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REVIEW

Lessons learned from protein aggregation: toward technological and biomedical applications

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Abstract The close relationship between protein aggregation and neurodegenerative diseases has been the driving force behind the renewed interest in a field where biophysics, neurobiology and nanotechnology converge in the study of the aggregate state. On one hand, knowledge of the molecular principles that govern the processes of protein aggregation has a direct impact on the design of new drugs for high-incidence pathologies that currently can only be treated palliatively. On the other hand, exploiting the benefits of protein aggregation in the design of new nanomaterials could have a strong impact on biotechnology. Here we review the contributions of our research group on novel neuroprotective strategies developed using a purely biophysical approach. First, we examine how doxycycline, a well-known and innocuous antibiotic, can reshape α-synuclein oligomers into non-toxic high-molecular-weight species with decreased ability to destabilize biological membranes, affect cell viability and form additional toxic species. This mechanism can be exploited to diminish the toxicity of α-synuclein oligomers in Parkinson's disease. Second, we discuss a novel function in proteostasis for extracellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in combination with a specific glycosaminoglycan (GAG) present in the extracellular matrix. GAPDH, by changing its quaternary structure from a tetramer to protofibrillar assembly, can kidnap toxic species of α-synuclein, and thereby interfere with the spreading of the disease. Finally, we review a brighter side of protein aggregation, that of exploiting the physicochemical advantages of amyloid aggregates as nanomaterials. For this, we designed a new generation of insoluble biocatalysts based on the binding of photo-immobilized enzymes onto hybrid protein:GAG amyloid nanofibrils. These new nanomaterials can be easily functionalized by attaching different enzymes through dityrosine covalent bonds.

Keywords Amyloid · Amyloid functionalization · Alzheimer's disease · Parkinson's disease · Protein aggregation · Cross-beta structure · Glycosaminoglycan

Introduction

Amyloid aggregates

Amyloids are a special type of protein aggregate. Once considered to be physiologically irrelevant, they are now known to play both physiological and pathological roles. This has sparked a renewed interest in the field, and such different disciplines as biophysics, biochemistry and neurology have converged to generate the exponential growth in knowledge of protein aggregation that we see today. This transdisciplinary experience is paving the way for applications in biomedicine and nanotechnology, by preventing protein aggregation or exploiting its features, respectively.

Even though the term "amyloid" refers to sugars, there is a historical basis to its current use. The Latin term "*amylum*" was coined almost two centuries ago to label plant starch

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that tested positive in the iodine–sulphuric acid test. Around the mid-nineteenth century, the pathologist Rudolph Virchow introduced the term amyloid into the medical literature to describe deposits in the nervous system that also tested positive in the iodine–sulphuric acid assay (Cohen 1986; Virchow 1854). Shortly after the term became widely accepted, August Kekulé demonstrated the presence of proteins in the amyloid mass (Friedreich and Kekulé 1859; Sipe and Cohen 2000). The presence of sugars surrounding these protein aggregates explained the iodine–sulfuric acid result. However, despite the well-proven polypeptidic nature of these aggregates, the term "amyloid" continues to be used today.

Expanding the structure–function paradigm

Knowledge obtained from amyloid biophysics prompted a necessary change in the well-known sequence-to-structure-to-function paradigm. This paradigm, initially developed for enzymes and transport proteins, is too simplistic for understanding the full universe of all protein structures, and even more so their associations. A more comprehensive landscape of protein conformations is represented in Fig. 1. The functional form of a protein is usually associated to the most populated state in solution, referred to as the native state. However, other functional forms can be found in the unfolded and aggregate states. In the past, proteins with no tertiary structure, as well as aggregated proteins, were considered to be non-functional. However, intrinsically disordered proteins (IDPs), which lack well-defined three-dimensional structures but are still capable of playing important biological roles (Uversky 2010), and functional amyloids, are currently a clear example of this paradigm shift (Nuallain and Mayhew 2002).

The unfolded state comprises polypeptidic chains recently synthetized, IDPs and some intermediates of the folding reaction. The aggregate state encompasses a heterogeneous population as well, which ranges from amorphous structures without any order to highly structured fibrils that result from distinct aggregation pathways (Dunker et al. 2002). Proteins have been shown to form both types of aggregates in vitro (Dobson 2003). Novel functions are being continuously discovered for proteins in the amyloid state (Chiti and Dobson 2017). For example, amyloid A is present throughout different animal kingdoms, and new functions are reported continuously (An et al. 2017; Fowler et al. 2007). The amorphous aggregate has also been assigned functions; for example, α-crystallin amorphous aggregates are related to the development of cataracts in humans. In addition, the non-amyloids TDP-43 are pathological hallmarks of amyotrophic lateral sclerosis (ALS), a fatal motor neuron disorder, and frontotemporal lobar degeneration (Johnson et al. 2009). It is important to note that proteins that move from the native to aggregate state cannot only lose their function but also change it. A good example of this transition is glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that will be discussed further in this review.

The amyloid aggregation pathway

Amyloid aggregates are produced in a sequential multistep reaction. For globular proteins, this process begins with the misfolding and self-aggregation of native monomeric proteins (Chiti and Dobson 2009), while for IDPs this step has been related to subtle conformational changes leading from compact ensembles to extended ones that are more prone to aggregation (Levine et al. 2015). This initial aggregation

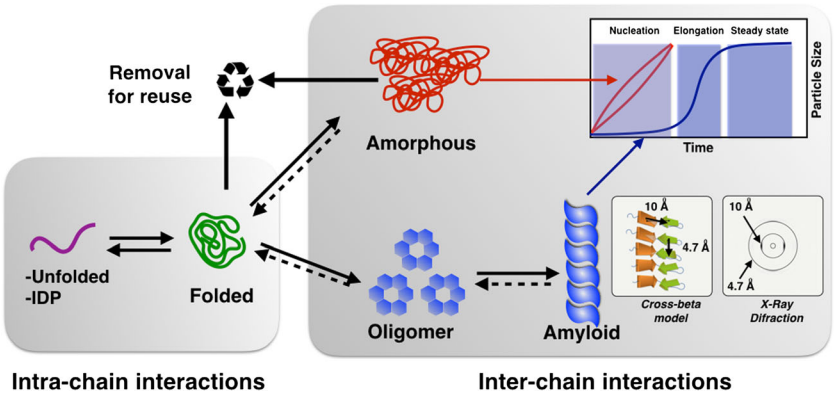


Fig. 1 A simplified representation of a protein aggregation pathway: Nascent and intrinsically disordered proteins (IDP) (unfolded, in violet) fold into a native functional state (folded, green), which is thermodynamically favored in globular proteins. Small destabilizing fluctuations in the intracellular medium can shift the equilibrium and increase the population of partly folded molecules. Under normal conditions, these are refolded by molecular chaperones or cleared by the ubiquitin–proteasome machinery (removal for reuse). Should these machineries be impaired or the populations of misfolded molecules overwhelm their buffering possibility, the equilibrium could lean towards amyloid fibrils (blue) or amorphous aggregates (red). The assembly of oligomers (blue) precedes that of amyloid fibrils, which are characterized by a specific X-ray diffraction pattern due to their cross-beta structure (inset). Kinetics data of particle size increase help distinguish between these two forms of aggregates

leads to the formation of nuclei or oligomers capable of recruiting other monomeric units. The growth of these oligomers results in larger structures known as protofilaments, the second intermediate transient species. The polymerization process continues by adding new polypeptidic chains on both ends until the number of free ends governs the overall reaction. Until the fibrillar endpoint state is achieved, metastable intermediates are commonly observed. The dynamic nature of these intermediate species hinders the isolation of homogeneous subpopulations for characterization. Therefore, there is no absolute consensus on the characteristics, size and morphology by which to define the different intermediate species that are frequently referred to in the literature, although one classification introduced by Kodali and Wetzel (2007) is among the most cited. According to this classification, the term "oligomer" is reserved for the first spherical smaller units. As the elongation process progresses, the first non-spherical species that appear are called protofibrils. Short protofibrils are sometimes called "rod-like" fibrils and longer protofibrils "worm-like" fibrils. In turn, when individual strands of protofibrils are twisted, they are called protofilaments. Finally, multiple protofilaments twisted around an axis constitute an amyloid fibril. When the amyloid fibril reaches its stable state, it is referred to as a mature fibril, and has usually reached around 10 nm in diameter and several micrometers in length (Fandrich 2007; Meinhardt et al. 2009).

Most amyloid fibrils are produced through a nucleation-dependent mechanism. The aggregation kinetics can be described by a sigmoidal function with a lag phase, where stable nuclei of polymerization are formed. Prefibrillar or fibrillar species can bypass this lag phase by seeding the polymerization reaction with preformed nuclei. On the contrary, amorphous aggregates do not have fibrillar forms nor sigmoidal reactions since they can be rapidly formed without a lag phase. Seeding cannot accelerate their formation. This kinetic characterization is valuable when the aim is to differentiate amorphous aggregates from amyloids. This is a very important distinction, not only from a basic scientific point of view but also in the pathophysiological context. In fact, with the exception of the TDP-43 amorphous aggregation in ALS and frontotemporal lobar degeneration (Kwong et al. 2008), all other protein conformational diseases are related to fibrillar amyloid aggregation (Aguzzi and O'Connor 2010). In this context, the aggregation kinetics of a protein, as measured by the increase in particle size as well as by its seeding ability, provides an excellent means to quickly discern the type of aggregation that the protein is undergoing. Obviously, the detection of a cross-beta structure as well as results from studies using advanced microscopy technology [scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM)] allow these aggregates to be differentiated with relative ease. Thus, protein aggregation kinetics, in addition to structural characterization, can be useful to distinguish these different kinds of aggregates (Yoshimura et al. 2012).

Structural fingerprint of amyloids

The basic structural motif of any amyloid fibril consists of β-strands oriented perpendicular to the fibril axis. This quaternary structure was initially interpreted from pioneering X-ray diffraction patterns of amyloid fibrils extracted from tissues (Eanes and Glenner 1968). This structure is currently referred to as "cross-beta" due to its characteristic diffraction signals at 4.7 and 10 Å (Fig. 1). The longitudinal and transverse ("cross") diffraction pattern caused by the inter-strand and stacking distances of the β-strands not only define this structure, but also gives it its name. The diffraction pattern indicates that amyloid fibrils share a common core structure, which consists of stacked β-strands where hydrogen bonds run parallel to the fiber axis, while the β-strands remain perpendicular to it (Fig. 1).

The generic character of the cross-β structure is also evident, based on the observation that the packing interactions by which the constituent β-strands are assembled were similar for different peptides without any evident sequence similarity (Fitzpatrick et al. 2013). The extensive hydrogen bond network developed between the cross-β and side-chain interactions in steric-zippers of tightly packed structures can account for the extraordinary stability (Giurleo et al. 2008; Gras 2007; Gras et al. 2008).

Amyloid fibril crystals were obtained using sequence-designed polypeptides. These crystals were found to be able to diffract at high resolution (1 Å), leading to a more detailed view of the cross-β structure (Makin et al. 2005). In subsequent studies, a number of short amyloidogenic peptides were crystallized and their atomic structure solved by X-ray crystallography, which increased our knowledge of this particular quaternary structure (Nelson and Eisenberg 2006; Sawaya et al. 2007).

This structural arrangement is also responsible for the specific binding of amyloids to dyes such as Congo red (CR) (Klunk et al. 1999) and thioflavin T (ThT) (LeVine 1999). An apple-green birefringence is produced under cross-polarized light upon the binding of amyloid to CR. These two dyes fluoresce only when they bind to cross-β structures and are therefore useful for spectroscopic monitoring of fibril growth and kinetics. It is important to note that CR and ThT are mainly considered not to bind to monomeric proteins/peptides, even if these contain β-sheets in their structures, although some exceptions have been reported (Khurana et al. 2001).

ThT, currently the most commonly used dye for amyloid detection, is a so-called molecular rotor. In water, the two aromatic rings of this benzothiazole can rotate around the bond connecting them with the concomitant fast dissipation

of excitation energy. Thus, a short fluorescence lifetime and low quantum yield is observed in this solvent (Biancalana and Koide 2010; Sulatskaya et al. 2010). In amyloid fibrils, the dye fits between the stacked layers of the cross-β structure, and the rotation becomes restricted due to geometric constraints, thereby giving an increased quantum yield (Stsiapura et al. 2008). Moreover, it has been very recently demonstrated that the intensity and lifetime of ThT fluorescence is sensitive to morphological differences between fibrils, as determined, for example, by preparation methods, protofilament structure and mutant isoforms of the precursor protein (Lindberg et al. 2015).

Fourier transformed infrared spectroscopy (referred to as FTIR) holds an important place in terms of technologies for analyzing and comprehending complex aggregation mechanisms at the oligomeric and/or fibrillary levels. The cross-β structural fingerprint can be easily detected by FTIR since it has a specific vibration in the Amide I band (Braido et al. 2016; Fink 1998; Oberg et al. 1994). Although FTIR lacks the high resolving power of X-ray crystallography, it has the advantage of fast-time response that provides an almost continuous structural view of protein/peptide conversions during the aggregation process (Avila et al. 2014; Torres-Bugeau et al. 2012).

FTIR is particularly sensitive to β-structures. Moreover, it is now well established that infrared can distinguish oligomers from fibrils simply based on their spectral features. In fact, FTIR can distinguish between β-sheets from the precursor globular proteins and those from their amyloid aggregates (Zandomeneghi et al. 2004). Moreover, the sensitivity of this technique to β-structures is sufficient to differentiate parallel and antiparallel arrangements (Celej et al. 2012). The latter is essential when studying oligomeric species, as will be discussed later in this review, since the toxicity of these intermediates is related to their β-arrangement (Gonzalez-Lizarraga et al. 2017). Because of this feature, clinical applications of infrared spectroscopy using FTIR-microscopy to diagnose amyloid abnormalities have been proposed (Choo et al. 1996; Kazarian and Chan 2006). However, to our knowledge, no developments have yet been reported in the area.

Amyloids in disease

Disease-related amyloids have a profound impact on human health. In this respect, approximately 60 heterogeneous proteins have been identified which can form amyloids, of which about 30 are associated to human diseases, including type 2 diabetes mellitus, ALS, dialysis-related amyloidosis, Parkinson's disease (PD) and Alzheimer's disease (AD), among others (Baker and Rice 2012; Huang et al. 2015; Knowles et al. 2014). The misfolding and amyloid aggregation of certain proteins may trigger deleterious effects, including membrane permeabilization, mitochondrial dysfunction and oxidative damage, on living organisms including human beings, in some cases with fatal consequences (Huang et al. 2015). For this reason, the term amyloidosis was given to a group of diseases characterized by the pathological misfolding and subsequent deposition of highly structured amyloid fibrils with cross-β conformations that lead to organ and tissue dysfunction, and eventually death (Baker and Rice 2012; Picken 2010; Sayed et al. 2015).

Compelling evidence has shown that soluble oligomeric intermediates constructed during the amyloid fibrillation pathway can alter membrane integrity, thereby triggering deleterious effects, which can ultimately lead to cell death. This suggests that prefibrillar aggregates, rather than mature amyloid fibrils, are the cytotoxic species responsible for the damage observed in amyloid-related diseases (Avila et al. 2014; Bennett 2005; Conway et al. 2000; Cremades et al. 2012). Moreover, it has been proposed that the passage from soluble toxic oligomers to innocuous insoluble fibril may constitute a cellular detoxification mechanism that protects cells from the detrimental effects induced by soluble oligomeric species (Avila et al. 2014; Cremades et al. 2012; Haass and Selkoe 2007).

Interfering with α-synuclein aggregation as a therapeutic target in PD

Synucleinopathies are progressive neurodegenerative disorders characterized by abnormal aggregation of the presynaptic protein α-synuclein. It is now accepted that cytotoxic α-synuclein aggregates are implicated in the neuronal demise observed in these pathologies. Currently, there are no effective therapeutic strategies that target α-synuclein aggregation or neurotoxicity.

A large number of recently published studies have shown that tetracyclines have remarkable neuroprotective properties in animal models of PD (Cho et al. 2009; Lazzarini et al. 2013). The antibiotic doxycycline, a well-tolerated drug that crosses the blood–brain barrier and possesses anti-inflammatory properties, has proved effective in modulating the aggregation process of some disease-associated proteins (Costa et al. 2011; Giorgetti et al. 2011; Manthripragada et al. 2011).

Our research group has recently shed light on the mechanism by which doxycycline interferes with α-synuclein aggregation to abolish its toxic effect on cells (Gonzalez-Lizarraga et al. 2017). Self-association of α-synuclein into fibrils is inhibited by the presence of doxycycline, as monitored with the ThT fluorescence assay. Examination of the samples by electron microscopy revealed that globular rather than fibrillar aggregates are formed when doxycycline is present in the aggregation assay. A deeper look into the aggregation pathway of α-synuclein using small angle X-ray scattering readily revealed that in the presence of doxycycline the pathways of

early oligomeric species are diverted, such that spherical off-pathway aggregates are formed instead of their rod-like on-pathway counterparts. The distinctive arrangement of α-synuclein in off-pathway assemblies was also evidenced by the decrease in the accessible surface of the hydrophobic solvent, as monitored by the fluorescent probe bis-ANS and by the changes in secondary structure content. Indeed, FTIR spectra of aggregating species showed a shift toward the formation of parallel β-sheet structures in off-pathway α-synuclein aggregates instead of the antiparallel β-sheet arrangements adopted by on-pathway oligomers. Importantly, these structural changes were observed to abolish the ability of α-synuclein prefibrillar aggregates to destabilize biological membranes, induce cytotoxicity and seed the formation of additional toxic species. It is also important to note that doxycycline binds only to aggregated species of α-synuclein, not to the monomeric form, as observed using nuclear magnetic resonance spectroscopy. In this way, doxycycline would not interfere with the physiological function of monomeric α-synuclein.

The involvement of GAPDH in neurodegenerative disorders

Glyceraldehyde-3-phosphate dehydrogenase is a glycolytic enzyme mainly known for its role in energy production. In addition to its canonical function, GAPDH also displays a myriad of other capabilities, such as activation of transcription (Zheng et al. 2003), membrane fusion (Morero et al. 1985), microtubule binding (Huitorel and Pantaloni 1985) and apoptosis (Tatton et al. 2000). These auxiliary functions have positioned GAPDH as a paradigmatic example of a "moonlighting" protein (Chauhan et al. 2017; Huberts and van der Klei 2010).

Many independent studies have pointed out that GAPDH may also be involved in neurodegenerative diseases. Genome-wide association studies have identified single nucleotide polymorphisms in GAPDH genes associated with increased or decreased risk of late-onset AD (Allen et al. 2012; Lee et al. 2008; Li et al. 2004; Lin et al. 2006) and, more recently, of Parkinson's disease (Liu et al. 2015). Even though the exact role of GAPDH in these afflictions remains unknown, a number of mechanisms have been proposed. Sunaga et al. showed that overexpression of GAPDH is associated with age-induced apoptotic death of neurons in culture (Sunaga et al. 1995). Deprenyl, a mono-aminooxidase B inhibitor used as a symptomatic treatment for PD, has been shown to exert neuroprotection by binding to GAPDH and reducing apoptosis independently of monoamine oxidase B inhibition (Carlile et al. 2000). Interestingly, nuclear accumulation of GAPDH was found to be associated with apoptotic death in PD *nigra*, as well as in AD (Tsuchiya et al. 2004).

However, GAPDH's role in neurodegenerative diseases is not limited to its involvement in apoptosis. GAPDH has been demonstrated to directly bind to several amyloidogenic proteins involved in neurogenerative conditions, including β-amyloid precursor protein (Sunaga et al. 1995), β-amyloid protein (Oyama et al. 2000) and tau in AD (Chen et al. 2000), huntingtin in Huntington's disease (Burke et al. 1996) and α-synuclein in PD (Tsuchiya et al. 2005). By immobilizing amyloid β-peptide on an affinity column, Oyama et al. (2000) were able to isolate amyloid-β binding proteins from a soluble fraction of rat brain and found that GAPDH was among the isolated proteins. Cumming et al. (2005) showed that amyloid-β induces the aggregation of GAPDH in rat primary cortical neurons in a similar fashion to that found in brain tissue from patients with AD. Indeed, GAPDH has been found to be associated to amyloid β-peptide in senile plaques (Wang et al. 2005), but it was not clear from the results of that study whether GAPDH was actively involved in the formation of the amyloid structure or if it was just adsorbed onto the fibrils after their deposition. To shed light on this, Naletova et al. (2008) tested the interaction between GAPDH and amyloid β-peptide by direct and indirect methods. The authors demonstrated that only non-native forms of GAPDH interact with soluble amyloid β-peptide, suggesting that GAPDH might be involved in the formation of amyloid structures.

By applying proteomic analyses, Wang et al. (2005) also demonstrated that GAPDH colocalizes with tau in neurofibrilar tangles and plaque-like structures in temporal cortices of patients with AD. In vitro, tau has also been found to bind to denatured GAPDH, suppressing its refolding and improving its aggregation in solution (Chen et al. 2000). It has also been reported that Lewy bodies in post-mortem brains from patients with PD are immunoreactive for GAPDH (Chen et al. 2000). Tsuchiya et al. (2005) showed that GAPDH could serve as a cofactor for α-synuclein aggregation in cultured cells. Indeed, coexpression of GAPDH with aggregation-prone A53T α-synuclein leads to the formation of intracytoplasmatic inclusions, reminiscent of Lewy bodies. Immunohistochemical examination of post-mortem material from patients with PD also demonstrated that GAPDH colocalizes with α-synuclein in the periphery of Lewy bodies.

Taken together, these data show the propensity of GAPDH to aggregate or interact with well-known aggregate-prone proteins. Still, the process by which it accumulates, and its relationship to neuronal apoptosis, remains unclear. As a matter of fact, both protective and pathological roles have been proposed for GAPDH aggregation. It seems plausible that these seemingly contradictory effects of GAPDH aggregation on the evolution of neurodegeneration may depend on the particular stimuli leading to aggregation, as detailed below.

Oxidative stress induces harmful aggregation of GAPDH

Oxidative stress is a common phenomenon in neurodegenerative diseases. Consequently, in these afflictions there is an

increase in the modification of proteins associated to oxidation damage (Widmer et al. 2006). There is also a correlation between the levels of oxidative stress and the progression of these diseases (Finkel and Holbrook 2000; Moreira et al. 2005; Przedborski and Jackson-Lewis 1998). Being one of the most abundant proteins in the cell, GAPDH is also very sensitive to oxidation (Schmalhausen et al. 2003; Suzuki et al. 1992) and has been proposed as a sensor of nitric oxide (NO) stress (Hara et al. 2006). Immunocytochemical analysis of post-mortem brain tissue from patients with AD and PD, respectively, revealed that GAPDH aggregated in neurons of the affected brain region (Tatton et al. 2000; Tsuchiya et al. 2004). These GAPDH aggregates appear as detergent-insoluble disulfide-linked high-molecular-weight species in brain extracts from patients with AD and aged transgenic mice models of AD (Cumming and Schubert 2005). The accumulation of aggregated GAPDH with disulfide bonding pattern was reproduced in primary culture neurons treated with amyloid β-peptide or subjected to oxidative stress (Cumming and Schubert 2005). Oxidative stress also affects GAPDH in models of PD. Rotenone is an insecticide used to reproduce the major pathological features of PD that inhibits mitochondrial respiratory chain complex I and is known to trigger oxidative stress. Exposure to rotenone induces the formation of intracytoplasmatic disulfide-bonded aggregates of GAPDH in rat adrenal pheochromocytoma cells cultures (PC12 cell line) (Huang et al. 2009) as well as in nigrostriatal cells in animal models (Huang et al. 2011).

The molecular events leading to oligomerization and aggregation of GAPDH upon oxidation was first described by Nakajima et al. (2007). According to their study, GAPDH oligomerization proceeds through the formation of intermolecular disulfide bonds, where the active site cysteine (Cys^{149} in rabbit muscle GAPDH or its equivalent Cys^{152} in recombinant human GAPDH) plays a central role. In addition, oxidation of Cys^{281}, which is only present in rabbit muscle GAPDH, accelerated the aggregation process. Indeed, mutation of the equivalent Ser^{284} to Cys in human GAPDH led to an increase in the propensity of the protein to aggregate (Nakajima et al. 2009). In a follow-up study, Samson et al. (2014) challenged the concept of cysteine oxidation being the primary step in GAPDH aggregation and proposed that the oxidation of Met^{46} was the rate-limiting step in aggregation. Mutation of this residue to leucine rendered recombinant human GAPDH highly resistant to aggregation under oxidative stress.

Based on circular dichroism spectra, upon oxidation the oligomerization of GAPDH occurs together with an augmentation of secondary structures, an increase in β-sheet content from 18 to 26%, a decrease in coil content from 67 to 56% and a slight increase in the α-helical content from 15 to 18% (Nakajima et al. 2007). Upon further incubation, oligomers evolve into aggregates with features characteristic of amyloid-like fibrils. Aggregated GAPDH exhibits CR binding under non-polarized light, typical orange–green birefringence under polarized light and an increase of fluorescence of the amyloid-binding dye thioflavin S (Nakajima et al. 2007). Examination of samples using electron microscopy revealed long helical fibrils that were 10 nm in width and up to 10 μm in length. To the contrary, Samson et al. (2014) reported large branched amorphous-looking aggregates up to 200 μm in diameter composed of repeating spherical units with diameters ranging from 0.5 to 2 μm. The difference in morphology was postulated to be due to the contribution of multiple concurrent aggregation events.

Since the discovery of GAPDH aggregates in patients with neurodegenerative diseases, many studies have focused on the functional consequences of this protein. The question of whether aggregation has pathological or beneficial consequences or is simply a by-stander in the evolution of the disease remains openly debated. In the case of oxidative-induced aggregation, many pieces of evidence point to an active pathological role. Overexpression of GAPDH in HeLa cells enhances death under oxidative stress, which is correlated to the extent of GAPDH aggregation (Nakajima et al. 2007). In addition, expression of the S284C aggregation-prone mutant GAPDH in SH5Y-5Y cells accelerates death associated with oxidative stress in comparison to cells overexpressing the wild-type protein (Nakajima et al. 2009). Also, a decapeptide designed to inhibit GAPDH aggregation was found to provide protection, thereby augmenting the survival of cells exposed to oxidative stress (Itakura et al. 2015b).

Some hints on the possible mechanism by which GAPDH would exert a toxic effect have been reported. Aggregates of GAPDH were found to enhance Aβ-40 amyloidogenesis, with a marked change in the morphology of the aggregates (Itakura et al. 2015a). These new aggregates exhibited a potentiated neurotoxicity both in vitro and in vivo, accompanied by mitochondrial dysfunction. While the addition of GAPDH aggregates to cell culture did not affect cell viability, its addition to isolated mitochondria induced swelling and depolarization by opening of the permeability transition pore (Nakajima et al. 2017).

Glycosaminoglycans induce the formation of protective aggregated GAPDH

Glycosaminoglycans (GAGs) are one of the main components of the extracellular matrix, constituting part of the proteoglycans. GAGs are unbranched polysaccharides made up of repetitive units of disaccharides bearing a large negative charge. This characteristic allows for electrostatic interactions with positively charged residues on proteins. GAGs have been implicated in neurodegenerative diseases based on experimental observations that they are present in almost all amyloid deposits in both the intracellular and extracellular space (Diaz-Nido et al. 2002). Moreover, they were shown to interact with

the main proteins involved in amyloid pathology, such as prion protein (Vieira et al. 2014; Wong et al. 2001), Aβ-42 (Castillo et al. 1997), tau protein (Goedert et al. 1996) and α-synuclein (Cohlberg et al. 2002).

Upon interaction, GAGs affect aggregation kinetics (Motamedi-Shad et al. 2009) and even protect protein aggregates from proteolysis (Gupta-Bansal et al. 1995). With respect to the evolution of PD, GAGs have been shown to accelerate α-synuclein fibrillation. Even though GAGs are found in Lewy bodies, which are the pathological hallmark of PD and associated to aggregated α-synuclein and GAPDH (Anderson et al. 2006; Spillantini et al. 1997; Tatton et al. 2000), the consequences of the interplay among these three molecules remained obscure.

The results of our experiments prove that specific GAGs, such as heparin or heparan-sulfate, triggers amyloid-like aggregation of GAPDH (Torres-Bugeau et al. 2012). This interaction seems to depend on the position of the sulfate groups since chondroitin sulfate, also a GAG but one differing from GAPDH, does not affect GAPDH quaternary structure. Following the interaction of GAPDH with GAG, there is a rapid increase in the fluorescence emission of ThT even though amyloid-like fibrils are only observed after 5 h of incubation. This shows that with respect to GAPDH, the dye not only reports the presence of fibrils but also indicates the presence of prefibrillar aggregates, as reported for other systems (Carrotta et al. 2001; Eakin et al. 2004). Aggregation proceeds by the dissociation of the native tetramer into dimers that preserve most of the secondary structural elements present in the native state, as determined by FTIR, while increasing the exposure of hydrophobic patches. The dimers readily reorganize into larger rod-like structures, which are compatible with protofibrils that elongate in one direction. Using small-angle X-ray scattering (SAXS) we characterized the protofibrils during the early stages of aggregation as cylinders with an average length of 21 nm and a diameter of 12 nm (Avila et al. 2014). Using structural constrains derived from experimental observations we modeled GAPDH protofibrils at atomic detail that were further validated using cross-linking followed by trypsin digestion and mass spectrometry characterization (Fig. 2). In this model, the native-like dimers are arranged into layers of hexamers stacked along the elongation axis. Aggregation-prone regions were revealed as hydrophobic patches at the edge of the protofibrils, which could serve as a scaffold to recruit additional aggregation species.

Indeed, we showed that GAPDH protofibrils, but not dimeric or tetrameric forms of the protein, were able to accelerate the aggregation kinetics of α-synuclein, resulting in the formation of mixed fibers. More interestingly, we observed that when added to dopaminergic cell cultures, GAPDH protofibrils abolished the deleterious effect associated to α-synuclein aggregates by sequestering the toxic oligomeric species (Avila et al. 2014). Also, in contrast with oxidation-induced aggregates, heparin-induced GAPDH aggregates were not harmful to the cells. Based on our results we believe that upon being released from the cell and interacting with the glycosaminoglycans present on the extracellular matrix, GAPDH can form protofibrils that play a protective role, acting as scavengers of toxic aggregated species (Fig. 3). Indeed, such high-molecular-weight species of GAPDH have been described as naturally occurring in the serum of healthy patients (Kunjithapatham et al. 2015). In this way, modification of the sulfation pattern of GAGs (Zhang et al. 2014) or alterations in the expression levels of GAPDH (Mazzola and Sirover 2005) associated with aging could enhance the risk of developing neurogenerative diseases.

Acidic membranes initiates yet another pathway of GAPDH aggregation

Lipid membranes are also known to affect the rate of formation of amyloid-like aggregates (Gorbenko and Kinnunen 2006; Stefani 2007). It has been postulated that they can catalyze aggregation by providing a surface for nucleation (Stefani 2006). Negatively charged membranes seem to play a role in aggregation catalysis, since they can provide both an acidic and hydrophobic environment that could aid in partially unfolding proteins (Rochet and Lansbury 2000), a key step in many aggregation process. Indeed, Zhao et al. (2004) showed that membranes containing acidic lipids, such as phosphatidylserine, efficiently triggered amyloid aggregation of GAPDH, lysozyme, insulin and myoglobin, among other proteins that had been previously shown to bind to negatively charged membranes. Using trace amounts of fluorescent phospholipids, these authors also showed that lipids get incorporated into the fibrils. In a follow-up study, we looked more deeply into the mechanisms leading to the lipid-induced aggregation of GAPDH (Cortez et al. 2010). We observed that upon interaction with acidic lipids, GAPDH suffers from a partial unfolding that increases the solvent accessible surface, as revealed by the hydrogen–deuterium exchange rate monitored using infrared spectroscopy. Aggregation then proceeds by dissociation of the native tetrameric form accompanied by an increase in the content of β-sheets and β-turns. Although, this aggregation pathway for GAPDH clearly differs from the one induced by GAGs (Avila et al. unpublished observations), the functional or pathological consequences of this aggregation pathway still remain to be probed.

Promises of self-assembling peptides and proteins as nanomaterials

Although amyloids have been associated with human diseases for over a century, their beneficial aspects have only been identified within the past decade. Amyloid fibers constitute

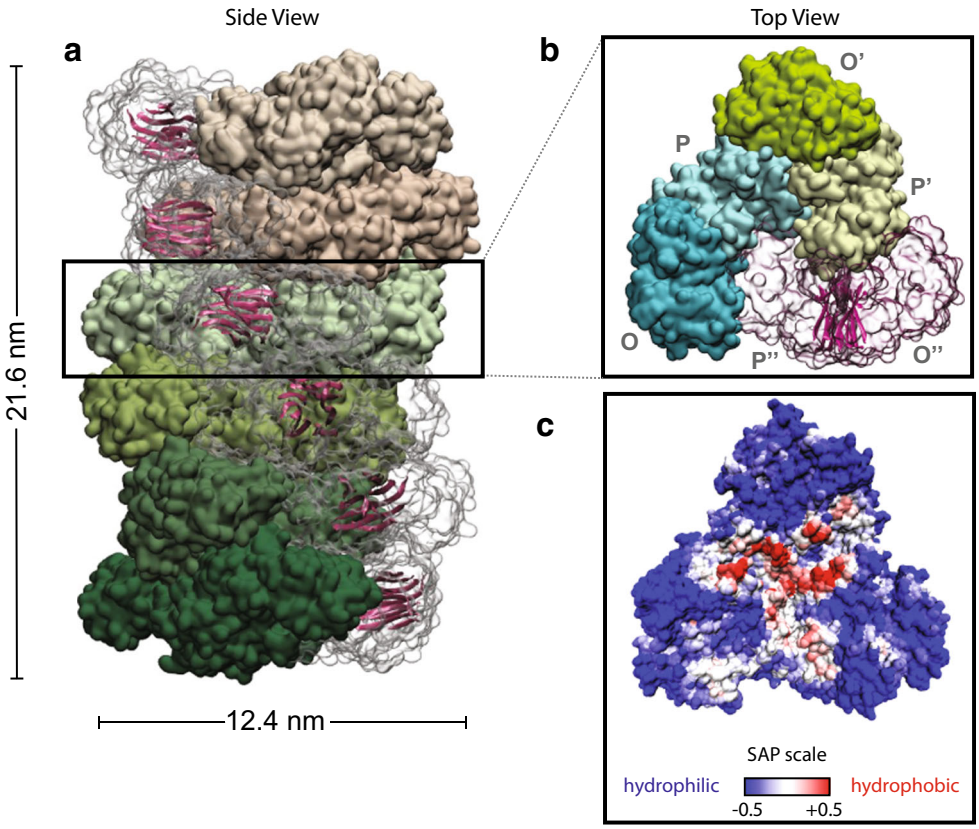


Fig. 2 Three-dimensional model of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protofibril: **a** Surface representation of a GAPDH protofibril formed in the presence of heparin. **b** Top view of the protofibril. The building block is represented as a transparent surface while the β-sheets that might be involved in the formation of cross-β structures in the mature fibril are shown in cartoon representation. **c** Spatial aggregation propensity mapped onto the protofibril solvent accessible surface. The red regions indicate aggregation prone sites with hydrophobic patches exposed. Adapted from Avila et al. (2014)

one of the most abundant and important naturally occurring self-associated assemblies (Chapman et al. 2002; Iconomidou et al. 2000). Inspired by how nature has applied them, scientists began to consider amyloids to be nanomaterials. The extensive hydrogen bond network of the cross-β structure and the tight side-chain interactions endow amyloids with exceptional properties. Amyloid fibrils have the potential to be one of the most versatile of nanomaterials as relevant properties, such as shape and morphology, could be controlled merely by regulating the polymerization conditions (pH, temperature, agitation, presence of ligands, etc.) in a very simple way (Mankar et al. 2011). In the same way, amyloids can be subjected to protein-engineering techniques to optimize these proteins for different requirements. This adaptability of protein self-assembly, together with their low cost of production, may result in the widespread use of amyloids as a nanomaterial.

It is also important to note that amyloid fibrils have considerable physical and chemical stability. In fact, amyloid fibrils are stable over a wide range of pH, salt concentrations and pressure (up to 1.3 GPa) and are also resistant to proteolysis and dehydration. Amyloid fibrils have also been found to be stable at temperatures of up to 100 °C (Meersman and Dobson 2006).

Amyloid fibrils have been successfully used as nanoscaffolds for enzyme immobilization (Phillips et al. 2014; Sasso et al. 2014; Verma et al. 2013). In addition to the well-known benefits of enzyme immobilization, such as reusability and increased stability, amyloids also offer a large surface area, increasing noticeably the active enzyme area (Brady and Jordaan 2009). Furthermore, amyloids are easily functionalized by exploiting the chemical properties of their different amino acid side chains (Hartgerink 2004) via cross-linking mature fibrils and enzymes of interest.

These peptide assemblies serve as a great "bottom–up" approach for the design and development of nanomaterials with desired functionality. Potential applications also cover other possibilities, such as nanobiosensors, or even as carriers in drug delivery for biomedical purposes (Scheinberg et al. 2017; Yu et al. 2016). A key to the successful use of the growing field of amyloid nanomaterials as technological and biomedical agents will be to appropriately match the biophysical features of the molecule to the system in which it will be applied.

Amyloid fibrils as nanoscaffolds for enzyme immobilization: the novel hybrid nanomaterial

The overall goal of industrial biotechnology is to develop new and more sustainable products to improve different processes. In this scenario, biocatalysis is an important cornerstone. However, despite the advantages of enzyme catalysts, such

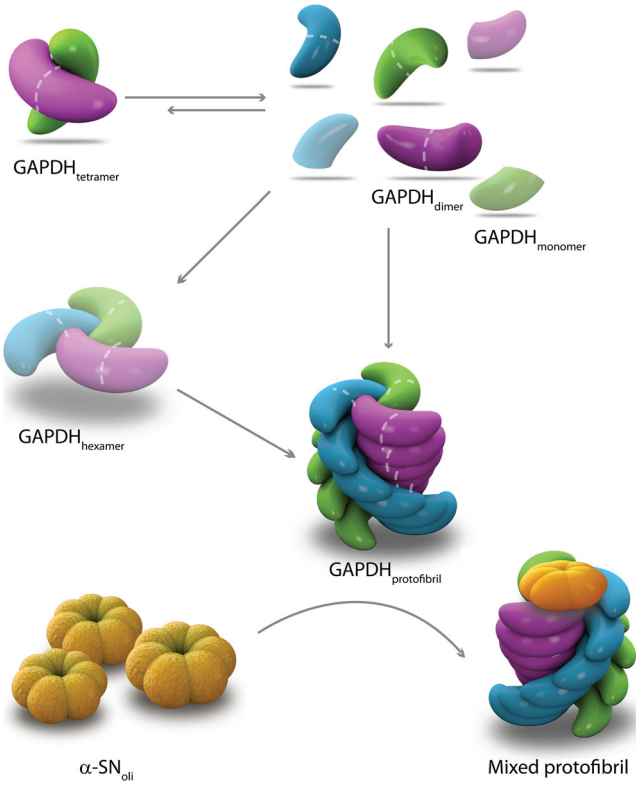


Fig. 3 Mechanism of α-synuclein sequestration by GAPDH protofibrils. In the extracellular space, glycosaminoglycans (GAGs) induce GAPDH dissociation into dimers, which then reassemble into high-molecular weight species such as protofibrils. GAPDH protofibrils are able to sequester α-synuclein oligomers into a mixed fibril through the hydrophobic patch present at the edge of the protofibril. Reproduced from Avila et al. (2014)

as effectiveness and eco-efficiency, their use is not as widespread as expected, mainly due to their (high) cost. Strategies to easily separate enzymes from their products and reuse them in many catalytic cycles are an essential requirement to impose these biocatalysts on biotechnological processes. The immobilization of enzymes on a readily separable support of the reaction products has proved to be a promising alternative and, as as already mentioned in this review, amyloids provide interesting advantages over other kinds of supports and can be exploited in such applications.

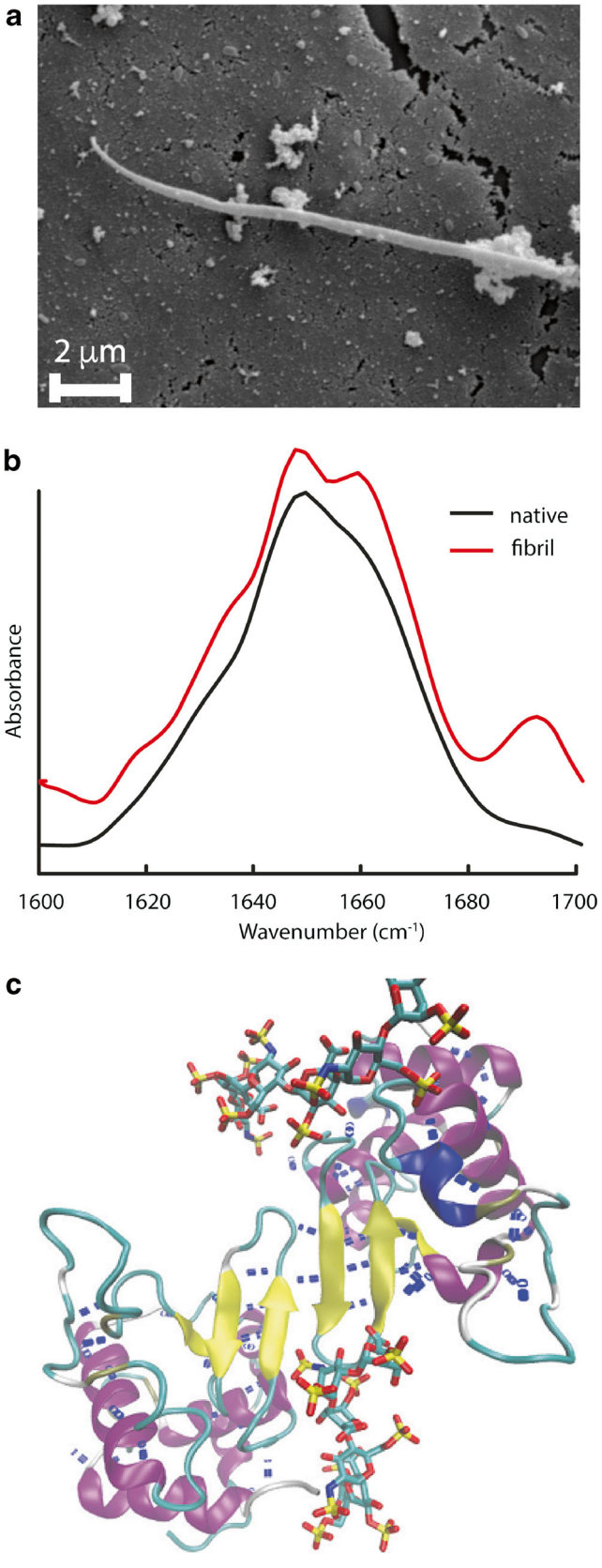
Although many enzyme immobilization protocols are available (Brena et al. 2013; Lopez-Gallego et al. 2013), novel methods capable of reducing production costs while remaining eco-friendly are urgently required. Accordingly, we have developed an interesting strategy to design a novel hybrid sugar:peptide nanosupport for obtaining amyloid-like nanofibrils without the addition of any chaotropic agent or solvent, or the application of extreme pH or high temperature.

Hen egg-white lysozyme (HEWL) is a relatively low-cost protein that has been widely used to study the mechanism of amyloid aggregation in vitro (Booth et al. 1997). It is a soluble globular protein that maintains its globular state at physiological temperature and pH. Amyloid fibrils of lysozyme can be obtained at pH 2.0 (Mossuto et al. 2010) and in the presence of trifluoroethanol (Liu et al. 2004) ethanol (Yonezawa et al. 2002) or guanidine hydrochloride (Vernaglia et al. 2004). However, none of these conditions are inexpensive or eco-friendly. For this reason, and based on our work to induce amyloid aggregation through the interaction of proteins with complex sugars, we decided to study the interaction of HEWL with heparin (Chaves et al. 2016). According to our results, heparin can induce HEWL amyloid aggregation, as assessed by the classical ThT fluorescence assay. We were unable to adjust the aggregation reaction to a sigmoid function since the lag phase of the process was almost undetectable under our working conditions. It is interesting to note that the amount of cross-β structures formed is proportional to the amount of polysaccharide used. To take into account that ThT fluorescence increments could be false positive results (Nilsson 2004), we also used second-order light scattering measurements to confirm the formation of larger size aggregated species. The SEM image in Fig. 4a shows the morphology and size (width 300 nm, length 13 mm) of the mature fibril obtained after incubation for 5 days at 37 °C and pH 7.4.

Infrared spectroscopy proved to be an extremely useful technique to study the HEWL amyloid-like fibrils. After heparin addition, the presence of a new band located near 1612 cm^{-1} was detected and assigned to a cross-β structure. However, the overall fold of the protein remained the same (Fig. 4b). These data are consistent with an amyloid-like supramolecular arrangement (Arrondo et al. 1994). It is important to note that, according to our results, the polysaccharide molecule also forms part of the fibril structure. We are currently characterizing this novel hybrid nanomaterial. We have proposed that heparin can act as a crowding surface, aligning and adjusting the HEWL conformation to the new physicochemical environment, thereby facilitating new inter-chain interactions (Chaves et al. 2016).

In order to understand the structural arrangement of this new hybrid nanomaterial, we applied molecular dynamics simulation techniques. The results showed that after heparin docks to the protein, novel hydrogen bonds between β-strands of different HEWL molecules appeared in an antiparallel pattern (Fig. 4c), in accordance with observations derived from FTIR spectroscopy measurements. The orientation of this novel inter-molecular β-strands allow the formation of classical amyloid nanofibril cores. These analyses provide an insight into the role that heparin (and other GAGs) may play in enhancing both the formation and stabilization of amyloids.

Using cross-linking methods, such as photo-induced cross-linking of unmodified proteins (PICUP) or copper and hydrogen peroxide induced cross-linking of unmodified proteins (CHICUP) (Fancy et al. 2000; Williams et al. 2016), the fibrils were successfully functionalized by linking the exposed Tyr residues of both the fibril and enzyme. We have even obtained improvements in stability and/or enzymatic activity in some



◀ **Fig. 4** **a** Variable-pressure-scanning electron microscopy image of lysozyme fibrils formed in the presence of heparin. **b** Time evolution of deconvolved amide I infrared spectra lysozyme incubated with heparin. **c** Putative model of heparin inducing dimerization of lysozyme in the first step of aggregation. Figures adapted from Chaves et al. (2016)

enzymes, such as lipase (Chaves et al. 2016), urease and invertase (Chavez et al. unpublished results).

In conclusion, by applying protein self-assembly expertise, we have been able to develop a novel generation of insoluble biocatalysts. Compared to conventional techniques, our procedure is a cleaner and faster method to obtain easily functionalizable fibrils in an eco-friendly way.

Non-amyloid self-assembly of peptides

The self-assembly of peptides is not limited to amyloid aggregation, and other driving forces can intervene to give rise to diverse macromolecular arrangements, such as nanotubes (Mishra et al. 2011), nanoribbons (Lin et al. 2011), nanospheres (Matsuura et al. 2016), nanotapes (Miravet et al. 2013) and hydrogels (Fichman and Gazit 2014), among others. These are interesting nanomaterials as they could serve as scaffolds for protein immobilization, drug encapsulation, tissue engineering or the deposition of metals to form nanowires.

An interesting example to illustrate the nature of the driving forces for self-association is represented in the study of the assembly of growth hormone releasing hexapeptide (GHRP-6) into nanotubes (Santana et al. 2014). GHRP-6 is a synthetic peptide that is used to induce the release of growth hormone in many animals, including humans (Cheng et al. 1989). While studying the storage conditions, Santana el al. (2014) found that the peptide is able to form stable nanotubes in aqueous solutions at room temperature and physiological pH. Analysis of the samples using TEM and cryo-TEM revealed that the peptides assemble into bundles of long linear structures with lower electron density along the longitudinal direction. Supplementary studies using SAXS also showed that the aggregates could be modeled as hollow cylinders with an external cross-section of 13.4 nm and internal cross-section of 7.4 nm, which is in accordance with the measures derived from TEM and cryo-TEM. Studying the assembly process using molecular dynamics the authors were able to postulate a putative model of the organization of the peptides within the aggregate in which they proposed that the peptides behave as amphiphilic molecules and are arranged within the cylinder into a bilayer-like structure with the amino terminal exposed to the water and the amide-capped carboxy terminus buried within the core of the cylinder. It is important to note that in the case of the GHRP-6 no structural features related to amyloid formation were detected.

Concluding remarks

The idea that one sequence ➔ one structure ➔ one function has dominated the way we think about protein function. This is true to such a degree that we immediately associate folded native states with canonical function. Alternative states, such as amyloids and other aggregates, have often been regarded as non-functional, biologically inert waste products. However, surmounting evidence suggests otherwise. For example, the intrinsically disordered protein α-synuclein plays a role in vesicle fusion and trafficking, while the transition to amyloid has fatal consequences for cells. Understanding the biophysics that drive state transitions would allow targeting the toxic species without affecting the function of the physiological state. It is tempting to speculate that nature has evolved transitions between quaternary structures to amplify the protein functional repertoire. In this framework we propose that the moonlighting protein GAPDH shifts its quaternary structure into cross-β to gain a new function in proteostasis. As the landscape of novel protein conformations continues to amaze us, it also offers the unique opportunity to harness the untapped potential of alternative structure–function states for biomedical and biotech purposes.

Compliance with ethical standards

Conflict of interest César L Avila declares that he has no conflicts of interest. Silvina Chaves declares that she has no conflicts of interest. Sergio B Socias declares that he has no conflicts of interest. Esteban Vera-Pingitore declares that he has no conflicts of interest. Florencia González-Lizárraga declares that she has no conflicts of interest. Cecilia Vera declares that she has no conflicts of interest. Diego Ploper declares that he has no conflicts of interest. Rosana Chehín declares that she has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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