Response to crowded conditions reveals compact nucleus for amyloid formation of folded protein

QRB Discovery

www.cambridge.org/qrd

Response to crowded conditions reveals compact nucleus for amyloid formation of folded protein

Tony E.R. Werner, Istvan Horvath and Pernilla Wittung-Stafshede*

Department of Biology and Biological Engineering, Chalmers University of Technology, S-41296 Gothenburg, Sweden

Research Article

Cite this article: Werner TER, Horvath I, Wittung-Stafshede P (2021). Response to crowded conditions reveals compact nucleus for amyloid formation of folded protein. *QRB Discovery*, **2:** e2, 1–8
https://doi.org/10.1017/qrd.2020.17

Received: 10 October 2020
Revised: 23 November 2020
Accepted: 04 December 2020

Key words:
Amyloid formation; macromolecular crowding; osmolyte; β-parvalbumin; protein aggregation

Author for correspondence:
*Pernilla Wittung-Stafshede,
E-mail: pernilla.wittung@chalmers.se

Abstract

Although the consequences of the crowded cell environments may affect protein folding, function and misfolding reactions, these processes are often studied in dilute solutions *in vitro*. We here used biophysical experiments to investigate the amyloid fibril formation process of the fish protein apo-β-parvalbumin in solvent conditions that mimic steric and solvation aspects of the *in vivo* milieu. Apo-β-parvalbumin is a folded protein that readily adopts an amyloid state *via* a nucleation–elongation mechanism. Aggregation experiments in the presence of macromolecular crowding agents (probing excluded volume, entropic effects) as well as small molecule osmolytes (probing solvation, enthalpic effects) revealed that both types of agents accelerate overall amyloid formation, but the elongation step was faster with macromolecular crowding agents but slower in the presence of osmolytes. The observations can be explained by the steric effects of excluded volume favoring assembled states and that amyloid nucleation does not involve monomer unfolding. In contrast, the solvation effects due to osmolyte presence promote nucleation but not elongation. Therefore, the amyloid-competent nuclei must be compact with less osmolytes excluded from the surface than either the folded monomers or amyloid fibers. We conclude that, in contrast to other amyloidogenic folded proteins, amyloid formation of apo-β-parvalbumin is accelerated by crowded cell-like conditions due to a nucleation process that does not involve large-scale protein unfolding.

Introduction

The intracellular environment is highly crowded, with an estimate of 80–400 mg ml^{-1} macromolecules (proteins, lipids, polysaccharides), corresponding to a volume occupancy of 5–40% (Ellis and Minton, 2003; Kuznetsova *et al.*, 2014). Also extracellular space is crowded, where for example blood plasma has a protein concentration of 60–80 mg ml^{-1} (Leeman *et al.*, 2018). High concentrations of macromolecules restrict the available molecular space by their mutual impenetrability and repulsive interactions, often termed excluded volume effect. The excluded volume effect is accompanied by increased opportunities for non-specific (chemical or 'soft') interactions, increased (heterogeneous) viscosity, reduced macromolecule dynamics, as well as changes in solvent polarity and water activity, which in total describes the macromolecular crowding effect (Gao and Winter, 2015; Davis and Gruebele, 2018). The excluded volume effect is an entropic effect in which (due to steric restrictions) compact and assembled protein species are favoured (Ralston, 1990; van den Berg *et al.*, 1999; Minton, 2001, 2005*a*; Kuznetsova *et al.*, 2014). Many *in vitro* studies of excluded volume effects (using so-called macromolecular crowding agents, for example, sugar-based polymers such as Ficoll and dextran) on protein folding and stability have shown increased folded-state stability and slower unfolding reactions in the presence of macromolecular crowding agents (van den Berg *et al.*, 2000; Ellis, 2001; Christiansen and Wittung-Stafshede, 2013; Christiansen and Wittung-Stafshede, 2014). Notably, macromolecular crowding agents such as Ficoll and dextran are reported to be inert agents with little or no protein interactions; thus, they are useful to specifically study excluded volume effects (Christiansen and Wittung-Stafshede, 2014; Gao and Winter, 2015).

Cellular environments also contain high concentrations of small organic molecules termed osmolytes; so-called 'protective' osmolytes are often upregulated (de novo synthesis or import) to cope with water stress, there aiding in equilibration of cellular osmotic pressure (Yancey, 2005; Ignatova and Gierasch, 2006; Burg and Ferraris, 2008; Sukenik *et al.*, 2013). High amounts of protecting osmolytes increase the viscosity but they also stabilize folded proteins *via* enthalpic effects as they are specifically excluded from protein surfaces (Timasheff, 1993, 2002). Thereby, protective osmolytes affect protein hydration and this results in stabilization of folded and compact protein structures that minimize interactions with the osmolyte, as compared to the many more interactions that are possible in the expanded unfolded state (Liu and Bolen, 1995; Ignatova and Gierasch, 2006; Auton and Bolen, 2007). Taken together, excluded volume effects are entropic in nature (and can be probed using macromolecular crowding agents), whereas

CAMBRIDGE UNIVERSITY PRESS

solvation effects (probed *via* small molecule osmolytes) have enthalpic origin. Nonetheless, these systems are complicated and there is more to be revealed; there are for example studies implying that also osmolytes act *via* entropic forces (Ishrat *et al.*, 2018).

Typically, macromolecular crowding conditions (large molecules), as well as osmolyte presence (small molecules), thermodynamically favour protein–protein interactions as assembled states will reduce the volume occupancy and number of species in solution. However, the effect on reaction kinetics will depend on conformation and surface-area exposure of rate-limiting steps. Here amyloid formation is interesting as this assembly process involves multiple steps and protein-conformational changes (Chiti and Dobson, 2017). Amyloid fibrils are biological polymers composed of monomeric protein units non-covalently assembled through β-strands arranged perpendicularly to the fibril axis, thereby forming a cross-β structure (Chiti and Dobson, 2017). Many proteins in all kingdoms of life (Otzen, 2010) can form amyloid fibrils at certain solvent conditions (Chiti and Dobson, 2017), and this process is directly related to several human diseases, such as Alzheimer's disease, Parkinson's disease and type 2 diabetes (Cooper *et al.*, 1987; Jarrett *et al.*, 1993; Wakabayashi *et al.*, 1997). But there are also proteins that form functional amyloids: for example, biofilms are structures used by bacteria to adhere to surfaces which contain amyloids made of curli proteins (Andersson *et al.*, 2013; Evans *et al.*, 2015). Human functional amyloids include proteins in the signaling pathway leading to necrosis (Li *et al.*, 2012) and in melanosome ultra-structures (Dean and Lee, 2019). Amyloid formation reactions of proteins that start from an intrinsically disordered monomeric state will necessarily involve compaction as the extended polypeptide will form β-structure. Thus for disordered/unfolded proteins, it is reasonable that macromolecular crowding *via* the excluded volume effect accelerates amyloid formation and this has been observed for aggregation of, for example, α-synuclein (Shtilerman *et al.*, 2002; Uversky *et al.*, 2002; White *et al.*, 2010), the amyloid-β peptide (Lee *et al.*, 2012) and a tau fragment (Zhou *et al.*, 2009).

Folded proteins may become amyloidogenic as a result of ligand release, thermal fluctuations, as well as by local or global unfolding (Kuznetsova *et al.*, 2014; Chiti and Dobson, 2017). It is also possible that assembly of folded proteins (native-like aggregates) occurs prior to structural rearrangements leading to amyloid fibers (Bouchard *et al.*, 2000; Plakoutsi *et al.*, 2004; Soldi *et al.*, 2005; Chiti and Dobson, 2017). For folded proteins, it is less straightforward to predict the macromolecular crowding effect on amyloid formation, as the folded states will be stabilized (with respect to unfolding) by macromolecular crowding agents (Jahn and Radford, 2005; Kuznetsova *et al.*, 2014). Indeed, several folded proteins exhibit increased resistance towards amyloid fibril formation in the presence of macromolecular crowding including carbonic anhydrase (Mittal and Singh, 2014), core histones and insulin (Munishkina *et al.*, 2008). However, when placed in denaturing conditions, that is, low pH that partially unfolds the proteins and, for core histones and insulin, dissociates the native oligomeric states, macromolecular crowding was found to accelerate aggregation of the proteins mentioned above (Munishkina *et al.*, 2008; Luo *et al.*, 2016). Acceleration of aggregation by crowding was also shown for partially unfolded apolipoprotein C-II in the absence of lipids (Hatters *et al.*, 2002). Notably, two of these three examples involve proteins with *oligomeric* native states, and amyloid formation involved oligomer dissociation into monomers as an initial requirement.

To test the effect of macromolecular crowding on an amyloidogenic protein that is *folded* and *monomeric*, we turned to *G. morhua* β-parvalbumin (β-PV). β-PV is an α-helical folded protein capable of binding Mg^{2+} and/or Ca^{2+} through two active EF-hand domains (Rall, 1996). Upon loss of the metal-ion ligands, which may occur due to low pH protonating the coordinating residues, or the chelating agent, ethylenediaminetetraacetic acid (EDTA), apo-β-PV readily forms amyloid fibrils (Martinez *et al.*, 2015; Castellanos *et al.*, 2018). We recently showed that apo-β-PV amyloid formation follows a nucleation–elongation mechanism where the nucleation step involves folded dimers connected by intermolecular disulfide bonds (Werner *et al.*, 2020). The dimers are on path but need to further assemble and/or structurally convert to form competent nuclei that can then be elongated (by monomer and dimer additions) to amyloid fibrils. Thus, the amyloid formation process of apo-β-PV, initiated by assembly of folded proteins, is similar to that reported for only a few other proteins (Bouchard *et al.*, 2000; Plakoutsi *et al.*, 2004; Soldi *et al.*, 2005). Since apo-β-PV is folded, one would predict that macromolecular crowding will slow down amyloid formation by stabilization of the folded monomer. Still, as a folded dimer is on path towards amyloid fibers (Werner *et al.*, 2020), the presence of macromolecular crowding may favor dimers and thereby accelerate nucleation of the amyloid process.

The macromolecular crowding agent Ficoll 70 is a sucrose-based branched polymer often used to mimic excluded volume (Zhou *et al.*, 2008; Wang *et al.*, 2010). Although there may sometimes be exceptions (Nasreen *et al.*, 2020), Ficoll 70 has been shown to only provide steric, excluded volume effects without chemical interactions with proteins (Christiansen *et al.*, 2010; Christiansen and Wittung-Stafshede, 2013, 2014). Using Ficoll 70, we here show that amyloid formation of apo-β-PV, in contrast to data for folded *oligomeric* proteins, is promoted by macromolecular crowding. From additional protective osmolyte (glycerol and sucrose) kinetic experiments, it appears that the competent nucleus for amyloid formation is compact, and its formation does not involve large-scale conformational changes. However, addition of monomers during amyloid fibril elongation must involve larger structural conversions (resulting in increased exposure of surface area) as this is retarded by protective osmolytes.

Materials and method

Chemicals

Powders of sucrose (BioXtra, Sigma, St Louis, MO, USA), Ficoll PM 70 (Sigma, St Louis, MO, USA) (70 kDa) and poly(ethylene glycol) (PEG) 35,000 (BioUltra, Sigma, St Louis, MO, USA) (35 kDa) were dissolved in 25 mM Tris-HCl buffer, pH adjusted for 7.4 at 37°C and volume adjusted to desired concentration. Molecular biology grade glycerol (Fisher Scientific, Waltham MA, USA) was used.

Protein preparation

Just prior to aggregation assays of *G. morhua* β-parvalbumin (A51874, Gad m1), expressed and purified as described in Werner *et al.* (2020), the his-tag was removed by treating β-PV with thrombin (35.5 NIH units $\mu mole^{-1}$) for 4–5 h at room temperature and 800 RPM shaking followed by size exclusion chromatography (Superdex 75 10/300, GE healthcare, Uppsala, Sweden) in 25 mM Tris-HCl, supplemented with 1 mM $CaCl_2$ to stabilize the monomeric state.

Thioflavin T-aggregation assay

The ThT assay was performed in a quiescent condition at 37°C in 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 5 mM

EDTA, 7.7 mM NaN_3 and 20 μM recrystallized ThT in 96 well plates. Fluorescence was measured in a plate reader (Fluostar Optima or Fluostar Omega; BMG Labtech, Ortenberg, Germany). Emission was recorded at 480 nm with excitation at 440 nm every 20 min.

Atomic force microscopy

Samples of apo-β-PV fibrils were diluted with Milli-Q water (10 times) and incubated on a freshly cleaved mica for 15 min. The mica was subsequently rinsed five times and dried under a nitrogen gas flow. Intermittent contact mode atomic force microscopy (AFM) in air was used to record the images (NTEGRA Prima setup, NT-MDT) with a single crystal silicon cantilever (TipsNano, NSG01, force constant of ~5.1 N m^{-1}) with a resonance frequency of ~180 kHz. 512-pixel images were acquired with a 0.5-Hz scan rate. Images were analyzed using the WSxM 5.0 software (Horcas *et al.*, 2007).

Circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectra (190–260 nm) of PV monomers (apo- and holo-β-PV) were collected at room temperature (21 ± 1°C) using Chirascan CD spectropolarimeter (Applied Photophysics) in a 1 mm pathlength quartz cuvette. Three scans were collected and averaged with a time-per-point of 2 s, 1 nm bandwith and step size of 1 nm. The buffer signal was subtracted, and intensities converted from millidegrees to mean residue molar ellipticity (degrees M^{-1} m^{-1}). CD spectra of amyloid fibrils were collected on post-aggregation samples that had been centrifuged at 100 000 *g* for 5 h, resuspended in 10 mM Tris-HCl, followed by 3 h centrifugation again and final resuspension into 10 mM Tris-HCl (pH 7.8).

Results and discussion

Amyloid formation of apo-β-PV (EDTA added at time zero to remove metal ions) in the presence of Ficoll 70 at 37°C was monitored by the Thioflavin T (ThT) fluorescence assay (Naiki *et al.*, 1989; Xue *et al.*, 2017). A significant increase in the speed of aggregation of apo-β-PV was observed when incubated in the presence of 100 and 200 mg ml^{-1} Ficoll 70 (Fig. 1*a*), with a reduction of the half-time (time point when ThT fluorescence has reached 50% of its final value) from 32 ± 3 h (buffer), to 11 ± 1 h (100 mg ml^{-1} Ficoll 70) and 4.5 ± 0.2 h (200 mg ml^{-1} Ficoll 70). The resulting β-PV amyloid fibrils formed in the presence of Ficoll 70 were found to share the curvilinear appearance of apo-β-PV amyloid fibrils formed in buffer (Fig. 1*b*,*c*) and the far-UV CD spectra of amyloid fibers formed with and without Ficoll 70 were identical (Fig. 1*d*). From inspection of the data in Fig. 1*a*, it is evident that the lag time is drastically reduced and the slope of the fluorescence rise (i.e. tangent at half-time point) is larger for the two Ficoll 70 conditions. This implies that both nucleation and elongation processes are accelerated in macromolecular crowded solutions. This is in excellent agreement with excluded volume theory that predicts that assembled species are favored when the volume is reduced (Minton, 2005*b*; Ellis and Minton, 2006). Also more elaborate modelling (including both scaled-particle and transition-state theories) predicts this for systems where the size of the monomer is smaller when in the amyloid fiber than when in the free form (Schreck *et al.*, 2020). Notably, 200 mg ml^{-1} Ficoll 70 will occupy 13% of the volume based on its partial specific volume value (Christiansen *et al.*, 2010; Aguilar *et al.*, 2011) and thus the effective concentration of apo-β-PV is not 30 μM but 34 μM in the presence of 200 mg ml^{-1} Ficoll 70. However, based on the earlier-determined concentration dependence of apo-β-PV aggregation (Werner *et al.*, 2018), this small difference in protein concentration does not increase the aggregation speed significantly.

Importantly, the Ficoll 70 result implies that the expected excluded-volume effect on the folded-to-unfolded transition of apo-protein monomers is not affecting amyloid formation. Unfortunately, we could not test the effect of Ficoll 70 on apo-β-PV monomer thermal or chemical equilibrium stability, as the apo-form is prone to aggregation during the required experimental time scales. Nonetheless, one expects macromolecular crowding to stabilize the compact folded state relative to the more expanded unfolded state (Christiansen *et al.*, 2010; Stagg *et al.*, 2011; Christiansen and Wittung-Stafshede, 2013; Aden and Wittung-Stafshede, 2014). Because some studies have shown macromolecular crowding to also modulate folded-state structure (Perham *et al.*, 2007; Stagg *et al.*, 2007; Homouz *et al.*, 2008), we tested the influence of Ficoll 70 on the secondary structure of folded apo-β-PV using far-UV CD within time scales without aggregation. However, we found that the presence of Ficoll 70 did not affect the secondary structure of apo-β-PV (Fig. 1*f*). Taken together, the effect of Ficoll 70 on apo-β-PV aggregation agrees with folded dimers, stabilized by disulfide bonds (Werner *et al.*, 2020), being key players in a nucleation process that occurs without monomer unfolding. The excluded volume effect due to Ficoll 70 presence favors dimer assembly and, thus, overall aggregation of apo-β-PV into amyloids is accelerated. Also, another macromolecular crowding agent, PEG, accelerated apo-β-PV amyloid formation (Fig. 1*e*) without affecting apo-β-PV folded structure (Fig. 1*f*). Although PEG may interact differently with proteins, and not only provide steric effects (Sukenik *et al.*, 2011; Gao and Winter, 2015), the similar results found for PEG and Ficoll 70 (two agents with different chemistry) support that the observed kinetic acceleration is due to excluded volume. Notably, if attractive chemical (soft) interactions between protein and crowding agent were occurring, as noted in a few cases (Mikaelsson *et al.*, 2014), such interactions would stabilize the folded monomers and thereby reduce aggregation (which is opposite to our findings).

To specifically test the effect of macromolecular crowding on the amyloid elongation step of apo-β-PV, we performed seeded reactions in which various amounts of preformed sonicated apo-β-PV amyloid fibril seeds were added to monomers. When high amounts of fibril seeds are added, only elongation takes place at early time points and therefore elongation can be exclusively probed. In Fig. 2, we show that seeded reactions (7.5, 15 and 24 μM monomer equivalents of fiber seeds added to 30 μM monomers) is accelerated in the presence of Ficoll 70, as compared to the corresponding seeded reactions in buffer. Again, this can be explained by the excluded volume effect (due to the presence of Ficoll 70) favoring assembled protein states. To elongate, apo-β-PV monomers must structurally convert from the native α-helical structure to the β-strand arrangement in the amyloid fibrils. The observed kinetic acceleration thus show that the macromolecular crowding effect on apo-β-PV monomer stability (folded *versus* unfolded states) does not kinetically limit the conformational transitions needed for the amyloid fiber elongation process. Analysis of the initial rates in the seeded apo-β-PV experiments reveals that the elongation rate constant is increased by a factor of 1.4–1.5 in the presence of 50 mg ml^{-1} Ficoll 70 as compared to in buffer (Fig. 2*d*). This magnitude is in accord with scaled particle theory predictions (thus, based on entropic effects/excluded volume) of amyloid fibril growth: in (White *et al.*, 2010) it was reported that experimental and predicted elongation rates for some different proteins were between 1.2 and 1.8 times

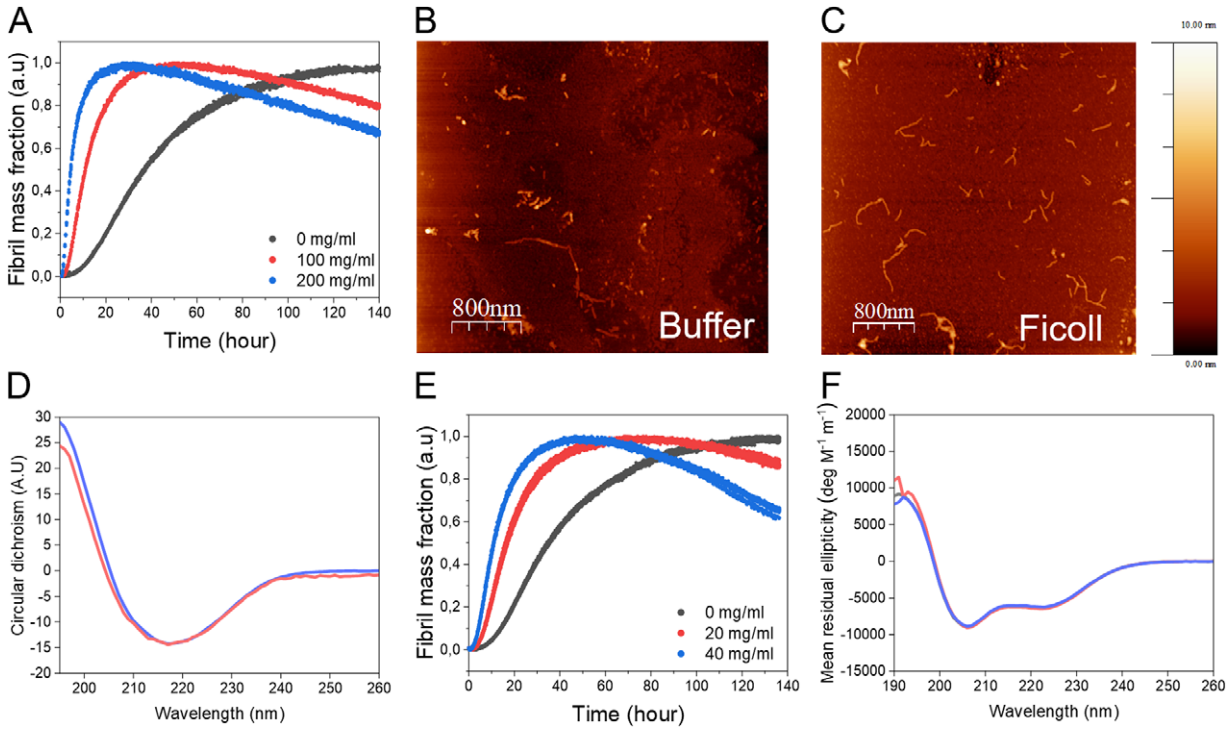

Fig. 1. Macromolecular crowding agents accelerate amyloid formation but do not affect fiber morphology. ThT fluorescence curves of 30 μM apo-β-PV at 37°C under quiescent conditions in 150 mM NaCl, 1 mM $CaCl_2$, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4 and 0–200 mg ml^{-1} Ficoll 70 (*a*) or 0–40 mg ml^{-1} PEG 35,000 (*e*). AFM images of fibrils obtained from apo-β-PV alone (*b*) or in the presence of 100 mg ml^{-1} Ficoll 70 (*c*). (*d*) CD spectra of resulting apo-β-PV amyloid fibers formed with (red) or without (blue) 100 mg ml^{-1} Ficoll 70 (curves normalized to the same signal at the minimum). (*f*) CD spectra of 15 μM apo-β-PV at 21°C in 10 mM Tris-HCl, pH 7.8, 1 mM EDTA and 96 μM $CaCl_2$, with (red) 50 mg ml^{-1} Ficoll 70, or (blue) 20 mg ml^{-1} PEG, or (grey) without addition (i.e. all prior to aggregation).

faster in 50 mg ml^{-1} of a macromolecular crowding agents as compared to in buffer.

To complement the Ficoll 70 results, which focus on excluded volume, we tested the effect of small molecule protective osmolytes (Kim *et al.*, 2006) to assess solvent effects on apo-β-PV amyloid formation. Somewhat surprising, we found that the presence of both sucrose and glycerol (two protective small molecule osmolytes with different chemistry) resulted in faster apo-β-PV amyloid formation (Fig. 3*a*). The effect was concentration dependent, with higher sucrose or glycerol levels accelerating amyloid formation more. Importantly, the resulting apo-β-PV amyloid fibrils formed in presence of osmolytes appear like those formed in buffer and Ficoll 70 conditions according to far-UV CD (Fig. 3*b*) and AFM analysis (Fig. 3*c*,*d*) Notably, comparing the curves in Fig. 3*a* and Fig. 1*a* reveals that the aggregation lag times are not reduced as much, and the slopes (tangents) at the half time points are not as steep, as when apo-β-PV is placed in Ficoll 70, despite the fact that the concentrations of sucrose and glycerol used (in mg ml^{-1} scale) are higher than the concentrations of Ficoll 70. For example, 380 mg ml^{-1} sucrose reduced the half time point by a factor two, whereas 200 mg ml^{-1} Ficoll 70 reduced the half time point by a factor of seven. Thus, excluded volume appears to have a larger effect than osmolyte-mediated hydration changes on the amyloid formation reaction. To better understand this difference, we assessed the effect of one of the osmolytes, sucrose, on the amyloid fibril elongation process of apo-β-PV by performing seeded amyloid formation reactions. We found that in contrast to Ficoll 70, the presence of sucrose reduces the elongation rate as compared to the corresponding reaction in buffer at all three amyloid fiber seed concentrations tested (Fig. 2).

To explain the protective osmolyte results, that is, that non-seeded apo-β-PV amyloid formation is accelerated but apo-β-PV amyloid elongation is retarded by osmolytes, the nucleation step in the amyloid formation process must be promoted by the osmolytes. Protecting osmolytes such as glycerol and sucrose stabilize folded proteins due to their preferential exclusion from the protein surface (Timasheff, 2002; Ignatova and Gierasch, 2006; Kim *et al.*, 2006). This effect will destabilize expanded unfolded states (that expose more surface area and thus require more osmolyte exclusion) but stabilize compact states of proteins. With this in mind, nucleation of apo-β-PV amyloid formation appears to involve compact structures that are stabilized by the osmolytes. The rate-limiting step for nuclei formation (i.e. the transition state) must also be stabilized by osmolyte presence to result in faster kinetics; thus, nucleation cannot involve large-scale protein unfolding. Again, this result is in accord with *folded* dimers being key species in apo-β-PV nuclei formation (Werner *et al.*, 2020).

Fibril elongation involves the addition of monomers to the ends of growing amyloids with β-structure. Here structural conversions are needed as apo-β-PV monomers, to be added to growing fibers, are folded with α-helical structure. Conversion from α-helical to β-sheet structure will require transient exposure of an increased protein surface area, which will be disfavored in the presence of the osmolytes due to preferential exclusion effects. The retarding osmolyte effect on apo-β-PV amyloid fiber elongation kinetics agrees with findings on insulin and lysozyme at conditions where the proteins retained some folded structure. For both proteins, slower amyloid fibril elongation was detected in the presence of the protective osmolyte glycine (White *et al.*, 2010).

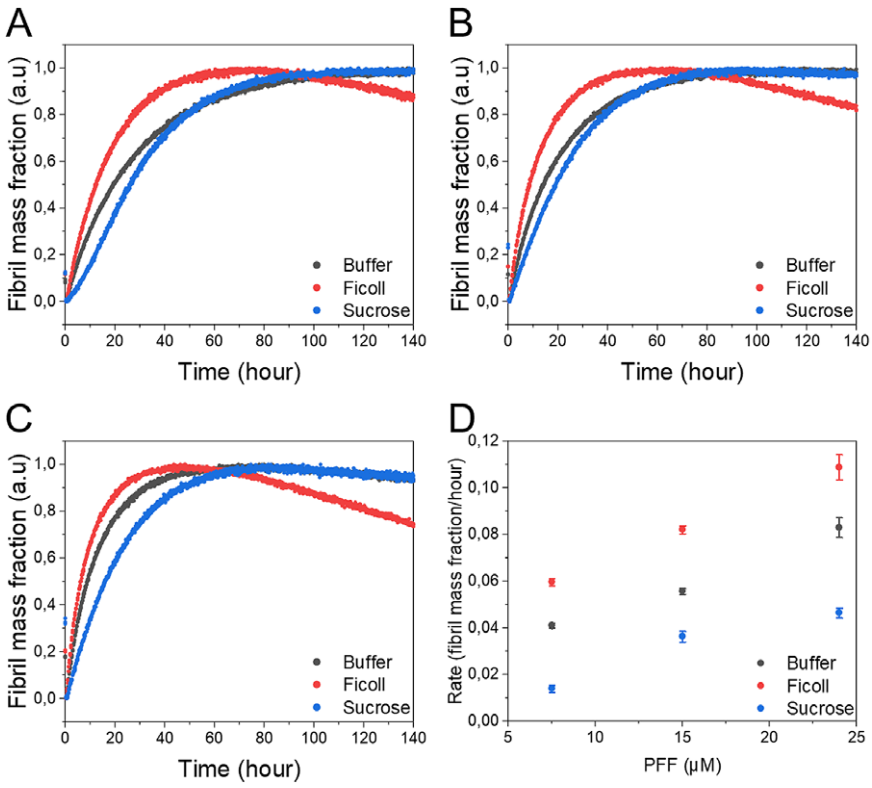

Fig. 2. Ficoll 70 accelerates, but sucrose slows down, amyloid fiber elongation. ThT aggregation curves of 30 μM apo-β-PV at 37°C under quiescent conditions in 150 mM NaCl, 1 mM $CaCl_2$, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4, with or without 50 mg ml^{-1} Ficoll 70 or 380 mg ml^{-1} sucrose, in the presence of 7.5 μM sonicated pre-formed fibrils (PFF) (*a*), 15 μM PFF (*b*) or 24 μM PFF (*c*). (*d*) Initial slope analysis of the curves (*a*–*c*) during the first 2.3 h.

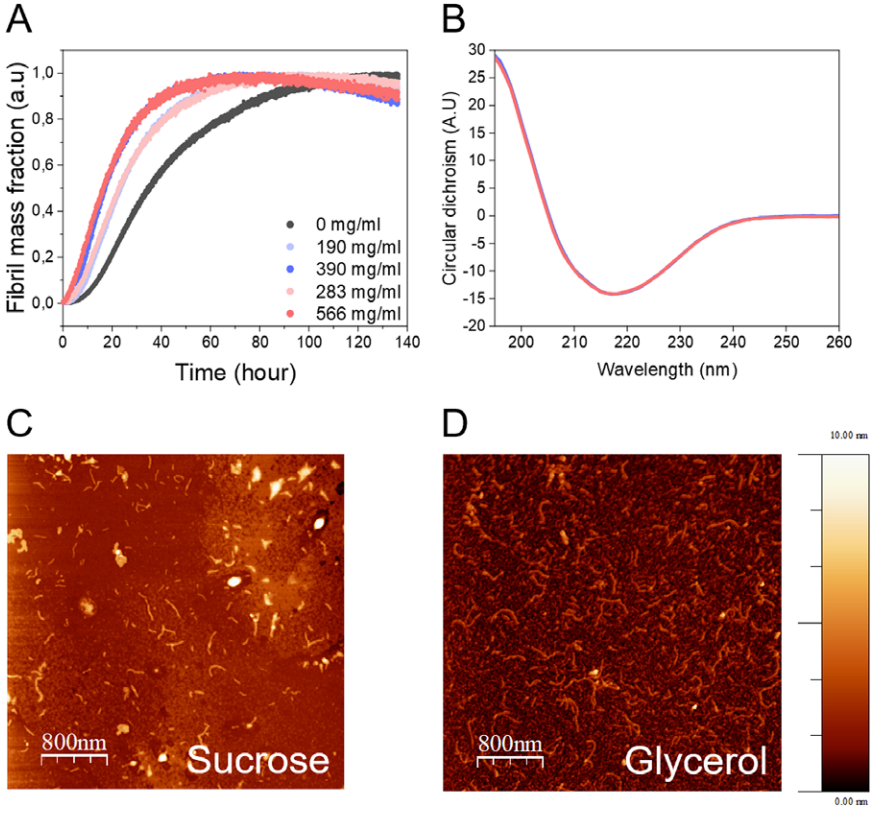

Fig. 3. Osmolytes accelerate the overall amyloid reaction but do not affect amyloid fiber morphology. ThT fluorescence curves of 30 μM apo-β-PV at 37°C under quiescent conditions in 150 mM NaCl, 1 mM $CaCl_2$, 5 mM EDTA, 25 mM Tris-HCl, pH 7.4 and 0–390 mg ml^{-1} sucrose (blue shades) or 0–566 mg ml^{-1} glycerol (red shades) (*a*). CD spectra of resulting apo-β-PV amyloid fibers formed with (red) or without (blue) 190 mg ml^{-1} sucrose (curves normalized to the same signal at the minimum) (*b*). AFM images of fibrils obtained from apo-β-PV in the presence of 380 mg ml^{-1} sucrose (*c*) or 566 mg ml^{-1} glycerol (*d*).

Conclusions and outlook

Folded proteins, in addition to intrinsically disordered proteins, may form amyloid fibrils and this sometimes results in human diseases. In most cases, *folded multimeric* proteins must first dissociate to monomers, and the monomers must become structurally perturbed, for amyloid formation to occur. In such cases, macromolecular crowding, as found in living systems, will reduce the aggregation reaction as it stabilizes both folded proteins and multimeric states. Here we analyzed a *folded monomeric* protein that can assemble to amyloids. We show that the macromolecular crowding agent Ficoll 70 speed up amyloid formation although we start from the folded protein. The results are explained by the fact that apo-β-PV amyloid formation is not initiated by monomer unfolding but is instead promoted by folded dimers (Werner *et al.*, 2020). The complementary experiments with small molecule osmolytes support the conclusion that no unfolding is involved in amyloid nucleation and further demonstrates that the nuclei are compact species stabilized in the osmolyte conditions. The fact that amyloid fiber elongation is accelerated by macromolecular crowding agents but retarded by osmolytes is explained by favorable excluded volume effects and unfavorable solvation effects, respectively, on monomer additions to growing amyloid fibers. Taken together, our study shows the first example of a *folded monomeric* protein that is augmented to adopt amyloid structures at conditions mimicking those found in living systems. The consequences of cell-like conditions on amyloid formation will depend crucially on mechanisms and structures of involved transient species: in the case of apo-β-PV, aggregation into amyloids is favored by the excluded volume effect as the process involves a compact nucleus that is reached without large-scale polypeptide unfolding.

Excluded volume, here mimicked by macromolecular crowding agents, is definitely an important factor of *in vivo* conditions. However, other aspects of the cell environment are also at play and may in some cases dominate. In addition to excluded volume and solvation effects (studied here), also nonspecific chemical interactions may take place between macromolecules and hydrophobic forces within macromolecules, for example, DNA, were shown to be affected by cell-like conditions (Feng *et al.*, 2019). Many more systematic studies of these effects on the molecular-mechanistic level are desired, including studies using mixtures of macromolecular crowding agents, protein crowders, as well as assessing combinations of macromolecular crowding agents and osmolytes; all in combination with new theoretical approaches (Schreck *et al.*, 2020). So far, known amyloidogenic proteins involved in human diseases are intrinsically disordered proteins or folded (often oligomeric) proteins that require partial unfolding/dissociation (i.e. some destabilization) to initiate amyloid formation. Perhaps we may eventually discover that folded proteins, without the need for destabilization, also participate in amyloid diseases, with amyloid initiation triggered by, for example, oxidative stress that cause covalent bonds between folded proteins. In fact, it may emerge that what we today consider 'well-characterized' human diseases may involve yet undiscovered amyloid formation that, in turn, is modulated by the cell environment (e.g. crowding, osmolytes).

Author contributions. T.W. and P.W.S. designed the study. T.W. and I.H. performed experiments. T.W., I.H. and P.W.S. analyzed data. P.W.S. wrote the draft article. T.W., I.H. and P.W.S. edited the article.

Financial support. We thank the Knut and Alice Wallenberg Foundation, the Swedish Research Council and the Chalmers Foundation for funding.

Conflicts of interest. The authors declare no conflicts of interest.

Data availability statement. All data are included in the manuscript.

References

Aden J and Wittung-Stafshede P (2014) *Folding of an unfolded protein by macromolecular crowding* in vitro. Biochemistry **53**(14), 2271–2277.

Aguilar X, Weice CF, Sparrman T, Wolf-Watz M and Wittung-Stafshede P (2011) *Macromolecular crowding extended to a heptameric system: the co-chaperonin protein 10*. Biochemistry **50**(14), 3034–3044.

Andersson EK, Bengtsson C, Evans ML, Chorell E, Sellstedt M, Lindgren AE, Hufnagel DA, Bhattacharya M, Tessier PM, Wittung-Stafshede P, Almqvist F and Chapman MR (2013) *Modulation of curli assembly and pellicle biofilm formation by chemical and protein chaperones*. Chemistry & Biology **20**(10), 1245–1254.

Auton M and Bolen DW (2007) *Application of the transfer model to understand how naturally occurring osmolytes affect protein stability*. Methods in Enzymology **428**, 397–418.

Bouchard M, Zurdo J, Nettleton EJ, Dobson CM and Robinson CV (2000) *Formation of insulin amyloid fibrils followed by FTIR simultaneously with CD and electron microscopy*. Protein Science **9**(10), 1960–1967.

Burg MB and Ferraris JD (2008) *Intracellular organic osmolytes: function and regulation*. Journal of Biological Chemistry **283**(12), 7309–7313.

Castellanos M, Torres-Pardo A, Rodriguez-Perez R and Gasset M (2018) *Amyloid assembly endows gad m 1 with biomineralization properties*. Biomolecules **8**(1), 11.

Chiti F and Dobson CM (2017) *Protein misfolding, amyloid formation, and human disease: a summary of progress over the last decade*. Annual Review of Biochemistry. R. D. Kornberg. Palo Alto, Annual Reviews. **86**, 27–68.

Christiansen A, Wang Q, Samiotakis A, Cheung MS and Wittung-Stafshede P (2010) *Factors defining effects of macromolecular crowding on protein stability: an* in vitro/in silico *case study using cytochrome c*. Biochemistry **49**(31), 6519–6530.

Christiansen A and Wittung-Stafshede P (2013) *Quantification of excluded volume effects on the folding landscape of* Pseudomonas aeruginosa *apoazurin* in vitro. Biophysical Journal **105**(7), 1689–1699.

Christiansen A and Wittung-Stafshede P (2014) *Synthetic crowding agent dextran causes excluded volume interactions exclusively to tracer protein apoazurin*. FEBS Letters **588**(5), 811–814.

Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB and Reid KBM (1987) *Purification and characterization of a peptide from amyloid-rich pancreases of type-2 diabetic-patients*. Proceedings of the National Academy of Sciences of the United States of America **84**(23), 8628–8632.

Davis CM and Gruebele M (2018) *Non-steric interactions predict the trend and steric interactions the offset of protein stability in cells*. Chemphyschem **19**(18), 2290–2294.

Dean DN and Lee JC (2019) *pH-Dependent fibril maturation of a Pmel17 repeat domain isoform revealed by tryptophan fluorescence*. Biochimica et Biophysica Acta – Proteins and Proteomics **1867**(10), 961–969.

Ellis RJ (2001) *Macromolecular crowding: obvious but underappreciated*. Trends in Biochemical Sciences **26**(10), 597–604.

Ellis RJ and Minton AP (2003) *Cell biology – join the crowd*. Nature **425**(6953), 27–28.

Ellis RJ and Minton AP (2006) *Protein aggregation in crowded environments*. Biological Chemistry **387**(5), 485–497.

Evans ML, Chorell E, Taylor JD, Aden J, Gotheson A, Li F, Koch M, Sefer L, Matthews SJ, Wittung-Stafshede P, Almqvist F and Chapman MR (2015) *The bacterial curli system possesses a potent and selective inhibitor of amyloid formation*. Molecular Cell **57**(3), 445–455.

Feng B, Sosa RP, Martensson AKF, Jiang K, Tong A, Dorfman KD, Takahashi M, Lincoln P, Bustamante CJ, Westerlund F and Norden B (2019) *Hydrophobic catalysis and a potential biological role of DNA unstacking induced by environment effects*. Proceedings of the National Academy of Sciences of the United States of America **116**(35), 17169–17174.

Gao M and Winter R (2015) *The effects of lipid membranes, crowding and osmolytes on the aggregation, and fibrillation propensity of human IAPP*. Journal of Diabetes Research **2015**, 849017.

Hatters DM, **Minton AP and Howlett GJ** (2002) *Macromolecular crowding accelerates amyloid formation by human apolipoprotein C-II*. Journal of Biological Chemistry **277**(10), 7824–7830.

Homouz D, **Perham M**, **Samiotakis A**, **Cheung MS and Wittung-Stafshede P** (2008) *Crowded, cell-like environment induces shape changes in aspherical protein*. Proceedings of the National Academy of Sciences of the United States of America **105**(33), 11754–11759.

Horcas IR Fernández JM, **Gómez-Rodríguez J**, **Colchero J**, **Gómez-Herrero J**, **Baro AM** (2007) *WSXM: a software for scanning probe microscopy and a tool for nanotechnology*. Review of Scientific Instruments **78**(1), 013705.

Ignatova Z and Gierasch LM (2006) *Inhibition of protein aggregation* in vitro *and* in vivo *by a natural osmoprotectant*. Proceedings of the National Academy of Sciences of the United States of America **103**(36), 13357–13361.

Ishrat M, **Hassan MI**, **Ahmad F and Islam A** (2018) *Sugar osmolytes-induced stabilization of RNase A in macromolecular crowded cellular environment*. International Journal of Biological Macromolecules **115**, 349–357.

Jahn TR and Radford SE (2005) *The Yin and Yang of protein folding*. The FEBS Journal **272**(23), 5962–5970.

Jarrett JT, **Berger EP and Lansbury PT** (1993) *The carboxy terminus of the beta-amyloid protein is critical for the seeding of amyloid formation – implications for the pathogenesis of Alzheimer's disease*. Biochemistry **32**(18), 4693–4697.

Kim SH, **Yan YB and Zhou HM** (2006) *Role of osmolytes as chemical chaperones during the refolding of aminoacylase*. Biochemistry and Cell Biology **84**(1), 30–38.

Kuznetsova IM, **Turoverov KK and Uversky VN** (2014) *What macromolecular crowding can do to a protein*. International Journal of Molecular Sciences **15** (12), 23090–23140.

Lee CF, **Bird S**, **Shaw M**, **Jean L and Vaux DJ** (2012) *Combined effects of agitation, macromolecular crowding, and interfaces on amyloidogenesis*. Journal of Biological Chemistry **287**(45), 38006–38019.

Leeman M, **Choi J**, **Hansson S**, **Storm MU and Nilsson L** (2018) *Proteins and antibodies in serum, plasma, and whole blood-size characterization using asymmetrical flow field-flow fractionation (AF4)*. Analytical and Bioanalytical Chemistry **410**(20), 4867–4873.

Li J, **McQuade T**, **Siemer AB**, **Napetschnig J**, **Moriwaki K**, **Hsiao YS**, **Damko E**, **Moquin D**, **Walz T**, **McDermott A**, **Chan FKM and Wu H** (2012) *The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis*. Cell **150**(2), 339–350.

Liu Y and Bolen DW (1995) *The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes*. Biochemistry **34**(39), 12884–12891.

Luo XD, **Kong FL**, **Dang HB**, **Chen J and Liang Y** (2016) *Macromolecular crowding favors the fibrillization of beta(2)-microglobulin by accelerating the nucleation step and inhibiting fibril disassembly*. Biochimica Et Biophysica Acta-Proteins and Proteomics **1864**(11), 1609–1619.

Martinez J, **Sanchez R**, **Castellanos M**, **Fernandez-Escamilla AM**, **Vazquez-Cortes S**, **Fernandez-Rivas M and Gasset M** (2015) *Fish beta-parvalbumin acquires allergenic properties by amyloid assembly*. Swiss Medical Weekly **145**, 11.

Mikaelsson T, **Aden J**, **Wittung-Stafshede P and Johansson LB** (2014) *Macromolecular crowding effects on two homologs of ribosomal protein s16: protein-dependent structural changes and local interactions*. Biophysics Journal **107**(2), 401–410.

Minton AP (2001) *The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media*. Journal of Biological Chemistry **276**(14), 10577–10580.

Minton AP (2005a) *Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations*. Journal of Pharmaceutical Sciences **94**(8), 1668–1675.

Minton AP (2005b) *Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: macromolecular crowding and protein stability revisited*. Biophysics Journal **88**(2), 971–985.

Mittal S and Singh LR (2014) *Macromolecular crowding decelerates aggregation of a beta-rich protein, bovine carbonic anhydrase: a case study*. Journal of Biochemistry **156**(5), 273–282.

Munishkina LA, **Ahmad A**, **Fink AL and Uversky VN** (2008) *Guiding protein aggregation with macromolecular crowding*. Biochemistry **47**(34), 8993–9006.

Naiki H, **Higuchi K**, **Hosokawa M and Takeda T** (1989) *Fluorometric determination of amyloid fibrils* in vitro *using the fluorescent dye, thioflavin T1*. Analytical Biochemistry **177**(2), 244–249.

Nasreen K, **Parray ZA**, **Ahamad S**, **Ahmad F**, **Ahmed A**, **Alamery SF**, **Hussain T**, **Hassan MI and Islam A** (2020). *Interactions under crowding milieu: chemical-induced denaturation of myoglobin is determined by the extent of heme dissociation on interaction with crowders*. Biomolecules **10**(3), 490.

Otzen D (2010) *Functional amyloid*. Prion **4**(4), 256–264.

Perham M, **Stagg L and Wittung-Stafshede P** (2007) *Macromolecular crowding increases structural content of folded proteins*. FEBS Letters **581**(26), 5065–5069.

Plakoutsi G, **Taddei N**, **Stefani M and Chiti F** (2004) *Aggregation of the acylphosphatase from* Sulfolobus solfataricus*: the folded and partially unfolded states can both be precursors for amyloid formation*. Journal of Biological Chemistry **279**(14), 14111–14119.

Rall JA (1996) *Role of parvalbumin in skeletal muscle relaxation*. News in Physiological Sciences **11**, 249–255.

Ralston GB (1990) *Effects of crowding in protein solutions*. Journal of Chemical Education **67**(10), 857–860.

Schreck JS, **Bridstrup J and Yuan JM** (2020) *Investigating the effects of molecular crowding on the kinetics of protein aggregation*. The Journal of Physical Chemistry B **124**(44), 9829–9839.

Shtilerman MD, **Ding TT and Lansbury Jr PT**. (2002) *Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease?* Biochemistry **41**(12), 3855–3860.

Soldi G, **Bemporad F**, **Torrassa S**, **Relini A**, **Ramazzotti M**, **Taddei N and Chiti F** (2005) *Amyloid formation of a protein in the absence of initial unfolding and destabilization of the native state*. Biophysics Journal **89**(6), 4234–4244.

Stagg L, **Christiansen A and Wittung-Stafshede P** (2011) *Macromolecular crowding tunes folding landscape of parallel alpha/beta protein, apoflavodoxin*. Journal of the American Chemical Society **133**(4), 646–648.

Stagg L, **Zhang SQ**, **Cheung MS and Wittung-Stafshede P** (2007) *Molecular crowding enhances native structure and stability of alpha/beta protein flavodoxin*. Proceedings of the National Academy of Sciences of the United States of America **104**(48), 18976–18981.

Sukenik S, **Politi R**, **Ziserman L**, **Danino D**, **Friedler A and Harries D** (2011) *Crowding alone cannot account for cosolute effect on amyloid aggregation*. PLoS One **6**(1), e15608.

Sukenik S, **Sapir L**, **Gilman-Politi R and Harries D** (2013) *Diversity in the mechanisms of cosolute action on biomolecular processes*. Faraday Discussions **160**, 225–237; discussion 311–227.

Timasheff SN (1993) *The control of protein stability and association by weak interactions with water: how do solvents affect these processes?* Annual Review of Biophysics and Biomolecular Structure **22**, 67–97.

Timasheff SN (2002) *Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components*. Proceedings of the National Academy of Sciences of the United States of America **99**(15), 9721–9726.

Uversky VN, **Cooper EM**, **Bower KS**, **Li J and Fink AL** (2002) *Accelerated alpha-synuclein fibrillation in crowded milieu*. FEBS Letters **515**(1–3), 99–103.

van den Berg B, **Ellis RJ and Dobson CM** (1999) *Effects of macromolecular crowding on protein folding and aggregation*. The EMBO Journal **18**(24), 6927–6933.

van den Berg B, **Wain R**, **Dobson CM and Ellis RJ** (2000) *Macromolecular crowding perturbs protein refolding kinetics: implications for folding inside the cell*. The EMBO Journal **19**(15), 3870–3875.

Wakabayashi K, **Matsumoto K**, **Takayama K**, **Yoshimoto M and Takahashi H** (1997) *NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease*. Neuroscience Letters **239**(1), 45–48.

Wang YJ, **He HW and Li S** (2010) *Effect of Ficoll 70 on thermal stability and structure of creatine kinase*. Biochemistry-Moscow **75**(5), 648–654.

Werner T, **Kumar R**, **Horvath I**, **Scheers N and Wittung-Stafshede P** (2018) *Abundant fish protein inhibits alpha-synuclein amyloid formation*. Scientific Reports **8**(1), 5465.

Werner TER, **Bernson D**, **Esbjorner EK**, **Rocha S and Wittung-Stafshede P** (2020) *Amyloid formation of fish beta-parvalbumin involves primary nucleation triggered by disulfide-bridged protein dimers*. Proceedings of the National Academy of Sciences of the United States of America **117**(45), 27997–28004.

White DA, **Buell AK**, **Knowles TP**, **Welland ME and Dobson CM** (2010) *Protein aggregation in crowded environments*. Journal of the American Chemical Society **132**(14), 5170–5175.

Xue C, **Lin TYW**, **Chang D and Guo ZF** (2017) *Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation*. Royal Society Open Science **4**(1), 12.

Yancey PH (2005) *Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses*. The Journal of Experimental Biology **208**(Pt 15), 2819–2830.

Zhou HX, **Rivas GN and Minton AP** (2008) *Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences*. Annual Review of Biophysics **37**, 375–397.

Zhou Z, **Fan JB**, **Zhu HL**, **Shewmaker F**, **Yan X**, **Chen X**, **Chen J**, **Xiao GF**, **Guo L and Liang Y** (2009) *Crowded cell-like environment accelerates the nucleation step of amyloidogenic protein misfolding*. Journal of Biological Chemistry **284** (44), 30148–30158.