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TOPICAL REVIEW

Protein aggregation and misfolding: good or evil?

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Abstract
Protein aggregation and misfolding have important implications in an increasing number of fields ranging from medicine to biology to nanotechnology and material science. The interest in understanding this field has accordingly increased steadily over the last two decades. During this time the number of publications that have been dedicated to protein aggregation has increased exponentially, tackling the problem from several different and sometime contradictory perspectives. This review is meant to summarize some of the highlights that come from these studies and introduce this topical issue on the subject. The factors that make a protein aggregate and the cellular strategies that defend from aggregation are discussed together with the perspectives that the accumulated knowledge may open.

Contents

1. Introduction

The importance of protein aggregation has increasingly gained interest in the last 15 years (Dobson 2003, 2004a, 2004b). This is, however, not due to the discovery of a new phenomenon. Physicists, chemists and biochemists have been aware since the early days of protein studies that, under stress conditions, proteins may aggregate, often in an irreversible manner. Physicists have described the phenomenon as being caused by the protein being trapped in local minima of the multidimensional conformational space which describes the energy landscape (Sheraga 1996). Chemists have often discussed aggregation in terms of properties such as solubility and hydrophobicity. Biochemists know well that any environmental stress condition such as a temperature or pressure increase, the presence of chaotropic agents, mutations, etc can lead to loss of the native structure with consequent denaturation (Petty 2001).

What is new in our modern perspective of protein aggregation is the realization that an increasing number of pathologies are associated with protein aggregation and that aggregation often occurs through misfolding rather than just unfolding. This implies a loss of the native structure to adopt a new structured state that often leads to formation of fibrillar species (Luheshi and Dobson 2009, Chiti and Dobson 2006). Dramatic examples of pathologies associated with misfolding include Alzheimer's and Parkinson's diseases, as well as several other rarer but not less devastating pathologies such as Huntington's, prion diseases and amyloid lateral sclerosis

(Bellotti *et al* 1999, Dobson 1999). The hallmark common to all these pathologies is the presence of amorphous or more often fibrillar protein aggregates, called amyloid fibres, that are invariably associated to cell toxicity (Stefani and Dobson 2003).

The interest in protein aggregation is, however, not limited to its medical implications. Many more proteins have been found to lead to fibres than those known to be implicated in human diseases, suggesting the hypothesis that most, if not all, amino acid sequences might be able to misfold in a similar ordered way. This evidence places protein misfolding into a much wider perspective.

It has also been shown more recently that protein aggregation is not always dangerous: formation of essential 'cellular bodies' such as melanosomes, dendritic cell aggresome-like structures, P-bodies and stress granules depends on protein aggregation (Huff *et al* 2003). It is also well established that some cellular protein interactions involve the formation of amyloid-like structures and that several protein and peptide hormones are stored in the cell in amyloid-like structures (Maji *et al* 2009). Understanding how proteins aggregate and misfold into fibrillar aggregates has therefore become an important goal of modern biology.

This topical issue of *Journal of Physics: Condensed Matter* is dedicated to the topic of protein aggregation. Different experimental and theoretical studies are presented here that address various aspects of our quest to understand aggregation. This review is not in any way meant to be an exhaustive account of the field given the large plethora of papers published on the topic over the last 15 years. We hope to cover, nevertheless, some of the most important milestones of the research on aggregation. Our sincere apologies go to the colleagues that will be unintentionally not mentioned.

2. Morphology of protein aggregates

Protein aggregates can have very different morphologies which may range from being amorphous to being well-ordered fibrillar structures (figure 1). In the latter case, the aggregated species are usually named amyloid fibres from the original idea of Rudolph Virchow who, around 1854, thought sugars could be an important component of the aggregates (reviewed by Cohen 1986 and Sipe and Cohen 2000). While the exact meaning of the amyloid term has changed through time, the current standing definition is 'an unbranched protein fibre whose repeating substructure consists of β strands that run perpendicular to the fibre axis, forming a cross-β sheet of indefinite length' (Greenwald and Riek 2010). This structure, which has been long since described in silk, would then be a universal fold that most if not all proteins may be able to adopt in given conditions (Astbury *et al* 1935). From the silk studies, it is known that the repeating cross-β sheet motif gives rise to a very characteristic x-ray fibre diffraction pattern with a meridional reflection at ca. 4.7 Å that corresponds to the inter-β strand spacing and an equatorial reflection at ca. 6–11 Å that corresponds to the distance between stacked β sheets (Astbury *et al* 1935, Sunde *et al* 1997). Because the same structural features have been observed in several *in vitro* and/or *in vivo* assembled aggregates this seems to be a very stable structure. Another important property of the amyloid deposits is that they have inherent birefringence that increases intensely after staining, for instance, with the Congo red dye. They are also very resistant to proteolytic attack.

There seem, however, to be other mechanisms by which proteins can assemble into ordered aggregates without invoking the cross-β motif. This includes domain-swapping and end-to-end stacking (Bennett *et al* 2006, Eisenberg *et al* 2006, Nelson and Eisenberg 2006).

2.1. Do all proteins really aggregate and misfold?

The medical (Powers *et al* 2009), biological (Fowler *et al* 2007) and fundamental (Zhang 2003) interest associated with protein aggregation and fibrillogenesis raises the important question of which characteristics are responsible for promoting fibrillogenesis and whether it is possible to predict, and ultimately modulate, the fibrillogenic propensities of different amino acid sequences. If clarified, this issue could make possible, for instance, the design of ways to encourage or suppress fibrillogenesis.

A set of principles has been identified. First of all, it is assumed that the information is stored linearly along the amino acid sequence (Chiti *et al* 2003). Second, the sequence must have some tendency to adopt structural features compatible with the semi-ordered structure observed in the repetitive assembly proper of fibres (Marshall and Serpell 2009, Nelson and Eisenberg 2006). This observation may be easily rationalized by remembering that essential elements in stabilizing β structures are not only the inter-strand hydrogen bonds but also packing of the side chains. Therefore, while polyproline is unlikely to form fibres since its conformation is hardly compatible with a β-structure, sequences with β-propensity could easily form amyloids. This assumption seems, however, not so stringent and the sequence does not necessarily have an elevated β-propensity: it seems sufficient that the sequences have some secondary structure propensity, no matter whether α or β. Sequences which adopt an α-helical structure in the context of a globular protein can act as chameleons and form efficiently packed fibrillar β-structures (Zou and Gambetti 2007, Atwood *et al* 2003, Uversky 2003, de Chiara and Pastore 2011).

Finally, the last aspect that seems important in fibre formation is that fibrillogenic sequences need to be at least partially if not completely accessible. This is easily obtained in peptides (such as the Alzheimer Aβ amyloid) and intrinsically unstructured proteins (such as α-synuclein) but can be more difficult in globular proteins, in which potential β-prone sequences are usually well buried in the protein core.

2.2. Why do proteins aggregate in vitro?

Why do proteins aggregate rather than reach the global minimum of their single-chain energy landscape? Much of what we know about protein aggregation and misfolding is based on biophysical studies *in vitro*. What we have learned from this work is that we can classify proteins into

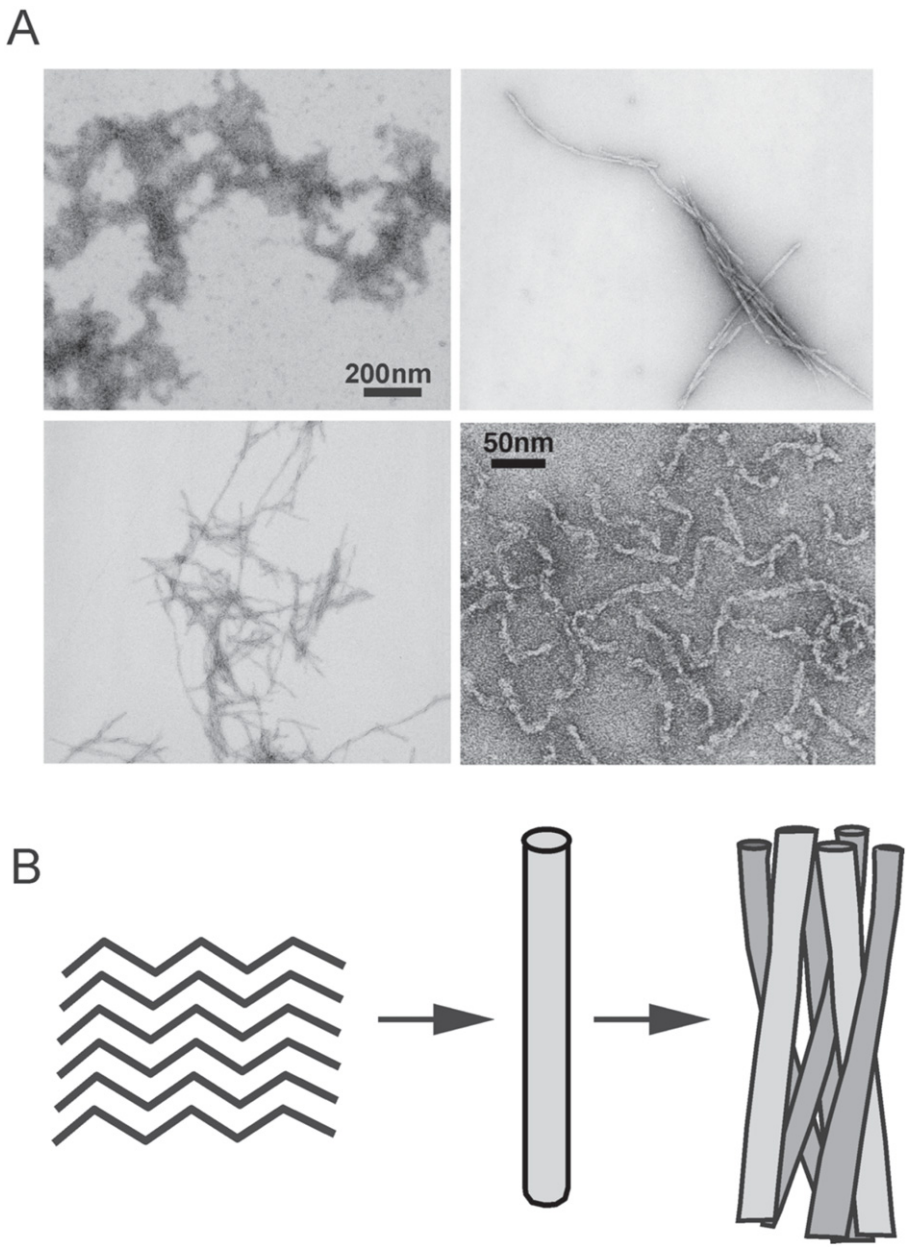

Figure 1. Aggregate morphology and fibre assembly. (A) Representative examples of amyloid fibres as observed by electron microscopy. From top to bottom and left to right: aggregates of FMRP, the Alzheimer peptide Aβ1–42, the H2H3 region of ovine PrP and the Josephin domain of ataxin-3. (B) Model of how amyloid fibres are thought to assemble. From left to right: formation of a *b*-rich oligomer, fibre formation, mature fibre.

two main families: one comprises the intrinsically unfolded proteins (Huang and Stultz 2009, Babu *et al* 2011), the second includes globular proteins or domains (Chiti and Dobson 2006) (figure 3). There is a distinct difference between the two families. Understanding how the former aggregate is conceptually easy: regions in the linear sequence more prone to aggregation start the event when their concentration in the cell is above their solubility products (Gsponer *et al* 2008, Jain *et al* 2011). It is more difficult to understand why globular proteins aggregate. In fact, they generally do not unless exposed to environmental perturbations (Chiti *et al* 1999). High temperature (Litvinovich *et al* 1998, Fändrich *et al* 2001), high pressure (Ferrão-Gonzales *et al* 2000, De Felice *et al* 2004), low pH (Guijarro *et al* 1998, McParland *et al* 2000), alcohols (Sassi *et al* 2011), chaotropic solvents and sample shaking (Apetri and Surewicz 2003, Morillas *et al* 2001) have widely been used for this purpose in *in vitro* studies. Under these conditions, proteins that have evolved to protect their hydrophobic core from the solvent open up and initiate the process of misfolding (Kelly 1996, Dobson 1999).

More recently, a subfamily of globular proteins was identified whose members have the tendency to aggregate spontaneously without the need of highly destabilizing conditions (Chiti and Dobson 2009). To explain this behaviour it was hypothesized that local structural fluctuations, rather than global changes, could trigger a conformational transition

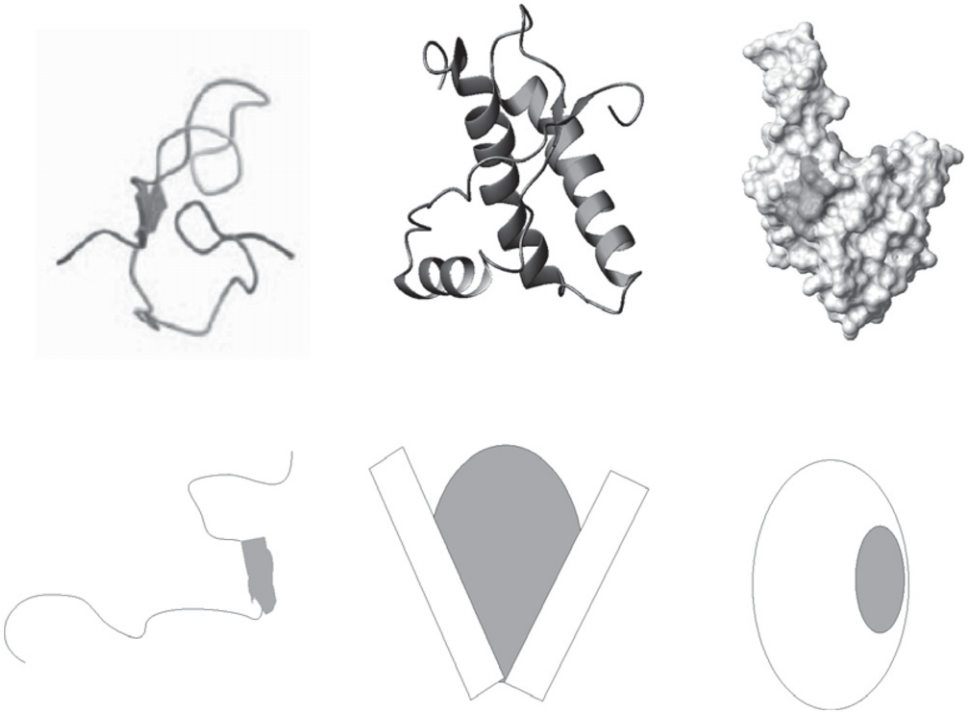

Figure 2. Classification of the current models of protein aggregation. From left to right: intrinsically unfolded peptides (bottom) exemplified with the Alzheimer Aβ(1–42) (top) (Tomaselli *et al* 2006); globular proteins that need to be destabilized to 'exposed' fibrillogenic regions as is the case for the C-terminal domain of prion protein (Adrover *et al* 2010) and proteins that aggregate spontaneously in the absence of a binding partner as is the example of the Josephin domain of ataxin-3 (Masino *et al* 2010).

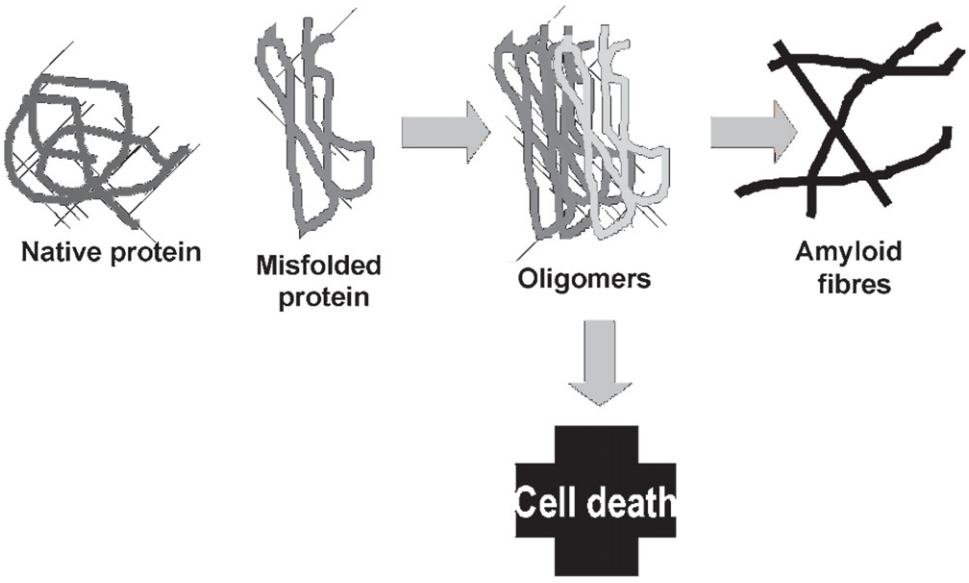

Figure 3. Schematic representation of the aggregation pathway and its consequences in misfolding diseases. Functional proteins (in red) may undergo mutations or alterations that lead to aggregation to form oligomeric species. Whether aggregation or a conformational change comes first needs to be established and might differ from case to case. The oligomeric soluble species usually forms an insoluble fibrillar species. The toxic species is thought to be the intermediate soluble aggregate rather than the mature fibre.

which would then initiate aggregation and misfolding. This hypothesis may well explain, for instance, the fibrillation of acylphosphatase from *S. solfataricus*, which retains its overall fold and enzymatic function under mildly destabilizing conditions (Plakoutsi *et al* 2004, 2005, 2006). It cannot, however, easily account for the properties of other proteins. One such example is constituted by ataxin-3 and its globular Josephin domain. Ataxin-3 is the protein responsible for the neurodegenerative disease spinocerebellar ataxia type 3 or Machado–Joseph disease (SCA3/MJD) (Paulson 2011, Matos *et al* 2011). This pathology is caused by the anomalous expansion of a tract of glutamine repeats (polyQ) within

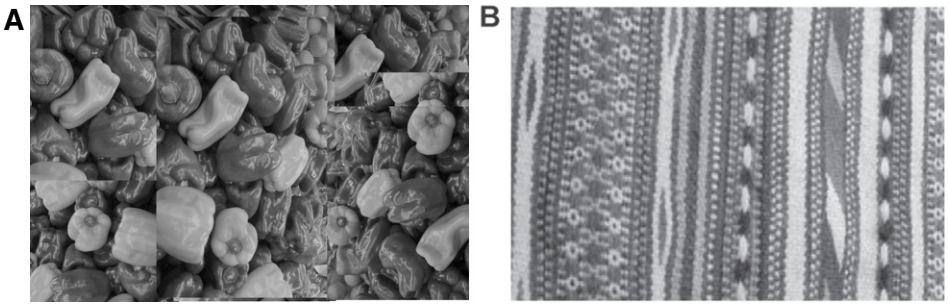

Figure 4. Metaphoric representation of the cell environment. On the left: a crowded basket of peppers that symbolizes a disordered crowded environment. On the right: a close up of a typical tapestry from Orune, Sardinia (Italy). There is still crowding but it is organized in several different patterns symbolizing the cellular compartmentalization.

ataxin-3. *In vitro* studies have shown that ataxin-3 has a strong intrinsic tendency to form fibrils even under native conditions. It has been shown that fibrillization is promoted not only by expansion of the polyQ sequence present in the C-terminus of the protein and essential for the development of the disease but by a second fibrillogenic signal involving residues within an *N*-terminal globular domain named Josephin. In the three-dimensional structure of Josephin, these residues form solvent-exposed patches that are also involved in the recognition of natural substrates, such as ubiquitin HHR23 proteins (Nicastro *et al* 2005, 2009). Indeed, designed mutations at these substrate-binding patches or the presence of protein partners significantly reduce the aggregation kinetics (Masino *et al* 2010). We shall discuss further how these results might bear important consequences for our understanding of the protective strategies against aggregation *in vitro* and *in vivo*.

2.3. Why do proteins aggregate in vivo?

While protein aggregation *in vitro* reflects the specific experimental conditions chosen by the researchers, a completely different question is why proteins aggregate *in vivo*.

A common cause is somatic mutations in the gene sequence that lead to the production of proteins unable to adopt the native folding because of missense mutations, early truncation, or shifts in reading frames. Well known examples are the mutations observed in type II diabetes, Huntington's disease and familial forms of Parkinson's disease and Alzheimer's disease (Powers *et al* 2009, Chiti and Dobson 2006, Ross and Poirier 2004). Alternatively, the mutations can affect components of the protein quality-control system, as in the case of mutations in the small HSP (sHSP) α-crystallin, that lead to cataract formation (Andley 2006) or of the E3 ubiquitin ligase Parkin, which results in an early onset form of Parkinson's disease (Kitada *et al* 1998, Olzmann *et al* 2007, Chin *et al* 2010).

Another cause is fragmentation: proteins may be attacked by proteases and cleaved, thus giving rise to non-physiologic fragments that can then migrate to hostile environments very different from those where they are normally located. One such example is that of Aβ peptides observed in Alzheimer's disease which are produced by cleavage of the transmembrane APP protein (De Strooper and Annaert 2000). Once released, they will no longer be protected by the lipid membrane and become highly susceptive to aggregation. Another example is huntingtin, the protein responsible for Huntington's chorea. The region encompassing huntingtin exon 1 is cleaved off by caspase 6 and produces a highly toxic fragment (Lunkes *et al* 2002).

Besides these causes, aggregation is the result of any defect in protein homoeostasis, that is the regulation of the protein levels in the cell. Despite the several control mechanisms developed by the cell (see below), when the concentration of misfolded proteins exceeds the refolding or degradative capacity of a cell, protein aggregates accumulate.

Environmental stress conditions, such as heat and oxidative stress, can also lead to aggregation. Excessive heat treatment above the optimal growth temperature of a particular organism may lead to unfolding of cellular proteins (Parsell *et al* 1994). Oxidative stress is a well recognized source of irreversible protein modifications triggered by reactive oxygen species (ROS). They include radical induced fragmentation of the polypeptide backbone and replacement of side chains of specific amino acid residues by carbonyl groups (Stadtman and Levine 2000). It has, for instance, been shown that carbonyl derivatives can be generated by a direct oxidation of Pro, Arg, Lys and Thr residues or in reactions involving Lys, Cys and His residues with reactive carbonyl compounds (Nystrom 2005). All these irreversible modifications can lead to cell toxicity.

The quality-control system seems anyway to become looser during ageing and senescence. It has, for instance, been shown that aggregation of polyglutamine model proteins in *C. elegans* and a misfolding-prone mutant of human superoxide dismutase 1 (SOD1) in mice is exacerbated during ageing (Morley *et al* 2002, Wang *et al* 2009). Similarly, carbonylated proteins accumulate progressively to form visible aggregation foci in the cytoplasm in aged yeast cells (Nystrom 2005, Erjavec *et al* 2007).

2.4. Strategies to defend proteins from aggregation in vivo

How does the cell as a whole prevent protein aggregation? Several strategies have been suggested and they all may be

considered a valid answer to this question as they tackle the problem from different perspectives. It was proposed that the first protection against aggregation might come at the level of primary sequence and that the presence of glycine and proline residues could prevent aggregation (Monsellier and Chiti 2007). This hypothesis certainly accounts for the fact that some residues are more prone than others to aggregation. It could, however, be argued that proteins do not fold to hide exposed hydrophobic residues but, rather, bury hydrophobic residues to fold. The view of a finalized selection of specific amino acids sounds attractive but, at the same time, is rather anthropomorphic.

A different perspective suggests that aggregation and folding are competing pathways (Dobson 2004a, 2004b, Tartaglia and Vendruscolo 2010). This is certainly true and offers an important reading frame for understanding misfolding as will be discussed in section 2.5. This view, however, has the limitation that it does not account for intrinsically unfolded proteins or peptides suggesting that it represents only one aspect of a wider picture. As a more generalized perspective, it has been suggested that the physiologic function, rather than just folding, is the alternative pathway to aggregation (Pastore and Temussi 2012).

It has also been argued that the best strategy for preventing aggregation is that of preventing the accumulation of aggregation-prone misfolded proteins by controlling protein concentration. Different groups have in fact proven a clear link between protein concentrations, solubilities and aggregation propensities (Castillo *et al* 2011, Tartaglia *et al* 2007, Tartaglia and Vendruscolo 2009, Gsponer *et al* 2008). Vendruscolo and co-workers also showed that proteins tend to be concentrated and soluble to a level inversely proportional to the volume of their subcellular localizations (Tartaglia and Vendruscolo 2009). These results suggest that the organization of a cell into compartments makes biochemical processes more efficient by concentrating the molecules that carry them out, but only by simultaneously ensuring that their solubility is kept at levels at which aggregation is avoided under normal circumstances.

Finally, it is of course important to mention the role of chaperones and the various degradation mechanisms which include the unfolded protein response and autophagy (for an extensive review see (Bukau *et al* 2006, Young *et al* 2004)). The main chaperone classes that prevent the accumulation of misfolded conformers include the heat shock proteins HSP60 and HSP70 (Hartl and Hayer-Hartl 2009, Bukau *et al* 2006, Horwich *et al* 2007, Spiess *et al* 2004). Misfolded proteins that cannot be refolded back are eliminated either by cytosolic ATP-dependent AAA+ proteases (i.e. the 26S proteasome) (Goldberg 2003) or transported into the lysosomal compartment and processed by acidic hydrolases (Kirkin *et al* 2009, Nakatogawa *et al* 2009, He and Klionsky 2009).

2.5. Complex-orphan proteins are prone to aggregation

A different perspective to understand the mechanisms of protection against aggregation is the view that proteins are hardly naked in the cell. As a consequence of specific attractive interactions, many proteins *in vivo* are part of large complexes (Srere 2000, Gierasch and Gershenson 2009). Interactions may thus play an essential role with the same protein being often involved in a complex network of interactions. This in turn may result in protection from aggregation. This view suggests the concept of 'complex-orphan proteins', that is proteins that would normally be present in the cell as part of large molecular assemblies that protect them from aggregation. When they are either produced in isolation (for instance *in vitro* by recombinant expression) or factors intervene to prevent this status, they will develop an elevated tendency to self-associate and/or unfold. This is also what we might observe in some pathologies.

The above mentioned ataxin-3 is an excellent example of this paradigm: the fibrillogenic regions of ataxin-3 reside on the protein surface and coincide with the binding sites of ubiquitin, the natural partner of this de-ubiquitinating enzyme (Masino *et al* 2010). Thus, the same regions are important for physiological functions but, if left exposed, promote aggregation.

Another example is the case of the fragile X mental retardation protein (FMRP). FMRP and its close homologues FXR proteins are components of different types of nuclear and cytoplasmic ribonucleoprotein granules in which they often co-localize (Moser *et al* 2010, Christie *et al* 2009, Huot *et al* 2005, Tassone *et al* 2004, Bakker *et al* 2000). Under stress conditions, high levels of transfected FMRP induce formation of granules in which mRNAs are trapped into repressed mRNP particles (Oostra and Willemsen 2009). Accordingly, FXR proteins and their fragments have an elevated tendency to aggregate *in vitro* (Sjekloća *et al* 2011).

2.6. The role of crowding and protein compartments in aggregation

Interactions with the environment can provide other ways to protect proteins from aggregation. There is a growing interest in the idea that, in the cell, proteins are not in diluted solutions but surrounded by a heavily crowded milieu: the interior of cells is characterized by a high concentration of macromolecules that makes the solution conditions drastically different from those typical of most biochemical *in vitro* studies (Luby-Phelps 2000); these conditions are often described as crowded or confined (Minton 2001). It has been suggested that crowding could have a strong effect on protein stability, interactions, and folding as a consequence, mostly, of excluded volume effects (Minton 2001, Zhou *et al* 2008).

At first sight crowding and confinement in the cell do not appear as useful means for protecting the cell against unwanted aggregation. The direct effect of either crowding or confinement should be to increase the local concentration of the aggregating protein. However, a combination of crowding and confinement may produce the opposite effect, i.e. dilution of the protein. If we imagine that a protein is confined to a small portion of the cell (a small cavity), the simultaneous presence of other (crowding) macromolecules can reduce the effective local concentration. Another obvious

consequence should be an increase in the stability of the folded species with respect to unfolded ones, although it has been pointed out that the increase in protein stability due to crowding alone is modest (Gierasch and Gershenson 2009). However, a combination of crowding and confinement might be surprisingly efficacious. Another, albeit more unlikely, possibility is that crowding could favour complexation of the aggregating protein with chaperones or other functional assemblies.

2.7. The insoluble aggregates may not after all be the toxic species

Despite the interest that amyloid fibres have attracted, they might not after all be the toxic species. Increasing evidence shows that other soluble intermediates formed along the aggregation pathway, now usually referred to as oligomers, seem to be responsible for toxicity much more than the insoluble amyloid fibrils (Bucciantini *et al* 2002, Walsh *et al* 2002). The first evidence of a role in neurodegeneration of soluble, nonfibrillar assemblies probably came from the Aβ peptide of Alzheimer's. In 1998, Lambert and colleagues presented the first experimental evidence that soluble, nonfibril forms of synthetic Aβ (which they called Aβ-derived diffusible ligands, or ADDLs) could be neurotoxic (Lambert *et al* 1998). This was supported by a significant statistical correlation between cortical levels of soluble Aβ and the extent of synaptic loss and severity of cognitive symptoms (Lue *et al* 1999, McLean *et al* 1999, Wang *et al* 1999). Toxicity seemed to be triggered by any form of 'soluble Aβ' that remained in aqueous solution following high-speed centrifugation of brain extracts (Kuo *et al* 1996, Lue *et al* 1999, McLean *et al* 1999, Wang *et al* 1999), starting already from Aβ dimers (Li *et al* 2009). The work on Aβ was followed by the demonstration of cytotoxicity for amyloid oligomers from the prion protein and α-synuclein (Sokolowski *et al* 2003, Conway *et al* 2000). Transgenic mouse models of Alzheimer's and Huntington's disease have also provided results that are consistent with the idea that oligomers rather than fibrils are toxic (Lesné *et al* 2006). Behavioural learning and memory deficits in mouse models occur far earlier than the appearance of fibrils or inclusion bodies, thus indicating that the toxic effects of smaller aggregates on cellular function arise prior to the production of amyloid fibrils.

Although often adopting a β-rich structure (Chimon *et al* 2007), the oligomers may have structural motifs distinct from that adopted in the mature fibrils, as evidenced by the establishment of both oligomer-specific and fibril-specific antibodies (Kayed *et al* 2003). These elusive species may be at the very heart of the toxicity problem and might therefore be more interesting targets for further studies.

2.8. A positive role for amyloids

It has been suggested that, rather than being the toxic species, amyloid fibrils could even have a beneficial function in diseases and could represent the cellular defence response that sequesters the toxic oligomers into nontoxic mature amyloid fibrils (Hardly and Selkoe 2002). This view is convincingly supported by studies of the inclusion bodies containing extended aggregated polyglutamine tracts in Huntington's disease (Arrasate *et al* 2004) and by evidence that Lewy body formation in Parkinson's disease is protective to dopaminergic neurons.

A positive role of amyloid fibres is also supported by recent evidence of amyloids in non-pathologic environments and functions. It has been, for instance, shown that peptide hormones are stored in secretory granules in an amyloid fibril-like state (Maji *et al* 2009). In this way they are insoluble but ready to be dispensed by granule exocytosis into the extracellular space.

E. coli and other gram-negative enteric bacteria also produce extracellular amyloid species known as curli (Wang *et al* 2008). These fibres are thought to be critical for growth in biofilms and to play a key role in binding to host cells and enabling bacteria to persist within their local environment. The gram-positive bacterium *S. coelicolor* produces functional amyloid fibres known as chaplins (Capstick *et al* 2011). The functional role of these fibres seems to be that of reducing surface tension at the air–water interface and permitting the growth of aerial hyphae.

3. Conclusions

In this review we have dealt with protein aggregation *in vitro* and *in vivo*, starting from basic structural and physicochemical considerations and moving on to various aspects of why aggregation occurs in the cell. We have discussed the sensitive issue, which has come for many researchers to be regarded as a dogma, of whether, given proper conditions, all proteins aggregate and misfold. We have also considered different strategies that prevent protein aggregation *in vivo*. Competition between aggregation and folding offers an important reading frame for understanding misfolding. A wider perspective that would account also for intrinsically unfolded proteins or peptides suggests instead that protein aggregation is competed out by functional interactions and compartmentalization and confinement.

Where do we go from here? Overall, the picture that comes out from ca. 20 years of studies on protein aggregation and misfolding illustrates the subtle but very tight mechanisms evolved by the living cell to reach a precise balance. It is therefore clear that any even small diversion from such conditions can result in an imbalance that might be lethal or pathologic. The lessons learned in this way do not only allow us to understand more about disease but might eventually help us to turn 'evil into good' and exploit some of the remarkable properties of the unwanted amyloid aggregates in useful new tools aimed at improving the human condition.

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