of EGCG. *Biomolecules* 9:1–17. DOI: 10.3390/biom9120855.

Sulatskaya AI, Rychkov GN, Sulatsky MI, Rodina NP, Kuznetsova IM, Turoverov KK. 2018. Thioflavin T Interaction with Acetylcholinesterase: New Evidence of 1:1 Binding Stoichiometry Obtained with Samples Prepared by Equilibrium Microdialysis. *ACS Chemical Neuroscience* 9:1793–1801. DOI: 10.1021/acschemneuro.8b00111.

Törnquist M, Michaels TCT, Sanagavarapu K, Yang X, Meisl G, Cohen SIA, Knowles TPJ, Linse S. 2018. Secondary nucleation in amyloid formation. *Chemical Communications* 54:8667–8684. DOI: 10.1039/C8CC02204F.

Wang S-H, Dong X-Y, Sun Y. 2012. Effect of (−)-epigallocatechin-3-gallate on human insulin fibrillation/aggregation kinetics. *Biochemical Engineering Journal* 63:38–49. DOI: 10.1016/j.bej.2012.02.002.

Xue C, Lin TY, Chang D, Guo Z. 2017. Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation. *Royal Society Open Science* 4:160696. DOI:
Zheng Q, Lazo ND. 2018. Mechanistic Studies of the Inhibition of Insulin Fibril Formation by Rosmarinic Acid. The Journal of Physical Chemistry B 122:2323–2331. DOI: 10.1021/acs.jpcb.8b00689.

Ziaunys M, Smirnovas V. 2019a. Emergence of visible light optical properties of L-phenylalanine aggregates. PeerJ 7:e6518. DOI: 10.7717/peerj.6518.

Ziaunys M, Smirnovas V. 2019b. Additional Thioflavin-T Binding Mode in Insulin Fibril Inner Core Region. The Journal of Physical Chemistry B 123:8727–8732. DOI: 10.1021/acs.jpcb.9b08652.

Ziaunys M, Sakalauskas A, Smirnovas V. 2020. Identifying Insulin Fibril Conformational Differences by Thioflavin-T Binding Characteristics. Biomacromolecules 21:4989–4997. DOI: 10.1021/acs.biomac.0c01178.

Ziaunys M, Sneideris T, Smirnovas V. 2020. Formation of distinct prion protein amyloid fibrils under identical experimental conditions. Scientific Reports 10:4572. DOI: 10.1038/s41598-020-61663-2.
Figure 1

Insulin aggregation curves and their derivatives.

Normal, sigmoidal (A) and anomalous, double-sigmoidal (B) insulin aggregation kinetics and their first order derivatives (C, D respectively).
Figure 2

Distribution of maximum insulin aggregation rate, the time at which it is reached ($t_r$) and final fibril fluorescence intensity.

Dependence of aggregation rate on $t_r$ (A), fluorescence intensity on $t_r$ (B) and aggregation rate on fluorescence intensity (C). Distribution of $t_r$ (D, G), aggregation rate (E, H) and fluorescence intensity (F, I) for normal and double-sigmoidal samples respectively. Color-coded numbers, displayed above peak-fit curves, indicate peak maximum values.
Figure 3

Normal, intermediate and double-sigmoidal sample AFM images, fibril height distributions, second order FTIR spectra and seeding kinetics.

AFM images of insulin aggregates during the first part of the double-sigmoidal kinetics (A) and at the end of normal (B) and double-sigmoidal (C) aggregation. Height distribution of double-sigmoidal aggregation intermediates and fibrils after normal and double-sigmoidal aggregation (D), where box plots indicate the interquartile range and error bars are one standard deviation. Second order FTIR spectra (E) and seeded aggregation kinetics (F).
Figure 4

Insulin aggregation kinetics and bound ThT fluorescence EEMs.

Insulin aggregation kinetics monitored by scanning ThT fluorescence EEMs (A), where each data point is the maximum value in the recorded EEM. EEM “center of mass” excitation (B) and emission (C) wavelengths over the course of aggregation. Top intensity values present in the ThT EEMs at different aggregation time points (D) during the first double-sigmoidal increase (darker green areas represent higher intensity zones). The red * symbol indicates the point where there is a sudden jump in ThT fluorescence intensity. Data in part (A) was fit using a Boltzmann’s sigmoidal equation with the anomalous aggregation phase data points omitted from the fitting procedure.