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The role of water in the primary nucleation of protein amyloid aggregation

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Highlights

· Biological water plays multiple roles in amyloid aggregation.

· The self-assembly of IDPs into amyloid structures can be thermodynamically driven by favourable water entropy contributions.

· Large desolvation energy barriers prevent primary nucleation in the typical amyloidogenic IDPs under hydrating conditions.

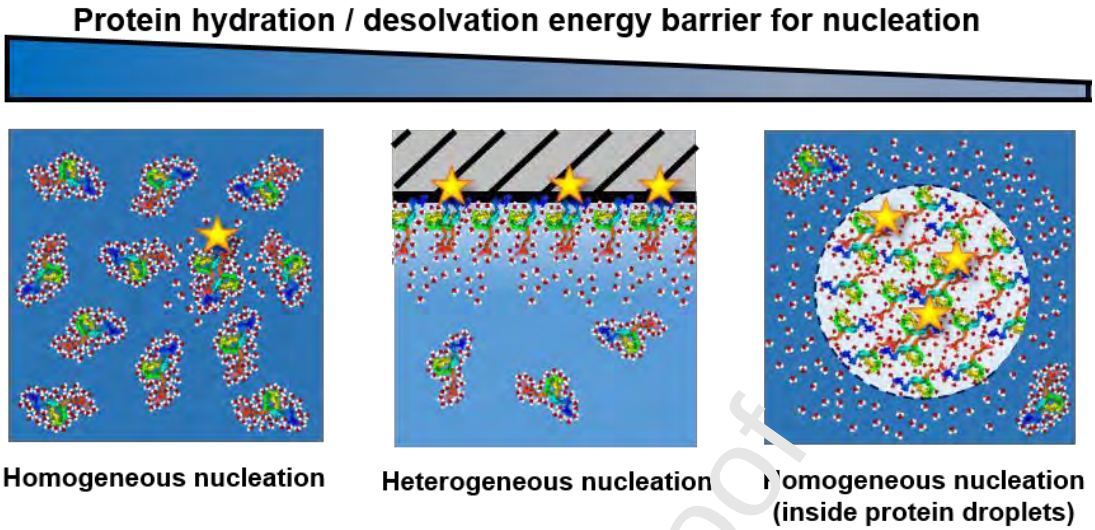
· Water strongly modulates the energy barrier for both heterogeneous and homogeneous amyloid nucleation.

·The extent of water activity of the protein microenvironment seems to be essential for dictating the preference for the type of primary nucleation and the type of structural amyloid polymorphs generated, favouring homogeneous nucleation under limited hydration conditions such as those found in the interior of membrane-less organelles.

Keywords

·Amyloid aggregation ·Primary nucleation ·Water activity ·Intrinsically disordered proteins ·Homogeneous nucleation ·Liquid-liquid phase separation

Graphical Abstract



ABSTRACT

The understanding of the complex conformational landscape of amyloid aggregation and its modulation by relevant physicochemical and cellular factors is a prerequisite for elucidating some of the molecular basis of pathology in amyloid related diseases, and for developing and evaluating effective disease-specific therapeutics to reduce or eliminate the underlying sources of toxicity in these diseases. Interactions of proteins with solvating water have been long considered to be fundamental in mediating their function and folding; however, the relevance of water in the process of protein amyloid aggregation has been largely overlooked. Here, we provide a perspective on the role water plays in triggering primary amyloid nucleation of intrinsically disordered proteins (IDPs) based on recent experimental evidences. The initiation of amyloid aggregation likely results from the synergistic effect between both protein intermolecular interactions and the properties of the water hydration layer of the protein surface. While the self-assembly of both hydrophobic and hydrophilic IDPs would be thermodynamically favoured due to large water entropy contributions, large desolvation energy barriers are expected, particularly for the nucleation of hydrophilic IDPs. Under highly hydrating conditions, primary nucleation is slow, being facilitated by the presence of nucleation-active surfaces (heterogeneous nucleation). Under conditions of poor water activity, such as those found in the interior of protein droplets generated by liquid-liquid phase separation, however, the desolvation energy barrier is significantly reduced, and nucleation can occur very rapidly in the bulk of the solution (homogeneous nucleation), giving rise to structurally distinct amyloid polymorphs. Water, therefore, plays a key role in modulating the transition free energy of amyloid nucleation,

thus governing the initiation of the process, and dictating the type of preferred primary nucleation and the type of amyloid polymorph generated, which could vary depending on the particular microenvironment that the protein molecules encounter in the cell.

Introduction

Many neurodegenerative disorders, including Alzheimer's, Parkinson's and the prion disease, are characterized by a conformational conversion of normally soluble, intrinsically disordered proteins (IDPs) or peptides into pathological aggregated species, by a process of self-assembly that leads ultimately to the formation of amyloid fibrils. These amyloid aggregates are primarily composed of one type of protein or peptide that adopts a characteristic and distinctive tertiary structure, named cross-β structure [1–6]. In this particular structure, typically described for long fibrillar aggregates, individual β-strands, allocated perpendicularly to the fibril axis, forms an array of β-sheets that run the length of the fibril [3,5,6]. The high stability of this protein conformation [7], as well as the high structural polymorphism found for the amyloid structure [8,9], arises primarily from the strength, and permutability, of the intermolecular hydrogen bond networks generated between groups of the main chain of the polypeptide chains. As a consequence, the amyloid cross-β structure is a generic feature of the polypeptide chain itself [10,11], and it can be, therefore, adopted by any peptide and protein regardless of their amino acid sequence or native fold under the appropriate environmental conditions. The predisposition of a given protein or peptide to form amyloid aggregates, however, is modulated by its primary sequence, and more specifically by its amino acid composition [12].

Amyloid aggregation depends on both intrinsic and extrinsic factors. Some intrinsic factors are the number, type and distribution of charge/polar and apolar residues along the polypeptide sequence and the propensity to adopt secondary structure elements [13–15]. IDPs generally lack or present a very low content of aromatic hydrophobic residues, which, together with the typical asymmetrical accumulation of positively and negatively charged residues in different regions of the protein, prevents their folding in the absence of other cellular components. In addition, they have a high net charge at neutral pH, typically negative, which maintains the monomeric conformation highly soluble and hydrated [16,17]. However, some IDPs show certain regions with an inherent propensity to aggregate into amyloid fibrils, which indeed can be predicted from some of the intrinsic factors listed above [14,15]. The initiation of the amyloid aggregation reaction also depends on the physicochemical properties of the environmental conditions in

which the polypeptide chains are located, such as protein concentration, pH, ionic strength, temperature, presence of nucleation-active surfaces and hydration degree.

Proteins have evolved in aqueous solutions for millions of years and water is ubiquitously indispensable for life on Earth. Indeed, water activity, the effective water content expressed as its mole fraction, in the interior of cells play a pivotal role in the physiology of living organisms, affecting both the thermodynamics and the kinetics of chemical and biochemical reactions. The solvating water molecules that surround the protein surface (the hydration shell) are fundamental in mediating protein folding, structure and function. The hydration shell or hydration layer of proteins typically extends to around 5-10 Å from the protein surface and the water molecules in this region (sometimes referred to as 'biological' water), because of their interactions with the protein surface, show different properties compared to 'bulk' water [18,19]. The density of water on the surface of proteins has been reported to be significantly higher than that of bulk water [20]. Consequently, the hydration shell water has slower reorientation times and, therefore, slower dynamics and can be highly structured, with some water molecules being so strongly bound to the protein surface that they can crystallize together during protein crystallization and are, thus, occasionally described as being part of the protein structure [19]. Protein function is also linked to its mobility, which, in turn, is coupled to water motion. While the bulk solvent fluctuations control the shape and global motions of proteins, the fluctuations of the dynamics of the hydration shell modulate the protein internal motions, which determine protein function [21–26]. In addition to the structure and function of proteins, water plays a major role in protein folding, ensuring that hydrophobic residues are predominantly sequestered in the protein interior and the water molecules are squeezed out from the hydrophobic core, resulting in an essentially dry, packed protein core [27–30]. Similarly, the folding accompanies a large decrease in the water-accessible surface area with the result that many of the water molecules forming the dense layer of the unfolded state are released to the bulk [31,32]. In addition, water participates in the stabilization of the folded structure through hydrogen bond networks with the protein and the screening of electrostatic interactions, and in some proteins it contributes also to the stabilization of hydrophobic cavities essential for the protein function [33].

In contrast, the effects of water on protein aggregation have been largely overlooked as many interpretations of experimental and computational studies have been carried out from a protein centric perspective. However, the need to include the crucial importance of water in order to understand the biophysical basis of amyloid formation is increasingly appreciated [26,34,35]. Water plays likely multifarious roles at different stages of protein amyloid aggregation, for

example during protofilament assembly, as water molecules from the hydration layer need to be released from the interaction surface of protofilaments in order to generate a dry interface [36,37]. A similar effect of water released together with a reduction of the monomer reconfiguration rate once water has been expelled that permits the locking of the monomer to the fibrillar seed, has also been described during the process of fibril elongation [38]. In this perspective we will discuss some of the fundamental effects of water on the first steps of amyloid self-assembly and the role that water activity plays in triggering the amyloid process and governing the type of preferred nucleation mechanism and amyloid pathway. The conclusions presented can help rationalise some aspects of primary nucleation of amyloid aggregation in the context of proteins embedded in aqueous solutions which can experience differences in water activity (i.e. effective water concentration) depending on the microenvironment that they encounter in the cell.

Protein amyloid formation and primary nucleation

Historically, the functional native state of a protein, be it a protein with a particular fold or an IDP conformation, was thought of as the most stable conformation they could adopt. Nowadays, the functional native state of proteins is considered to correspond to a local minimum of the protein conformational landscape, isolated from the more thermodynamically favoured cross-β sheet conformation by energetic barriers [39,40]. Indeed, the cross-β sheet conformation of the peptide backbone has been theoretically shown to be the thermodynamically most stable structure for all possible polypeptides both in vacuum and in aqueous environments by first principle calculations [41] and it has been demonstrated experimentally for a number of proteins and peptides [40].

The process whereby a protein transitions from its native state to the amyloid state implies a highly complex molecular rearrangement of the protein upon self-assembly. The formation of the first amyloid nuclei upon monomer assembly, termed primary nucleation [42–44], typically requires to overcome an extremely high energy barrier [45,46]. For the vast majority of amyloid-prone proteins under hydrated conditions, therefore, amyloid formation kinetics are characterised by a slow, rate-limiting nucleation step [47,48]. Once the first amyloid-competent nuclei are formed, aggregate elongation through monomer addition [47] and/or secondary nucleation at the surface of pre-existing fibrils [49,50] dominate the reaction [44], resulting in the characteristic sigmoidal kinetic traces with a long lag-phase followed by an

exponential growth phase that lasts until the equilibrium between aggregates and monomers is reached.

Primary nucleation can occur either in the bulk of the solution, as homogeneous nucleation, or at a particular interface able to catalyse the otherwise energetically disfavoured monomer-monomer interaction, as heterogeneous nucleation [51]. Hydrophobic/hydrophilic interfaces have been found to be critical for the aggregation of many amyloidogenic proteins and peptides at highly hydration conditions, including IDPs such as α-synuclein [52,53], involved in Parkinson´s disease, or Aβ peptide [51], involved in Alzheimer´s disease, as well as folded proteins such as insulin [54], involved in insulin-derived amyloidosis. As a matter of fact, currently established *in vitro* assays for studying the aggregation of these proteins necessarily involve a nucleation-inducing interface [55–58] and, therefore, provide insights into the heterogeneous nucleation mechanisms. The amphipathic nature of the sequences of these proteins and peptides, either when unfolded or in helical structural elements, prompt their preferential adsorption at hydrophobic/hydrophilic interfaces in order to simultaneously maximize the hydrophilic interactions in the aqueous environment and the hydrophobic force at the hydrophobic surface [59], and there, they initiate their self-assembly [51,53,57,60,61]. The initiation of primary heterogeneous nucleation, therefore, depends on the relative affinities of the different protein species (monomeric vs oligomeric) for the interface and the degree to which the interfacially-associated monomer lowers the free energy barrier for nucleation. As it has been suggested, this decrease in the nucleation energy barrier is likely associated in part to an increase in local protein concentration and a selection of suitable orientations and nucleation-prone conformations of the polypeptide chains at the interface [57]. In addition, the different behaviour of the water molecules at the interface, i.e. in the vicinity of the hydrophobic surface, with respect to the bulk might play an important role in decreasing the solvation free energy barrier for nucleation at the interface [58]. Under highly hydrating conditions, at physiological pH, temperature and ionic strength, Aβ peptide and α-synuclein heterogeneous primary nucleation has been shown to dominate over homogeneous nucleation [51,53,58]. Indeed, in the absence of hydrophobic solid surfaces, such as those from the sample containers or stirring bars, the air/water interface typically acts as amyloid nucleating interface in protein solutions *in vitro* [51,53,57,58]. Other surface-active materials such as polytetrauoroethylene (PTFE) beads or synthetic lipid vesicles of particular lipid compositions have also been used to accelerate primary nucleation in these systems [51,62–64].

Recently, it has been reported that certain amyloidogenic IDPs, such as tau [65,66], TDP-43 [67,68] and α-synuclein [58,69,70], are able to phase-separate from the aqueous solution generating protein droplets by a process of liquid-liquid phase separation (LLPS) both *in vitro* and *in vivo*, and that in such environment amyloid aggregation is a particularly favourable process. The aggregation into amyloid aggregates inside these phase separated protein droplets, also referred to as liquid-to-solid transition, has been suggested to play a role in the *in vivo* aggregation of amyloidogenic IDPs associated to neurodegenerative diseases [71].

The distinct hydration properties of IDPs

The influence of the hydration shell of proteins, particularly that of the IDPs, has been proposed to have a key role in protein aggregation and amyloid formation [35]. The properties of the hydration water molecules of IDPs have been shown to be remarkably different with respect to globular, folded proteins, with much faster dynamics, which in turn is reflected by faster protein dynamics [24,25]. As a consequence, small variations in the solution conditions strongly affect the structure and dynamics of the hydration shell and, therefore, the conformational ensemble of the IDPs, a property that has been suggested to be important for their functional plasticity [72].

In terms of protein self-assembly in IDPs, the consequence of having a highly dynamic conformational reconfiguration would in principle play against aggregation, as it is more difficult that various protein molecules in aggregation-competent conformations associate together for the required time to establish persistent interactions before any of the protein molecules reconfigures to a non-aggregation conformation. If the mobility of water is, however, decreased by the presence of ions [73], osmolytes [74,75], membranes or by cellular crowding or a cellular environment with poor water activity [76], IDP self-association would be, therefore, promoted. Accordingly, when the mobility of human prostatic acidic phosphatase (PAP) fragment PAPf39 and prion protein was reduced by temperature or protein sequence modification, respectively, the aggregation rates of both proteins increased [77,78]. A similar reduction of the monomeric reconfiguration rate was observed for α-synuclein when decreasing the pH of the solution [79]. In this study, the rate of intramolecular diffusion in monomeric α-synuclein was determined by tryptophan-cysteine quenching when the protein is freely diffusing in the bulk of the solution under solvent conditions that have been reported to accelerate or decelerate aggregation, and a correlation between the two parameters was found. The authors suggested that the relative rates between backbone reconfiguration of the

monomeric protein and bimolecular association is what dictates the probability and, thus, the rate of the initial amyloid self-assembly. However, this study on the dependence of α-synuclein monomer reconfiguration rate on its self-assembly rate does not take into account that the nucleation of the protein under the conditions used to correlate the propensity for amyloid formation occurs by heterogeneous nucleation at the air/water interface, where the protein backbone reconfiguration rates are likely to differ significantly from those the authors measured in the bulk of the solution. It would be interesting to correlate the conformational reconfiguration rates of the protein when adsorbed at hydrophobic/hydrophilic interfaces with protein aggregation rates, as the monomeric reconfiguration rate, strongly modulated by the protein hydration conditions, likely contributes to the rate of initial protein self-association.

In addition to having an intrinsic highly dynamic hydration shell, the monomeric conformation of amyloidogenic IDPs have been shown to possess a dramatically tuneable energy landscape depending on the particular properties of the solution conditions and thus the properties of the hydration shell. Indeed, some studies have suggested that certain monomeric conformations or intramolecular interactions of particular amyloidogenic IDPs are required for their self-assembly, and that those are favoured under limited hydration conditions. For example, Thirumalai and co-workers, using MD simulations, proposed that a particular intramolecular salt bridge in the Aβ peptide (both $A\beta_{1-40}$ and $A\beta_{1-42}$), essential for its initial self-assembly, requires the expulsion of discrete and tightly bound water molecules that are solvating the residues involved in this interaction [80]. The consequence is that the formation of this key salt bridge in the monomeric protein at highly hydration conditions is highly improbable. In contrast, conditions that favour protein desolvation would result in the stabilization of conformations with the intramolecular bridge formed that would favour oligomer nucleation [80]. Similarly, also using MD simulations, Balupuri *et al*. suggested that the properties of the water network of the hydration shell in the α-synuclein monomeric ensemble are important for the occurrence of an α-strand structure between residues 72-74, which the authors proposed to be relevant for the initial self-assembly of the protein [81]. An earlier experimental study on α-synuclein reported relevant structural changes in the monomeric structural ensemble under conditions of partial desolvation, which in turn promoted its aggregation [82]. Similar structural changes were observed for different desolvation conditions such as the addition of trifluoroethanol or the increase of temperature. Specifically, the authors reported a loss of polyproline-II and a gain in helical secondary structure, as a result of weakened water-protein interactions, and suggested that similar

structural changes might occur in other aggregation-prone IDPs [82].

The role of water in triggering amyloid self-assembly

Both experimental and computational studies indicate that amyloid formation, protofilament assembly and fibril growth can be thermodynamically driven by a favourable change in entropy associated with the release of confined water molecules from the protein surface to the bulk [37,83–86]. However, only a small number of studies have addressed the effects of water on amyloid primary nucleation [34,35,87]. Most of these studies have been performed by computer simulations, given the experimental difficulties in directly monitoring water activity during protein self-assembly. An interesting study of Aβ peptide ($A\beta_{1–42}$) used a combination of MD simulations with fluctuating thermodynamics analysis to investigate the dimerization of the peptide and found that the interaction of the protein with surrounding water, i.e. the solvation free energy, plays a critical role in its aggregation [88]. They observed that Aβ monomer release from a membrane environment, where it is initially located, to an aqueous phase after being processed is driven by favourable changes in protein potential energy and configurational entropy, but it is also accompanied by an unfavourable increase in solvation free energy, which the authors suggested is the driving force for dimerization in the bulk of the solution. According to the fluctuating thermodynamic analysis of $A\beta_{1–42}$ and that of other amyloidogenic proteins with varying propensities to aggregate, the authors suggested that proteins with higher solvation free energies are more prone to aggregate.

Few experimental studies have implicated dehydration in protein aggregation processes. One of the earliest studies used infrared band-shift to probe backbone hydration of bovine pancreatic ribonuclease A. The authors found a correlation between the status of backbone dehydration, modulated by the addition of ethanol, and protein aggregation [89]. Another example was reported by Mukhopadhyay´s group [90]. Using the bovine κ-casein model as amyloidogenic protein, the authors showed that the monomeric state of this protein represents a collapsed IDP globule that contains highly ordered water molecules with profoundly restrained dynamics in the interior of the globule, which would need to be released for β-sheet formation. In a following publication from the same group and using the same protein model, the authors used time-resolved fluorescence spectroscopy to monitor the water dynamics of the amyloid state as compared to the monomeric conformation, and found that there is a significant desolvation of the protein molecules upon amyloid formation [86]. Their results, therefore, strongly indicate that, at least for this system, amyloid formation can be driven by a

positive change in water entropy. Interestingly, other amyloidogenic IDPs such as Sup35, polyQ, α-synuclein and tau have been shown to adopt a collapsed globule conformation under amyloidogenic conditions [91–94]. Interestingly, the opposite behavior has also been observed for tau, where extended conformations of the protein were found responsible for amyloid aggregation [95,96].

A seminal experimental study demonstrating the role of protein dehydration on the aggregation kinetics was reported by Gai and collaborators [97]. They studied the dependence of the aggregation kinetics of two amyloid peptides, $A\beta_{16\text{-}22}$ and $Sup35_{7\text{-}13}$, on their hydration degree, which was modulated by the use of reverse micelles. The authors varied the number of water molecules inside the micellar core, which in turn varied the physicochemical properties of the entrapped water, and found that protein dehydration promotes aggregation by reducing the water shell protection of the monomeric peptides. In a different study Arya et al., [98] studied the dynamical behaviour of the hydration water molecules along the α-synuclein sequence, and observed differences in the properties of the interfacial water molecules in the central, amyloidogenic region of the protein (the NAC domain) with respect to the N-terminal and C-terminal regions. Concretely, they reported the presence of a relevant number of quasi-bound water molecules within the amyloidogenic region, with significantly restrained dynamics, and, consequently, much longer residence times, as a consequence of the highly hydrophobic character of this protein segment [99]. The fact that the same protein region that presented these trapped or confined water molecules coincides with the region proposed to initiate amyloid self-assembly [100] is in agreement with the idea of the presence of particularly high desolvation free energy barriers for α-synuclein initial self-assembly. In order to reduce the desolvation barrier, Anderson *et al.* [82] added co-solvents to α-synuclein solution, particularly trifluoroethanol, and found that aggregation was particularly enhanced under conditions where protective protein-solvent interactions are minimized. Consequently, both intramolecular and intermolecular protein interactions are favoured. More recently, Bokor et al. investigated the role the hydration shell plays in the structures of monomeric, oligomeric and fibrillar α-synuclein using a novel approach that evaluates wide-line ^{1}H NMR spectroscopy results through a thermodynamic framework [101]. Their results indicated that half of the mobile solvation water fraction of monomeric α-synuclein is lost upon oligomer or fibril formation. Similar conclusions showing expulsion of confined water molecules from the protein surface upon amyloid formation were also obtained for tau protein [84,85], the prion protein [102] and insulin [103].

Collectively, these studies show the relevance of water on the initiation of amyloid aggregation in different amyloidogenic systems. One of the reason why IDPs seem to be more prone to amyloid aggregation might be, thus, related to their larger solvent-accessible surface area as compared to globular, folded proteins. This, together with the presence of restrained water molecules around the solvent-exposed hydrophobic amyloidogenic protein segments and highly confined water molecules in the interior of the globule conformations of the typical amyloidogenic IDPs adopted under aggregation-prone conditions, would lead to a large contribution of the solvation entropy to the overall free energy of amyloid aggregation. Other factors, such as additional unfolding free energy barriers for nucleation in the case of globular proteins, also differentiates amyloid aggregation between globular proteins and IDPs [104]. The higher number of interactions between the polypeptide chains and the solvation water molecules in the IDPs, due to their overall higher proportion of polar and charged residues, however, also indicates a large desolvation energy barrier that would need to be overcome for the initial protein self-assembly. MD simulations showed that protofilament formation is 1,000 times slower for a highly hydrophilic polypeptide than for a highly hydrophobic one [37]. From these studies, therefore, it is evident that water has at least a dual effect on primary amyloid nucleation. On the one side, the initiation of self-assembly of an amyloidogenic protein or peptide is thermodynamically favoured in aqueous solutions due to a large solvation entropy contribution, so that the more solvated the monomeric conformation, the more thermodynamically favourable its self-assembly. On the other side, the rate of initial self-assembly, associated with the energy barrier that the protein molecules need to overcome for nucleation, might be very slow for highly solvated IDPs, as indicated by a number of experimental observations [57,58,97]. This is also in line with the conclusions obtained from the studies on the backbone reconfiguration rates of the monomeric forms of some amyloidogenic IDPs and their propensity to aggregate [51,78,79]. A reduction in the water activity of the protein microenvironment would result in a reduction of the free energy barrier for nucleation and thus an acceleration of the primary nucleation, and, consequently an acceleration of the overall amyloid aggregation process.

Reconciling ideas for the role of water in the heterogeneous primary nucleation

As introduced before, in most of the computational and modelling studies of initial intermolecular protein self-assembly of Aβ peptide or α-synuclein, two of the most important disease-associated amyloidogenic systems, the nucleation is typically assumed to occur in the

bulk of the solution (homogeneous nucleation), while the experimental information of protein aggregation used to correlate the theoretical approaches corresponds in most cases to aggregation processes that have been initiated at the surface of an active interface (heterogeneous nucleation), typically at the air/water interface. While these studies provide relevant insights into the homogeneous nucleation of the proteins/peptides, the analysis of heterogeneous nucleation requires the incorporation of the interaction of the proteins with the nucleation-active interfaces and the modulation of the conformational ensemble and dynamics of the monomeric protein when adsorbed to the interface.

We have recently studied the initiation of α-synuclein amyloid formation in the presence of the air/water interface, but in the absence of other nucleation-active interfaces, at quiescent conditions (without sample agitation) and found that, in agreement with previous studies [57,61], under high hydration conditions the protein is unable to form a significant number of aggregate nuclei either by homogeneous or heterogeneous nucleation. Consequently, no apparent amyloid aggregation was observed for more than 7-10 days of incubation, despite the interface being fully covered by the protein. In contrast, when the same experiments were performed with the addition of co-solvents to induce mild protein dehydration, we observed induction of aggregation within the first 1-2 days of incubation, independently of the type of co-solvent used to promote the thinning of the protein hydration shell [58]. These results complement those previously performed by the groups of Uversky [105] and Eliezer and Webb [82,106], which already suggested an important role of protein desolvation in triggering α-synuclein aggregation under conditions at which we now know nucleation occurs at the hydrophobic/hydrophilic interfaces, i.e. by heterogeneous nucleation. The heterogeneous primary nucleation of α-synuclein at the air/water interface at quiescent conditions requires, therefore, a reduction of the water activity of the protein solution, as compared to the highly diluted typical *in vitro* conditions, in order to form a significant number of nuclei at the interface to trigger the macroscopic reaction. The ideas and concepts extracted from the computational and modelling studies of amyloid self-assembly assuming homogeneous primary nucleation seems, therefore, to hold also for heterogeneous primary nucleation. In both cases, a reduction of the desolvation free energy barrier favours amyloid nucleation.

A significant reduction in water activity of the solution favours homogeneous nucleation

We have recently observed that α-synuclein can form amyloid aggregates without the need of a nucleation-active surface through homogeneous nucleation under limited hydration

conditions, such as those induced by the addition of certain co-solvents known to thinning the protein hydration shell, or those found in the interior of α-synuclein droplets by LLPS [58]. Interestingly, we have observed that when the protein undergoes this process, there is a preference for a remarkably different amyloid polymorph, with an antiparallel intermolecular β-sheet arrangement, in contrast to the parallel β-sheet architecture adopted when heterogeneous nucleation dominates. Indeed, we have proposed that the origin for the preference of the parallel or the antiparallel β-sheet structure in the α-synuclein amyloid aggregates is likely related to the type of primary nucleation favoured under the particular solution conditions. When α-synuclein aggregation is triggered by heterogeneous nucleation, the pre-nucleus of amyloid structure formed at a given hydrophobic/hydrophilic interface would inevitably adopt a parallel intermolecular β-sheet arrangement given the restrictions in the disposition and orientation of the polypeptide chains anchored through their N-terminal amphipathic region to the interface. When the aggregation is triggered by homogeneous nucleation, however, there is no restriction in the orientation of the protein molecules in the bulk, and the antiparallel orientation of the β-sheets would be preferred over the parallel arrangement, as the stability of the hydrogen bonds in such configuration is generally higher [107,108].

The formation of amyloid aggregates rich in intermolecular antiparallel β-sheets under limited hydration conditions has been also reported for other amyloidogenic peptides. Mukherjee *et al.* studied the aggregation of a series of amyloidogenic peptides, including A$\beta_{16\text{-}22}$ and Sup35$_{7\text{-}13}$, and found that when polypeptide aggregation was significantly accelerated under limited hydration conditions, the aggregates formed showed a preference for antiparallel *β*-sheet conformations [97,109]. This amyloid *β*-sheet configuration has also been found for hydrophobic peptides, such as the A$\beta_{16\text{-}22}$ or A$\beta_{24\text{-}36}$ peptide, when nucleating in the bulk under hydrated conditions [110], although for longer peptides both parallel [111–114] and antiparallel [74,115,116] configurations have been observed upon self-assembly by MD simulations.

The intermolecular antiparallel β-sheet structure has been previously proposed to be distinctive of stable, particularly toxic oligomers of α-synuclein and other amyloidogenic systems [117–120]. Due to the stability of such oligomers, as result of their slow elongation and disaggregation rates, and the differences in the β-sheet arrangement of their structure as compared to the typical amyloid fibrils generated by heterogeneous nucleation, these oligomers have been proposed to be off-pathway by some researchers. However, in the light

of our recent findings, at least some of these oligomers are best described as on-pathway species of an amyloid aggregation pathway triggered by homogeneous nucleation under limited hydration conditions. Indeed, a significant number of protocols to generate the stable antiparallel β-sheet oligomers reported to date include a lyophilisation step (or the peptide/protein stock is lyophilised) that is critical for their formation.

Interestingly, a multitude of a priori non-amyloidogenic proteins belonging to different structural classes, including ordered proteins, such as lactate dehydrogenase, phosphofructokinase, γ-interferon, bovine pancreatic trypsin inhibitor or chymotrypsin, and disordered peptides including poly(L-lysine) have been also reported to aggregate acquiring an antiparallel β-sheet structure during the lyophilisation process [121–123]. The degree of aggregation of the folded proteins under lyophilisation has been related to the degree to which the protein unfolds under dehydration, as addition of protein stabilizers that preserves the native structure during dehydration minimises aggregation [121,124]. Similarly, hydrophobic surfaces provide denaturing conditions for globular, folded proteins able to interact with such surfaces through solvent-exposed hydrophobic regions. Furthermore, at the same time, a local reduction of the water activity at the interface with the hydrophobic surface would facilitate the formation of intermolecular hydrogen bonds and, thus, surface-induced aggregation. This phenomenon has been observed for a number of folded proteins such as β-lactoglobulin, which when adsorbed to hydrophilic/hydrophobic interfaces suffers self-aggregation with the formation of intermolecular antiparallel β-sheet structure [125]. Other examples of aggregation with a preference for antiparallel β-sheet structure upon adsorption on hydrogels without apparent preferential protein orientations (which otherwise would favour the formation of intermolecular parallel β-sheet structure) have been reported for lysozyme, mucin and γ-globulin by Castillo *et al.* [126–128]. The authors also reported protein denaturation as a preliminary step for surface-induced aggregation, which at the same time was favoured in the presence of more hydrophobic hydrogels [126].

Conditions of limited protein hydration occurring at high temperatures have also been reported to induce the aggregation of a large number of proteins regardless of their native structural topology [129], including bovine serum albumin [130], azurin [131], β-lactoglobulin [132], cholera toxin [133], adenylate cyclase [134], cytochrome c [135], chymotrypsinogen [136], acetylcholinesterase [137] and ribonuclease A [138]. A common feature in all these studies is the formation of aggregates with an intermolecular hydrogen-

bonded antiparallel β-sheet structure, represented by the low-frequency band around 1620 cm^{-1} and associated weaker high-frequency band around 1685 cm^{-1} in the amide I region of the infrared spectra [129].

All these studies together, therefore, suggest that the formation of antiparallel β-sheet amyloid aggregates might be a general process of the polypeptide chains that is triggered under limited hydration conditions by a mechanism of homogeneous primary nucleation similar to that we have described recently for α-synuclein [58].

Amyloid aggregation inside protein droplets generated by LLPS

Liquid-liquid phase separation (LLPS), also referred to as demixing, has been long observed within certain aqueous mixtures of two or more components such as synthetic polymers, proteins, salts, nucleic acids or polysaccharides. More recently, growing experimental evidence indicates that this process can also occur inside cells giving rise to what has been referred to as membrane-less organelles. These are formed mostly by proteins, either specific multivalent modular folded proteins or particular types of IDPs, and, in some cases, also by RNA/DNA molecules [139–141]. The formation of these phase-separated cellular compartments, as well as the content and relative proportion of these condensates, is tightly regulated by the cell [141], in agreement with the relevant role these membrane-less organelles have in the context of important cellular functions such as biochemical catalysis, RNA transcription, cell cycle and autophagy control among others [142]. Consequently, a change in the phase-separating behaviour of the molecular components or a failure in the regulation of the formation/dissolution of these protein droplets can bring about pathological effects. One of such undesired consequences is the liquid-to-solid transition of the protein droplets with the formation of amyloid aggregates [141].

The driving force for LLPS in aqueous solutions has been proposed to be the relative strength of the interactions between macromolecules and between the macromolecules and water according to the Flory-Huggins theory [143,144]. However, the ordering of hydration water molecules has been proposed to be also key to phase separation [143,144]. Experimental observations of *in vitro* polymer-driven LLPS suggest that alteration of water properties, induced by either the polymers and salts of the solutions or factors such as temperature or pressure, modulates phase separation and it has been proposed that such alterations induced by IDPs play an important role in intracellular phase separation [145,146]. For example, salts have been shown to affect protein-driven LLPS, even in

systems in which electrostatics have been shown to represent a minor factor, with a relevant role of salt-mediated changes in hydration energies [145]. Also, hydrostatic pressure, which alters water structure, has been shown to modulate *in vitro* LLPS of lysozyme [147,148]. Another example is the role of water in the temperature-induced phase separation of a number of hydrophobic polymers [145], although a general effect of water in temperature-dependent phase separation processes of both hydrophilic and hydrophobic IDPs is expected [149,150]. LLPS, therefore, might be triggered by a balance of enthalpically favourable intermolecular interactions and the increase of solvent entropy by the release of water molecules from the solute surface to the bulk [151].

In the interior of the protein droplets, where protein-protein interactions are preferred over protein-water interactions and water has been largely expelled, a significant local concentration of the protein and consequently a significant reduction in water content is expected. For example, estimations of the protein concentration and water content of an elastin-like polypeptide droplet with respect to the typical diluted conditions indicated that inside the droplet the concentration of the protein increased ca. 100 times and the water content decreased to ca. 60% [152]. In such conditions, and according to our recently reported results [58], protein self-assembly with the formation of antiparallel β-sheet amyloid aggregates by a homogeneous primary nucleation could be favoured. Accordingly, we have observed such mechanism to occur inside α-synuclein droplets generated *in vitro* by LLPS. Under highly hydrated conditions, the desolvation free energy barrier for α-synuclein self-assembly in the absence of any hydrophobic surface that could accelerate nucleation (see Figure 1A-B) is remarkably high, and thus kinetically disfavoured (Figure 1C). Under conditions of protein phase separation, however, a significant number of water molecules of the protein hydration shell have been already expelled to the bulk of the solution in order to generate the protein droplets, and, consequently, amyloid aggregation is greatly enhanced by reducing the initial self-assembly energy barrier (see Figure 1D). While the local concentration of the protein in the interior of the protein droplets certainly favours aggregation, we have observed that the sole exposition of low micromolar concentrations of α-synuclein to limited hydration conditions is enough to dramatically accelerate amyloid homogeneous nucleation [58]. Recent work on the liquid-to-solid transition of tau after LLPS shows that the type of interaction promoting LLPS might be responsible for dictating whether the system proceeds to maturation and amyloid aggregation [153]. In particular, hydrophobic interactions and, indeed, interfacial dehydration, have been proposed to be

required for amyloid formation in this system.

Given the intrinsic ability of certain amyloidogenic IDPs to phase separate from the cytoplasm/nucleoplasm, and the general process of amyloid aggregation of the polypeptide chains by homogeneous nucleation under limited hydration conditions that we have described above, we propose that amyloid homogeneous nucleation might be a relevant *in vivo* amyloid pathway for α-synuclein and other amyloidogenic IDPs.

Conclusions

By understanding the mechanisms and factors that trigger self-association in amyloidogenic IDPs we may better interpret how amyloid aggregation initiates *in vivo* and how this process yields different amyloid polymorphs that could be related to distinct neurodegenerative disorders. The increasing number of recent studies on the nature of water structure and dynamics around IDPs and its influence on their interactions and ability to suffer LLPS and self-assembly strengthen the notion that water plays a central role in protein amyloid aggregation and in particular in its primary nucleation step. The initiation of amyloid aggregation likely results from a synergistic effect between both intermolecular interactions and the properties of the water hydration layer of the protein surface. While the self-assembly into amyloid structures of the typical hydrophilic IDPs would be thermodynamically favoured, due to a large water entropy contribution, a large desolvation energy barrier, however, prevents their nucleation under hydration conditions. This scenario seems to be the case for both heterogeneous and homogeneous primary nucleation. Indeed, the extent of water activity on the protein microenvironment seems to be essential not only for regulating the energy barrier of primary nucleation (either heterogeneous or homogeneous), but also for dictating the preference for the type of primary nucleation and the type of structural amyloid polymorphs generated. Under highly hydration conditions, the large desolvation free energy for the initial peptide assembly, largely due to the high content in polar and charged residues of the typical amyloidogenic IDPs, would maintain the peptides monomeric. But at lower hydration conditions, the reduction in the free energy barrier would trigger aggregation either by heterogeneous or homogeneous primary nucleation depending on the presence of hydrophobic surfaces and the extent of water content of the protein microenvironment. Interestingly, water may also have a crucial role in the formation of protein droplets by LLPS, and in this microenvironment of particularly low water activity amyloid aggregation is kinetically and thermodynamically highly favourable.

Under such conditions, therefore, the protein can initiate its self-assembly into amyloid aggregates by homogeneous primary nucleation.

The interior of a cell is a highly crowded environment with an overall restriction in the water accessibility and dynamics as compared to the highly diluted protein solutions of typical *in vitro* experiments. The majority of water inside cells has been proposed to be involved in slow (at least an order of magnitude slower than bulk water), collective motions, with only trace amounts of "bulk-like" water, despite 50-70% water content by volume [154]. Also, different cellular compartments and microenvironments have very different physicochemical properties and water activities, which likely has a direct impact on the structural conformation of IDPs [155] and on the magnitude of their energy barrier for amyloid nucleation. Water strongly modulates the energy barrier for nucleation, preventing nucleation at highly hydration conditions, favouring heterogeneous nucleation in the presence of nucleation-active surfaces at diluted conditions, or favouring homogeneous nucleation under conditions of limited hydration conditions such as those found in the interior of membrane-less organelles. Establishing whether homogeneous or heterogeneous primary nucleation occurs *in vivo* and their involvement in the formation of amyloid aggregates in disease requires further investigation. However, at this point, it is reasonable to speculate that the presence of multiple cellular microenvironments, with a range of water contents and dynamics, might lead to the formation of amyloid aggregates by both nucleation mechanisms. And that, in consequence, structurally different amyloid polymorphs could be formed depending on the cellular context that the protein encounters. Interestingly, a decrease in the water content in brain cells, likely as a consequence of an increased total intracellular protein concentration with advancing age has been reported [156–158], which may also contribute to the increased incidence of amyloid formation and, therefore, neurodenegerative diseases in the aged population.

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Author contributions

Nunilo Cremades: Conceptualization, Funding acquisition, Investigation, Supervision and Writing - original draft, review and editing. **José D. Camino** and **Pablo Gracia**: Investigation and Writing – original draft, review and editing.

Conflict of interest

The authors declare no conflicts of interest.

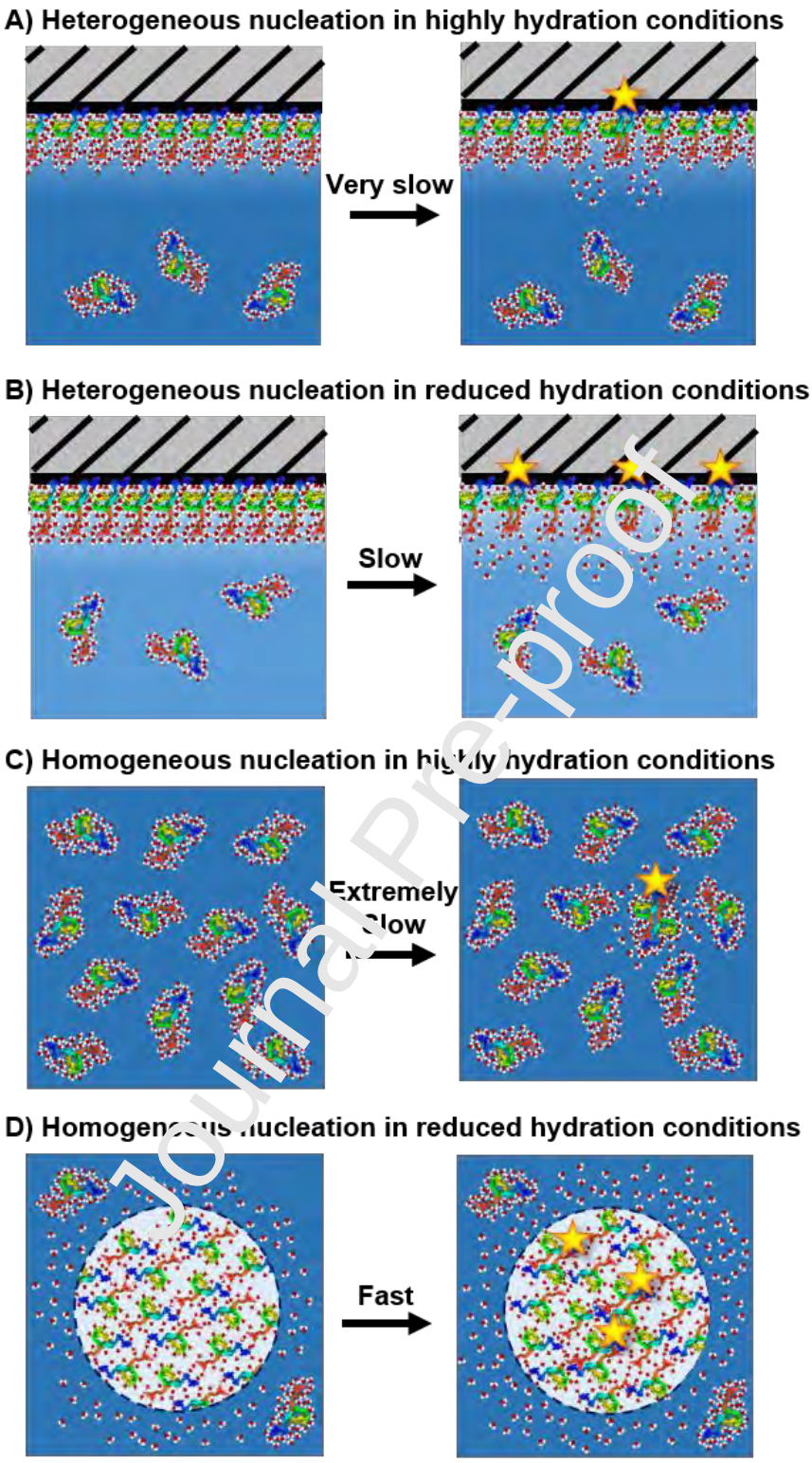

Figure 1. Scheme for the possible role of water in heterogeneous and homogeneous amyloid nucleation. The process of amyloid aggregation in IDPs, and particularly its primary nucleation step, can be thermodynamically favoured, due to a large water entropy contribution. The process, however, is kinetically disfavoured under highly hydration

conditions because of the large desolvation free energy barrier of the monomeric state of the typically highly polar, amyloidogenic IDPs. Hydrophobic surfaces can catalyse amyloid nucleation in aqueous solutions (heterogeneous nucleation), although it is typically a rather slow process under highly hydration conditions (panel A; highly hydration protein environment is depicted in dark blue), as for example described in α-synuclein, but can be accelerated under conditions of reduced protein hydration (panel B; protein environment with a reduced water activity is depicted in light blue). In the absence of nucleation-active surfaces, homogeneous amyloid nucleation is an extremely slow process under highly hydration conditions (panel C), but it is dramatically accelerated under conditions of limited hydration, such as those found in the interior of protein droplets generated by LLPS (panel D; the interior of the protein droplet, with a significantly reduced water activity, is depicted in very light blue).

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