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Structural features and cytotoxicity of amyloid oligomers: Implications in Alzheimer's disease and other diseases with amyloid deposits

Massimo Stefani *

Department of Biochemical Sciences and Research Centre on the Molecular Basis of Neurodegeneration, University of Florence, V.le Morgagni 50, 50134 Florence, Italy

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ABSTRACT

Amyloid diseases display the presence, in targeted tissues and organs, of fibrillar deposits of specific peptides or proteins. Increasing efforts are presently spent in investigating the structural features and the structure–toxicity relation of the soluble oligomeric precursors arising in the path of fibrillization as well as the importance of surfaces as triggers of protein misfolding and aggregation and as possible responsible for amyloid polymorphism. Presently, it is recognized that the unstable, heterogeneous pre-fibrillar aggregates are the main responsible for amyloid toxicity. Conversely, mature fibrils are considered stable, harmless reservoirs of toxic species, although direct fibril toxicity has been reported. Recent studies show that mature fibrils grown at various conditions can display different structural features, stabilities and tendency to disassemble with leak of toxic oligomers. Fibril polymorphism can result from protein aggregation at differing conditions populating misfolded monomers and oligomers with distinct conformational characteristics. Recent research has started to unravel oligomer structural and biophysical features and their relation to cytotoxicity. Increasing information supports the notion that oligomer–membrane interaction, disruption of membrane integrity and cell impairment results from both oligomer and membrane biophysical features; accordingly, the formation of the oligomer–membrane complex, often the first step of amyloid toxicity, can be the result of the interplay of these events. This view can help explaining the variable vulnerability of different cell types to the same amyloids and the lack of relation between amyloid load and severity of clinical symptoms; it also stresses the importance, for cell/tissue impairment, of the presence of fibrils conformers of reduced stability as a possible source of oligomers resulting from leakage possibly favored by the interaction with suitable macromolecular/lipid surfaces or by other environmental conditions.

Contents

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; FRET, fluorescence resonance energy transfer; CD, circular dichroism; DLS, dynamic light scattering; NMR, nuclear magnetic resonance; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of flight; NUP, natively unfolded/unstructured protein; PS, phosphatidylserine; ER, endoplasmic reticulum; APP, Amyloid beta precursor protein; GM1, ganglioside M1; EM, electron microscopy; AFM, atomic force microscopy; ESI-MS, electron spray ionization-mass spectrometry; SAXS, small-angle X-ray scattering; RAGE, receptor for advanced glycation end-products; NMDA, N-methyl-D-aspartic acid; AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid.

* Tel.: +39 055 4598307; fax: +39 055 4598905.
E-mail address: stefani@scibio.unifi.it.
URL: http://www.unifi.it/unifi/scibio/pers/stefani.html

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1. Introduction

Amyloid diseases are a number of familial or, more often, sporadic conditions characterized by the presence, in the affected tissues/organs of extracellular or intracellular proteinaceous deposits, generically known as amyloid, whose core constituents are fibrillar polymers of one out of a number of peptides/proteins, each characteristic of a given disease [reviewed in Stefani and Dobson, 2003; Chiti and Dobson, 2006; Sipe et al., 2010]. The amyloid history can be dated back to 1838, when the term amyloid was first introduced by Matthias Schleiden, a German botanist, to describe a normal amylaceous constituent of plants. Subsequently, in 1854, the term was used, albeit incorrectly, by the German physician Rudolph Wirchow to describe "cerebral corpora amylacea" in abnormal human brains staining blue by iodine and turning into violet by addition of sulfuric acid and thus considered to be identical to plant starch (Kyle, 2001; Puchtler and Sweat, 1965). A few years later (1859) Friedreich and Kekulé found that amyloid contained nitrogen and was made up of proteins rather than of carbohydrates (Friedreich and Kekule, 1859). At the beginning of the 20th century, based on optical microscope observation, scientists believed that amyloid was an amorphous material. The first clues on the existence of ordered structural organization with anisotropic optical properties within amyloid deposits came in 1927, when Divry and Florkin by polarized optical microscopy found orange or apple-green birefringence upon treatment of amyloid with Congo red (Divry and Florkin, 1927). After Divry and Florkin, congophilia and green birefringence became the signature used to recognize amyloid, whereas the fibrillar nature of the deposits was first recognized in 1959 by ultrastructural studies by Calkins and Cohen (Cohen and Calkins, 1959). Presently, it is known that the basic component of amyloid is a fibrillar network made up of polymeric unbranched fibrils with a beta sheet-rich core (the cross-beta structure) (Serpell et al., 2000) arising from ordered polymerization of specific proteins/peptides basically under conditions favoring a reduction of their thermodynamic stability resulting in misfolding [reviewed in Stefani and Dobson, 2003; Chiti and Dobson, 2006].

Among the 27 human amyloid diseases presently identified by the International Nomenclature Committee (Sipe et al., 2010), Alzheimer's disease (AD) is by far the most important for its large occurrence in the aged population. Therefore, it is not surprising that, after a large body of work done on systemic amyloidoses, the subsequent identification of Aβ as the main component of the AD plaques has driven much of the research on amyloid in the past twenty years. That polymeric fibrils of Aβ, the peptide arising from the proteolytic cleavage of the membrane amyloid precursor protein (APP), were the main component of the plaques found in the brain parenchyma of AD people was first stated in 1984 by Wong and Glenner (Glenner and Wong, 1984) and subsequently confirmed in 1985 by Colin Masters in collaboration with Konrad Bayreuther. Masters identified the amino acid composition and N-terminal sequence of the Aβ peptides and found that both corresponded to those of the main protein component of the amyloid deposits found in the brains of people affected by congophilic angiopathy, AD or Down syndrome (Masters et al., 1985). In the same year it was also shown that the prion protein, previously reported to possess properties of infectious proteinaceous material, assembled into polymeric fibrils deposited in the amyloid plaques found in scrapie affected brains (DeArmond et al., 1985).

Previous descriptions of systemic amyloidoses did not need to state an amyloid hypothesis to relate cell/tissue degeneration to the presence of amyloid deposits; in fact, there was no doubt that a relationship did exist between amyloid deposits and disease and that amyloid fibrils or, in some cases, non-fibrillar aggregates, were the pathological entities. At variance with systemic amyloidoses, the link between neurodegeneration and presence of amyloid plaques in AD brain was first proposed in 1990, when it was shown that aggregated Aβ was neurotoxic *in vitro* (Yankner et al., 1990). At the same time seminal discoveries in the AD genetics pave the way to the formulation of the "amyloid cascade hypothesis", stating that Aβ peptides originating from the proteolytic cleavage of their precursor protein (APP) are the main culprits of AD symptoms at the molecular level (Selkoe, 1991). Rapidly, yet with diffuse resistances in AD scientists, the "amyloid cascade hypothesis" replaced previous proposals such as the "cholinergic hypothesis"; yet, the latter has recently been re-proposed, though in a modified form (Craig et al., 2011).

Since 1990, the development of transgenic AD animal models has dramatically advanced our knowledge of several aspects of the AD pathophysiology and pathogenic mechanisms, even though a completely satisfactory mouse model of the pathology is not yet available. After 1990, huntingtin and α-synuclein were identified as the proteins polymerized in the fibrils deposited in the brains of people affected by Huntington's and Parkinson's disease, respectively (Mayers et al., 1993; Spillantini et al., 1998), even though the plaques deposited in the latter did not display the histologic characteristics of those found in the previously described amyloidoses. At the same time, data suggesting a basic shared neurotoxic mechanism of amyloids started to appear (Schubert et al., 1995).

Until the end of 1990s the data available and the genetics of amyloid diseases supported a quite general consensus that the basic toxic species in amyloid plaques were amyloid fibrils, even though no mechanistic data supporting fibril cytotoxicity had been clearly reported. Therefore, it appeared likely that the pathogenic features underlying AD and other neurodegenerative and systemic amyloidoses were the result of the presence of extracellular or intracellular deposits of amyloid type. Besides providing a theoretical frame to understand the molecular basis of these diseases, such a scenario stimulated the exploration of therapeutic approaches to amyloidoses that were mainly focused at hindering the growth and deposition of amyloid fibrils. However, at the end of the 1990s the attention shifted to the cytotoxicity of amyloid fibril precursors, notably amyloid oligomers and protofibrils (Lambert et al., 1998); data also started to appear indicating that neuronal synapses were the main target of cytotoxicity (Walsh and Selkoe, 2004) and that the impairment of the long-term potentiation was the main effect resulting from the oligomer–synapse interaction (Walsh et al., 2002). At the same time, on the basis of experimental evidence, the "generic hypothesis" of amyloid formation was proposed (Dobson and Karplus, 1999). The latter states that the ability to assemble into ordered cross-β structures cannot be considered an unusual behavior exhibited by a small sub-set of peptides and proteins with special sequence or structural properties, but as a property inherent to the basic structure of polypeptide chains. Soon after, in 2002, data were reported suggesting for the first time that cytotoxicity is a generic property of amyloid oligomers (Bucciantini et al., 2002) associated with a shared "toxic" fold (Kayed et al., 2003). This new view shifted the target of pharmacological research aimed at finding

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molecules useful to combat cell/tissue impairment in amyloid diseases from counteracting fibril growth to hindering the appearance of amyloid oligomers.

At the present, the pivotal role of amyloid oligomers as key players of amyloid cytotoxicity is widely recognized and the amyloid hypothesis has been extended to other amyloid diseases; presently the detrimental role of amyloid oligomers and other pre-fibrillar entities is supported by a large number of data on many *in vivo* and *in vitro* amyloidogenic proteins indicating a direct cytotoxic effect of amyloid aggregates (Selkoe, 2003). The cytotoxicity of pre-fibrillar amyloid assemblies has been confirmed for all proteins and peptides associated with amyloid diseases, including Aβ peptides, α synuclein, amylin, beta2-microglobulin, transthyretin and others (Walsh et al., 2002; Cleary et al., 2005; Bhatia et al., 2000; Conlon et al., 2001; Conway et al., 2000; Nilsberth et al., 2001; Sousa et al., 2001; Poirier et al., 2002; Chung et al., 2003a). Pre-fibrillar assemblies of many proteins and peptides not associated with disease have been found to be cytotoxic as well (Dobson and Karplus, 1999; Sirangelo et al., 2004; Anderluh et al., 2005).

The growing awareness that the early oligomeric species transiently arising in the fibrillization path of proteins/peptides are the most potent toxins to cells stems from a large number of studies on Aβ aggregation both *in vitro* and *in vivo* and Aβ aggregate neurotoxicity [reviewed in Walsh and Selkoe, 2004]. The idea that Aβ oligomers appear inside neuronal cells and are responsible for neuronal impairment was first proposed in 1998 by the William Klein group (Lambert et al., 1998). However, one of the first evidences that Aβ oligomers are really produced in cultured cells and impair neuronal cell physiology and viability came only in 2002, when the Dennis Selkoe group showed that Aβ oligomers are generated and secreted soon after peptide appearance in model cultured cells overexpressing APP containing the AD mutation V717F. The authors also showed that microinjecting into rat brains the cell medium containing these oligomers, but not Aβ monomers or fibrils, potently inhibited hippocampal long-term potentiation, one of the signs of the disease in animal models (Walsh et al., 2002). Since then, many studies have confirmed the Selkoe results eventually leading to assess (i) the nature of those Aβ oligomers as variously sized spherical dimer–hexamer to 24mer aggregates (ADDLs, amylospheroids) (Cleary et al., 2005; Townsend et al., 2006; Chromy et al., 2003; Hoshi et al., 2003); (ii) their phosphorylation state (Kumar and Walter, 2011); (iii) their presence inside AD brains (Gong et al., 2003; Lesné et al., 2006; Gouras et al., 2000); (iv) their formation within neuronal cells (Walsh et al., 2000; Billings et al., 2005; Mucke et al., 2000); (v) their ability to impair memory and cognitive function (Gong et al., 2003; Lesné et al., 2006; Gouras et al., 2000); and (vi) their involvement in physical degeneration of synapses (Mucke et al., 2000). Similar results have also been reported for amyloid oligomers grown from many other peptides/proteins [reviewed in Caughey and Lansbury, 2003] providing solid support to the idea that toxicity is a general feature of those species.

This review will focus some aspects of protein misfolding and aggregation as well as of amyloid oligomer growth, structure and cytotoxicity. It will also stress the importance of biological surfaces, notably macromolecules and phospholipid bilayers, as key players of protein misfolding and aggregation. The importance of the interplay between membrane and oligomer physicochemical features modulating amyloid cytotoxicity in the membrane–oligomer complex will also be taken into consideration.

2. Protein folding and aggregation are competing processes

Since 1998, when it was first reported that two proteins unrelated to any amyloid pathology were able to aggregate *in vitro* into fibrillar assemblies of amyloid type (Gujiarro et al., 1998; Litvinovich et al., 1998), it has increasingly been recognized that the tendency to aggregate into amyloid assemblies of proteins/peptides is a general property of the peptide backbone (Stefani and Dobson, 2003; Chiti and Dobson, 2006; Bucciantini et al., 2002; Fändrich et al., 2001). Such a property arises from the intrinsic tendency of polypeptide chains to self-organize into polymeric assemblies stabilized by inter-molecular hydrogen bonds established between the peptide bonds in parallel or anti-parallel polypeptide stretches in the beta-strand conformation. The resulting ordered arrangement, known as cross-beta structure, characterizes the amyloid fold [reviewed in Serpell et al., 2000]. The key role of the main-chain contacts does not reduce the importance, for amyloid fibril growth, of the side chain sequence, that determines the environmental conditions under which the polypeptide chain can aggregate. This view considers natural proteins as a group of amino acid polymers selected by evolution so as their amino acid sequences are optimized to disfavor aggregation whilst favoring folding into compact, yet not rigid, states; these result mainly from the tertiary interactions among the side chains that shield not only the hydrophobic core but also the peptide backbone. Conversely, protein aggregation into amyloid polymers, which are mainly stabilized by secondary interactions, can be considered the result of the emergence, under non-natural conditions, of the intrinsic primordial tendency of the peptide backbone to give secondary intermolecular interactions (reviewed in Stefani and Dobson, 2003; Chiti and Dobson, 2006). Such a paradigm considers peptides and proteins as polymers endowed with the intrinsic property to self-organize into higher order polymeric aggregates; it also suggests that protein folding and protein aggregation must be distinct but competing processes and that the environmental conditions dictate which one is favored for a given polypeptide chain (Fig. 1). Accordingly, extensive studies have been carried out *in vitro* to investigate the transition between natively folded states and soluble aggregate-precursor states, and between the latter and mature amyloid fibrils (Wiseman et al., 2005).

That protein folding and aggregation can be competing processes is suggested by several theoretical and experimental evidences. For example, the high intra/extracellular concentration of macromolecules, averaging 300–400 mg/ml inside the cell, appears one of the environmental conditions affecting the choice between folding and aggregation. This feature, often referred to as macromolecular crowding, affects thermodynamically the conformational states of proteins (Ellis, 2001). A very high macromolecular concentration means that the volume freely available to a macromolecule is only a fraction of the total volume where it is dissolved; therefore, the resulting excluded volume effects can favor thermodynamically, though to a different extent for any given protein, compact structures, including both natively folded and aggregated states. On this aspect, it has been calculated that an increase in macromolecular crowding from 30% to 33% (in terms of the volume of a given space occupied by macromolecules) could result in a rise of the molecular binding affinities by as much as an order of magnitude (Ellis, 2001). The excluded volume effects can also favor compact states resulting from aggregate nucleation when proteins and peptides are unable to undergo efficient fold into monomeric compact states. However, it must also be considered that the increased viscosity of the medium can lower the diffusion-limited growth rate of the aggregation nuclei by reducing the translational movements of the macromolecules. The general validity of these considerations must also take into account that they stem from studies carried out *in vitro* mainly with non-physiological crowding agents (PEGs, dextrans and others); these agents do not establish interactions with proteins similar to those occurring with the crowding molecules found inside the cell

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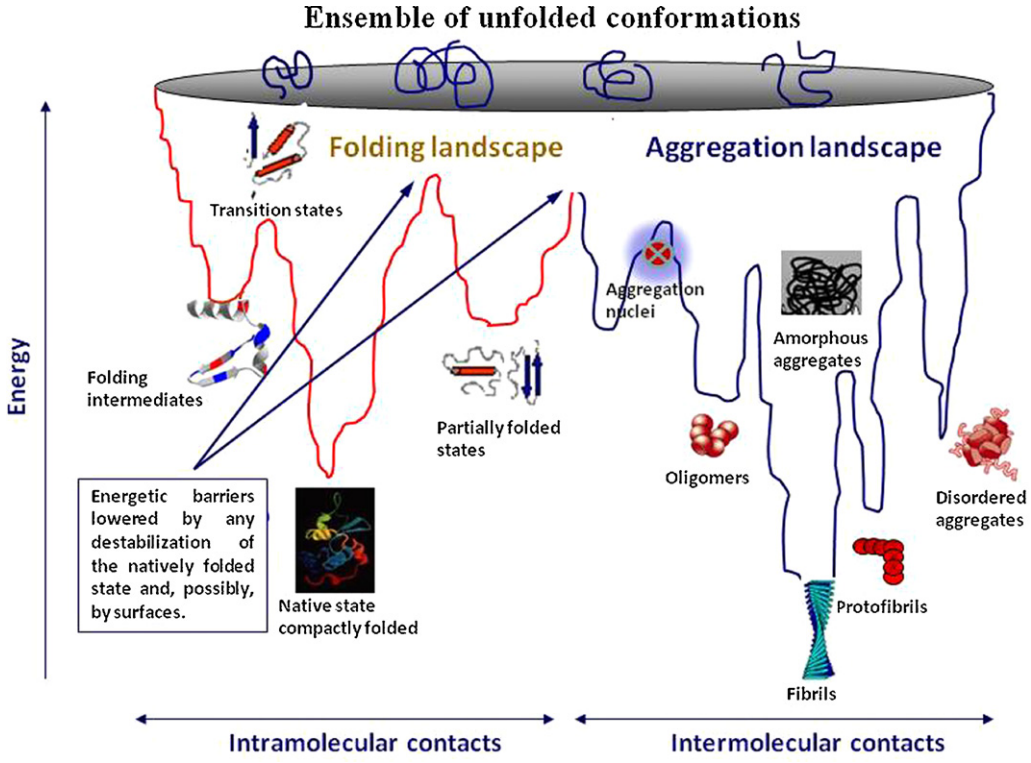

Fig. 1. The energy landscape of the conformational states available to a polypeptide chain in the folding and aggregation sections. Folding and ordered (amyloid) aggregation are processes relying on similar physicochemical parameters and hence in competition to each other.

(nucleic acids and proteins, including molecular chaperones) or in the extracellular milieu (glycosaminoglycans and proteins, including molecular chaperones).

Besides macromolecular crowding, a number of physicochemical parameters of the polypeptide chain affect similarly both protein folding and aggregation; these include a significant propensity to gain secondary structure, a low net charge and a relatively high content of hydrophobic residues (Chiti et al., 2003). The importance of these properties for protein folding and aggregation is supported by the structural adaptations found in the natively unfolded proteins (NUPs) (Uversky, 2002). It is also confirmed by the data indicating that mutations neutral to the thermodynamic stability but increasing the mean hydrophobicity or the propensity to generate beta structure or reducing the net charge of any protein/peptide can favor kinetically its aggregation from the unfolded states in dynamic equilibrium with the folded structure (Chiti et al., 2003). The case of natively unfolded proteins rises interesting considerations on the relation between stability and function in protein evolution. Protein thermodynamic stability cannot be considered an absolute goal of evolution independently from the environmental conditions (temperature, ionic strength, pH) at which a protein folds and performs its biological function. The example of natively unfolded proteins and of the proteins of the hyperthermophiles clearly show that each natural polypeptide chain is a product of evolution which displays specific thermodynamic features; these must ensure proper stability of the natively folded protein and its folding intermediates making it possible to be easily unfolded for correct degradation. Moreover, proper stability also results in optimal flexibility of the protein allowing it to perform its biological function at the best under physiological conditions.

Overall, the findings showing that protein folding and protein aggregation depend on the same physicochemical features of the polypeptide chain and of the medium where it is dissolved, confirm the idea that both pathways are in close competition; it results that any polypeptide chain can undergo either process depending on its structural and physicochemical features and the medium conditions. These considerations apply more correctly to protein domains than to whole proteins; in fact, it appears that in proteins containing potentially amyloidogenic domains, evolution has embedded those domains in larger intramolecular contexts hindering their tendency to aggregate. However, the aggregation propensity appears when the domain is removed from its "chaperoned" context, is mutated or cleaved. Actually, recent data suggest that natural proteins are engineered by evolution so that, at the conditions where they are synthesized and perform their functions, folding is favored over aggregation, even though the latter would be thermodynamically preferred; this can happen in several ways, including the presence of high kinetic barriers to unfolding and the evolution of eukaryotic genomes encoding both proteins with high molecular weights, that limit their propensity to grow into polymers of amyloid type (Uversky, 2002) and a large number of molecular chaperones.

The view that protein folding and aggregation are in competition to each other considers both as distinct yet not mutually excluding processes relying on a more general energy landscape including conformational states not directly involved in protein folding, yet potentially available to a polypeptide chain (Jahn and Redford, 2008) (Fig. 1, modified from Jahn and Redford, 2008). The two sides of the protein energy landscape highlight the competition between intramolecular (folding) and intermolecular (aggregation) interactions, which increases considerably the roughness of the whole landscape. The scheme depicted in Fig. 1 shows the complexity of the overall energy landscape of protein folding/aggregation, which includes some of the main conformational states a polypeptide chain can reach during its self-organization eventually culminating with the appearance of thermodynamically favored compact monomeric (folded) or polymeric (aggregated) states (Jahn and Redford, 2008). In a living environment (cell, tissue), either stable final state may be even more favored thermodynamically by the above mentioned macromolecular

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crowding, whose excluded volume effects favor stabilizing interactions of functional, or pure thermodynamic, significance. Even though there is a substantial lack of information on the energy barriers a folded or a partially unfolded protein must overcome to gain access to the conformational spaces allowing it to re-organize into aggregation nuclei, it is believed that these structural transitions can be favored, among others, by surfaces. This is a key issue, considering that in the intracellular environment an extremely large surface area is provided by macromolecules and phospholipid bilayers. Actually, either biological or synthetic surfaces, besides favoring, in special cases, protein folding (molecular chaperones), can also be potent enhancers of protein misfolding and accelerators of aggregation.

3. Protein folding/misfolding on surfaces

Most of the present knowledge on protein folding arises from a multi-disciplinary approach including the use of a variety of simulation and experimental measurements carried out on wild-type and side-directed mutants of the investigated proteins. The most widely used experimental tools include spectroscopic (CD, DLS, NMR), fluorimetric (fluorescence spectroscopy, FRET), diffraction (small angle X-ray diffraction scattering), mass spectroscopy (MALDI-TOF coupled to limited proteolysis) and mutational (protein engineering) techniques as well as theoretical and *in silico* studies (molecular dynamics simulations and model building). The experimental results provided by these techniques are of immense value, however, in general, they have been obtained in test tube experiments where a very simple environment is present (usually a buffer with a defined pH and ionic strength containing some co-solvent or denaturing agent). These conditions are very different from those found in the intracellular medium where protein folding takes place; in fact, they lack the presence of other factors potentially affecting protein folding, misfolding and aggregation such as specific protein ligands, molecular chaperones and the macromolecular crowding mentioned above, with its very large surface area including the overall macromolecular surface and that provided by the cell membranes (Ellis, 2001).

Surfaces can favor reversible unfolding/refolding of specific proteins, for example when they physiologically translocate across a membrane (Bychkova et al., 1998); in other cases, suitable surfaces or environments provided by specific macromolecules found inside a cell can favor folding of a newly synthesized or of a misfolded protein. This is the case of the large and heterogeneous family of the molecular chaperones, including the prokaryotic GroES/GroEL and DnaK/DnaJ systems as well as many eukaryotic assemblies, both intracellular (the Hsp70/Hsp40 system, other Hsp proteins and the crystallins) and extracellular (clusterin, α2-macroglobulin, haptoglobin). These molecular chaperones provide a suitable environment, in most cases a surface, where a protein can fold rapidly and efficiently still exploiting exclusively the information encoded in its amino acid sequence (Hartl and Hayer-Hartl, 2002). In most cases, the molecular chaperones are able to specifically recognize exposed highly aggregation-prone segments avoiding their inappropriate interactions with other cellular components or with similar segments exposed on other protein molecules that could possibly result in aggregate nucleation. A recent survey in the protein data bank has shown that these highly aggregation-prone segments are most often capped by basic residues. Besides hindering the mutual interaction of these stretches that could enhance the generation of aggregation nuclei, this also favors recognition of these stretches by molecular chaperones, thus featuring co-evolution of the latter (Rousseau et al., 2006a).

Another example of surfaces favoring protein folding concerns the large family of the NUPs (see above), which includes many proteins involved in key functions such as gene transcription, control of cell cycle and signal transduction (Romero et al., 1998). The molecular features of NUPs, including a low mean hydrophobicity and a high net charge, besides allowing these molecules to remain unfolded in the absence of binding partners, are also able to reduce their intrinsic tendency to aggregate in the highly crowded intracellular milieu (Minton, 1994). Here, the unstructured state of most NUPs favors their binding to the molecular chaperones during their short living time before they interact with their specific targets or are degraded (Uversky, 2002). Actually, many NUPs are "short lived" proteins and adopt specific three-dimensional structures only upon interaction with their specific targets that are thought to provide them a surface suitable to allow their folding in some "assisted" manner (Dedmon et al., 2002). It is also possible that the target protein provides some structural information needed for the specific NUP to reach its correct fold in the protein-protein complex, in particular charged and hydrophobic residues complementing its structural deficiencies. Finally, the unfolded state of NUPs favors their rapid intracellular turnover by the cellular clearance mechanisms (Wright and Dyson, 1999). The latter feature could be an advantage for certain cellular functions, providing a further level of control to enable the cell to respond rapidly, effectively and reversibly to perturbations of the cellular environment.

The huge surface extension in the intra/extracellular environment can also influence profoundly the tendency of a protein to lose its native fold and to gain alternative conformations (misfolding) favoring aggregate nucleation. For instance, peptides and proteins can interact with, and be actively recruited by, biological surfaces thus modifying their conformational states. Indeed, in the adsorbed state, the interaction of protein residues with surface-exposed hydrophobic or charged groups can induce local or more extensive unfolding populating non-native, aggregation-prone conformations (Bokvist et al., 2004). In fact, under these conditions many protein molecules will loosen their structure and externalize normally buried non-polar groups and the peptide backbone that will interact with the surface-exposed hydrophobic clusters in a process that can be different from that occurring in bulk solution and energetically favored (Sethuraman and Belfort, 2005). Such a view leads to propose that, similarly to catalyzed reactions, surfaces can favor amyloid aggregate nucleation and growth by a mechanism substantially different from that occurring in bulk solution (Zhu et al., 2002). Finally, besides populating aggregation-prone conformers possibly different from those arising in solution, surfaces can actively recruit them increasing considerably their local concentration; this effect can remarkably speed aggregate nucleation (Sethuraman and Belfort, 2005; Zhu et al., 2002) and induce structural alterations of membrane integrity (Yip et al., 2001; Porat et al., 2003; Knight and Miranker, 2004).

These considerations account for the increasing interest in investigating the physicochemical features of protein interaction with natural or artificial surfaces and the relation between the latter and protein aggregation. Indeed, many studies on heterogeneous interfaces carried out mainly using inorganic or synthetic surfaces have shown that basic physicochemical features of the surface such as net electrostatic charge, hydrophilicity or hydrophobicity affect the conformational properties of the interacting peptide/protein (Necula et al., 2002; Hoyer et al., 2004; Arce et al., 2011). These studies have raised increasing interest on the role performed in protein aggregation by biological surfaces, notably membranes, even in relation to their structure and lipid composition. In addition to membranes, other surfaces in cell or tissue can also favor protein aggregation. For example, it has been reported that beta2-microglobulin binds to the collagen triple helix and that binding affinity fluctuations could influence the

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concentration of both the wild-type and the N-truncated forms of the protein in the proximity of collagen fibers and hence its propensity to aggregate (Giorgetti et al., 2005). More recently, amyloid fibrils of beta2-microglobulin sprouting from the surface of collagen fibers have been imaged (Relini et al., 2006) providing mechanistic support to the proposed explanation of the tissue-specificity of dialysis-related amyloidosis (Homma, 1998). Other examples of biological surfaces favoring protein fibrillization include different anionic macromolecules such as glycosaminoglycans (Suk et al., 2006; Monsellier et al., 2010) and nucleic acids (Nandi and Nicole, 2004; Cherny et al., 2004). These molecules, by providing a true interface to protein molecules act as modifiers of the protein surface properties affecting protein stability and structure.

Membrane environment is also of fundamental importance for the regulation of membrane protein degradation upon co-localization of membrane proteases and their substrates; accordingly, in several cases membranes contribute to amyloid fibrillogenesis as the primary sources of the aggregating peptide monomers. Examples include the Abeta peptides resulting from APP processing, the ABri/ADan peptides resulting from BRI processing and the peptides medin and gelsolin arising from the partial proteolysis of lactadherin and wild-type gelsolin, respectively (Peng et al., 2005; Huff et al., 2003). On this aspect, the study of protein compartmentalization in discrete ordered membrane domains such as caveolae and lipid rafts can provide further clues to the regulatory effects of membrane dynamics to membrane protein turnover by proteolysis (Rushworth and Hooper, 2010). In addition, the hydrophobic interior of the plasma membrane favors structural changes in proteins and peptides and increases the strength of the secondary interactions, often leading to protein aggregation, as it has been shown for the prion protein, amylin and Abeta peptides (Bokvist et al., 2004; Yip et al., 2001; Kazlauskaite et al., 2003a; Engel et al., 2006; Lührs et al., 2006). Finally, lipid membranes can enhance protein aggregation also favoring the growth of supramolecular lipid protein complexes, as it has been reported for the islet amyloid polypeptide (Domanov and Kinnunen, 2008).

The ability of lipid membranes to unfold monomers and to nucleate and recruit amyloid oligomers suggests that membrane lipid composition is of relevance for these effects. Indeed, several reports highlight the key role of anionic surfaces and anionic phospholipid-rich membranes as triggers of protein/peptide fibrillization (Necula et al., 2002). Negatively charged membrane surfaces have also been proposed as potent inductors of beta-sheet structures by acting as conformational catalysts for amyloids (Jayakumar et al., 2004; Zhao et al., 2004). Furthermore, it has been shown that phosphatidylserine (PS)-containing liposomes induce amyloid aggregation of a variety of proteins *in vitro* (Engel et al., 2006) and that annexin 5 protects against Aβ-peptide cytotoxicity by competing at a common PS-rich site (Lee et al., 2002). Finally, it has been proposed that membranes rich in anionic phospholipids could interact with amyloid aggregates possibly by recognizing a shared fold (Zhao et al., 2004). These findings also suggest that amyloid aggregate toxicity can be different depending on the aggregate cellular localization. In fact, PS is almost exclusively found in the cytosolic leaflet of the plasma membrane in all cells other than apoptotic, cancer and vascular endothelial cells in tumors, where it moves to the outer leaflet (Utsugi et al., 1991; Ran and Thorpe, 2002); this can be of importance, considering that recent data suggest that Aβ peptide aggregates display their cytotoxic properties mainly in the intracellular location (Kaminski Schierle et al., 2011).

The above mentioned data could explain the selective anti-tumor specificity of either endostatin (Zhao et al., 2004, 2005) and the folding variants of α-lactalbumin (Svanborg et al., 2003) and apoptin (Zhang et al., 2003). It must also be reminded that amyloid aggregates are found in a wide variety of malignancies (Kazlauskaite et al., 2003a); the latter feature has led to suggest that the presence of PS at the surface of cancer cells and vascular endothelial cells in tumors could favor the formation of toxic aggregates by these anti-tumor proteins. Such idea is further supported by the observation that a number of antimicrobial peptides known to target phosphatidylglycerol in microbial membranes are also able to kill cancer cells (Hancock and Scott, 2000). Finally, recent research reports β-amyloid-induced modifications of the structure and permeability of the mitochondrial, lysosomal and ER membranes (Kaminski Schierle et al., 2011; Ferreiro et al., 2004, 2006; Aleardi et al., 2005; Caspersen et al., 2005), although a substantial lack of information still exists on the effective occurrence of the interaction of amyloid aggregates with cell organelles and on the molecular features of such interaction.

An increasing number of data highlight the importance of membrane lipid composition in modulating either protein aggregation at the membrane level and aggregate interaction with cell membranes also for what the content of cholesterol and gangliosides in concerned. It is known that APP and secretases preferentially localize into ganglioside and cholesterol-rich membrane microdomains (lipid rafts) (Lee et al., 1998; Ehahalt et al., 2003; Kakio et al., 2003). Several lines of evidence also support the idea that the pathological conversion of the cellular (PrP^c) to the scrapie (PrP^{sc}) form of the prion protein occurs at the lipid raft level (Prusiner et al., 1998; Harris, 1999; Kazlauskaite et al., 2003b; Sarnataro et al., 2004) and that the PrP^c conformation is stabilized upon association with lipid rafts in the secretory pathway (Sarnataro et al., 2004). It has therefore been proposed that aggregation of soluble Aβ peptides and the prion protein can be raft-associated processes (Ehahalt et al., 2003) and that any alteration of cholesterol homeostasis can be a shared primary cause of several neurodegenerative diseases (Kakio et al., 2003).

The interaction of Aβ peptides with the cell surface, particularly at sites rich in membrane cholesterol, has been considered an important requirement for neurotoxicity (Kakio et al., 2002; Koudinov and Koudinova, 2005). On the other hand, reduced levels of cholesterol are found in brains from AD patients (Mason et al., 1992) and a loss of cholesterol in the brain leads to neurodegeneration (Arispe and Doh, 2002). In this regard it is interesting to note that recent studies have shown that the expression of seladin-1, a desmosterol reductase involved, among others, in cholesterol synthesis, appears to be down regulated in AD brains (Benvenuti et al., 2006) even though many data indicate that increasing cholesterol in neuronal membranes results in enhanced production of Aβ peptides (reviewed in Stefani and Liguri, 2009). Recent reports also show that loss of cholesterol in neuronal membranes enhances amyloid peptide generation (Abad-Rodriguez et al., 2005) and that cell interaction with pre-fibrillar aggregates supplemented to the culture media and aggregate cytotoxicity are reduced upon enriching in cholesterol the cell membrane (Arispe and Doh, 2002; Cecchi et al., 2005). Finally, the importance of membrane cholesterol in cell membrane resistance against disruption by Aβ peptides has recently been reported by a molecular dynamics simulation study (Qui et al., 2011). The relation between cholesterol content, Aβ aggregate generation and cytotoxicity requires more extensive research; however, the data presently available support the idea that, in general, the presence of cholesterol in the cell membrane can modulate conformational changes of specific protein/peptides and that a higher membrane rigidity following increased cholesterol content can be protective against aggregate interaction with cell membranes and reduces their cytotoxicity arising from membrane integrity perturbation.

GM1, the most abundant raft ganglioside, particularly enriched in neuronal cell membranes, is the main carrier of the negative

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charge occurring on the outer leaflet of the plasma membrane of most cells. The content of gangliosides, particularly GM1 and GM2 is increased in detergent-resistant membranes (DRMs, considered to correspond functionally to the membrane rafts) purified from neuronal membranes of AD people, further supporting their importance in Aβ pathology (Molander-Melin et al., 2005). The latter is confirmed by a large body of evidence that GM1 and other gangliosides, together with their negatively charged sialic acid moieties and their lipid environment, are key sites not only for nucleation of Aβ peptide aggregates (Yuyama and Yanagisawa, 2010) but also for oligomer interaction at the cell membrane, possibly upon ganglioside clustering (Fujita et al., 2007). In particular, several studies have indicated that the preferential binding to GM1 of monomeric and oligomeric Aβ stimulates peptide fibrillization (Wakabayashi and Matsuzaki, 2007; Matsuzaki et al., 2010; Malchiodi-Albedi et al., 2010; Mclaurin et al., 1998). Finally, recent evidence indicates that Aβ deposition starts at presynaptic terminals in the AD brain and that the levels of GM1 are significantly increased in amyloid-positive synaptosomes obtained from AD brains, suggesting that age-dependent GM1 clustering at the presynaptic terminals of neurites can be a key step for Aβ deposition in AD (Yamamoto et al., 2008). Similar effects have been reported for other peptides such as amylin (Wakabayashi and Matsuzaki, 2009) and salmon calcitonin (sCTO) (Diociaiuti et al., 2006) suggesting the possibility that these effects can be somehow generalized. The importance of the negative charge carried by gangliosides is confirmed by the data indicating that treatment with neuraminidase, which removes from gangliosides the sialic acid moiety with its negative charge, inhibits amylin neurotoxicity and its associated biochemical modifications in exposed cells (Wakabayashi and Matsuzaki, 2009).

Taken together, the data on the effect of membrane cholesterol and gangliosides on protein/peptide misfolding and aggregation and oligomer interaction with the cell membrane further support the importance of lipid rafts as key sites where these processes occur. They also provide a possible rationale of the acute vulnerability of neuronal cells to amyloids considering the large surface area of these cells and the enrichment in membrane rafts of their plasma membranes.

4. The onset of protein aggregation

The generation of aggregation nuclei is considered the rate-limiting step in the onset of protein aggregation, accounting for the delay times of polymer appearance that are recorded in *in vitro* protein aggregation experiments. However, at variance with protein folding, where significant knowledge has been accumulated in the last decade, presently much less is known on the conformational states available to an aggregating polypeptide chain and on the atomic level structural features of the oligomeric assemblies arising in the early steps of aggregation. Actually, some of the energy minima in the aggregation side of the energy landscape (Fig. 1) are expected to be poorly defined due to the broad heterogeneity of unstable, highly dynamic early oligomeric states endowed with comparable free energies. On the contrary, the energy minima of the much more structurally defined, stable higher order species (protofibrils, protofilaments and mature fibrils) can be much more easily identifiable. For example, the stabilities, and hence the energy minima, of mature amyloid fibrils, and their structural variants grown at different conditions (Petkova et al., 2005) are expected to be more pronounced than those of their natively folded monomers considering the reduced molecular dynamics and the consistency of the ordered core structure of the fibrils. The nucleation-dependent polymerization mechanism of fibril growth, whose physical basis approaches that of the ordered assembly occurring in crystal growth, also supports fibril stability and represents a key difference between protein folding and aggregation.

A protein can be shifted from the folding to the aggregation side of its energy landscape by enhancing the factors that promote more consistently aggregation over folding (Fig. 1). For example, protein stability can be reduced upon modification of the environmental conditions (temperature, pH, medium polarity, ionic strength) or the structural features of the specific protein/peptide (mutations, truncations, chemical modifications), or even by merely increasing its concentration. The latter can result in aggregate nucleation when the concentration of the nucleation precursors exceeds a critical threshold (reviewed in Chiti and Dobson, 2006). In most cases, protein aggregation can be started in the presence of mildly destabilizing medium conditions; these include a mild shift of the temperature or the pH or the presence of moderate amounts of denaturing agents or of co-solvents such as trifluoroethanol. The latter modify the dielectric constant of the medium thus increasing the stability of the secondary contacts while reducing that of the tertiary interactions in the folded protein (Colon and Kelly, 1992; Chiti et al., 1999; McParland et al., 2000). Under these conditions, the destabilized protein opens its closely packed folded structure and populates partially unfolded states exposing hydrophobic patches and aggregation-prone regions normally buried into the hydrophobic core, including the peptide backbone, normally shielded by the side chains. These partially unfolded structures can bear similarities to the folding intermediates (Chiti et al., 2003; Apetri et al., 2004; Uversky et al., 2001) or to some of the near-native conformations in dynamic equilibrium with a fully folded protein. The link between native state dynamics and fibrillar aggregation of a protein has been highlighted by mass spectrometry experiments in the case of lysozyme. It has been shown that the relative instability of the partially folded precursors of the latter is the driving force allowing them to re-organize into still poorly stable, and often thermodynamically disfavored, transient aggregation nuclei rich in beta structure, established between stretches of polypeptide chains in the beta strand conformation (Dumoulin et al., 2005).

The kinetics of nucleation of the aggregates of a given protein/peptide can depend on the protein concentration and structure as well as on the medium conditions affecting the height of the energetic barrier to be overcome for amyloid aggregate nucleation can occur; at variance, the subsequent thermodynamically and kinetically favored nuclei elongation proceeds until completion of fibril assembly. In some cases, instead of aggregation nuclei, spherical oligomers and other pre-fibrillar forms, including curvy protofibrils, can be formed, apparently resulting from a nucleation-independent path in the absence of any lag phase (Harper and Lansbury, 1997; Modler et al., 2003; Gosal et al., 2005; Smith et al., 2006a). In most cases, it is not clear the difference between oligomers and aggregation nuclei nor whether oligomers are on-pathway products, growing by direct binding of monomers, or off-pathway entities representing dead-end reversible intermediates (Gosal et al., 2005; Bitan et al., 2003; Baskakov et al., 2002; Necula et al., 2007). For example, beta-2-microglobulin and other proteins can exist in different aggregation states depending on protein structural features and medium conditions. Some of these states (oligomeric species and beaded protofibrils) are off-pathway products (Petkova et al., 2005; Gosal et al., 2005; Kad et al., 2003) arising from the polymerization of partially folded species retaining significant amount of native structure and involving some of the native beta strands (McParland et al., 2002); the latter species are different from the oligomers appearing in the path of fibril formation, which involves extensive structural rearrangement until the stable cross-beta structure of amyloid fibrils is reached (Smith et al., 2006a).

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The presence of significant native structure in the conformational states of a protein/peptide undergoing the first steps of aggregation is further supported by recent findings showing that, at least in some cases, a protein can aggregate by initially populating monomeric or oligomeric states where it maintains substantially its natively folded structure before undergoing structural rearrangements into amyloids (see later). In addition to the above mentioned natively folded beaded protofibrils of beta-2-microglobulin, other proteins have been proposed to undergo ordered fibrillar polymerization retaining their native fold at least in the initial steps. These include the serpins (reviewed in Lomas and Carrell, 2002), where a domain swapping mechanism has been shown, transthyretin, for which a model with direct stacking of natively folded monomeric subunits has been proposed (Serag et al., 2002), T7 endonuclease I (Guo and Eisenberg, 2006), and p13suc1 (Rousseau et al., 2006b). Apart serpins, where the whole polymerization process occurs without gross modification of the protein globular structure, in all these cases marked modifications of the secondary structure accompany the final products of protein polymerization. A similar mechanism could also underlie the generation of native-like fibrils by the yeast prion Ure2p (Bousset et al., 2002) and the first step of the amyloid aggregation of the acylphosphatase from the hyperthermophile *Sulfolobus solfataricus* (Plakoutsi et al., 2005).

5. General structural features of amyloid oligomers

Due to the variable cytotoxicity of structurally different amyloid assemblies, the study of the amyloid structure is becoming a main focus in the investigation of the molecular basis of amyloid diseases. As reported above, amyloid nucleation is considered a key event in the onset of protein aggregation and often the rate-limiting step of fibril growth. However, reduced knowledge is currently available on the structural and biophysical features of pre-fibrillar assemblies, particularly amyloid oligomers grown at different conditions.

Many *in vitro* studies performed in the last decade have shown the morphological modifications occurring in amyloid assemblies grown from different disease-related and disease-unrelated peptides and proteins undergoing fibrillization (Quintas et al., 2001; Relini et al., 2004), and a theoretical frame of these modifications has been provided by molecular dynamics simulation studies (Cheon et al., 2007). These studies have shown that several more-or-less defined steps are involved in amyloid fibril growth. EM and AFM images have shown that, in many cases, at the onset of the aggregation process transient, unstable, roundish or tubular particles 2.5–5.0 nm in diameter generally, but with exceptions, enriched in β-structure, often called "amorphous aggregates" are present (Relini et al., 2004; Cheon et al., 2007; Lashuel et al., 2002; Poirier et al., 2002). These species are characterized as protein/peptide oligomeric assemblies that frequently associate to each other into bead-like chains, small annular rings ("doughnuts" or "pores"), or curvy protofibrils. The latter often appear to be precursors of highly organized amyloids such as large closed rings, ribbons (Relini et al., 2004; Lashuel et al., 2002; Poirier et al., 2002; Lin et al., 2001) or longer protofilaments eventually generating mature fibrils [reviewed in Caughey and Lansbury, 2003]. Overall, the data emerging from these and other studies depict amyloid fibril growth as a hierarchical process with "dead end" routes in which both the monomer basic structure and the environmental parameters concur to determine the general conformational properties of the misfolded/unfolded monomers initially present. These properties dictate, to a large extent, the structural modifications resulting in monomer arrangement into less or more compact oligomers with variable hydrophobic exposure and subsequently in oligomer re-organization into increasingly more complex, ordered and stable β-sheet-rich assemblies. That such a process can be physically approached to crystal growth is shown by the data indicating that seeds of polymorphic mature fibrils or their intermediates grown from the same peptide/protein or of fibrils grown from different monomers often are able to speed up the growth of oligomers and mature fibrils (Baldwin et al., 2011); furthermore, these species, though sharing the general basic cross-beta structure of amyloids, maintain the same specific structural and morphological features of the seeding assemblies.

The presence of pre fibrillar amyloids has been shown in the aggregation path of many proteins and peptides either associated (Aβ peptides, α-synuclein, superoxide dismutase, huntingtin, the androgen receptor, amylin, transthyretin, serum amyloid A, and others) (Quintas et al., 2001; Cheon et al., 2007; Lashuel et al., 2002; Poirier et al., 2002) or not associated (Bucciantini et al., 2002, 2004; Sirangelo et al., 2004; Relini et al., 2004; Ceru et al., 2008) with amyloid disease. Although most of these assemblies are considered intermediates arising in the path of fibrillization, some of them, such as the frequently imaged small annular oligomers ("doughnuts") mentioned above, could be "dead end" products of the process or, at any rate, structurally and functionally distinct types of amyloid oligomers (Kayed et al., 2009). Finally, soluble oligomers arising in the aggregation of several peptides and proteins have also been repeatedly detected in, and in some cases purified from, cultured cells and tissues where the monomeric precursors are expressed (Cleary et al., 2005; Lesné et al., 2006; Billings et al., 2005; Koffie et al., 2009). These data reinforce the idea that amyloid oligomers are really formed *in vivo* and are directly associated with cell/tissue impairment (Koffie et al., 2009).

The increasing body of knowledge supporting the cytotoxicity of early aggregates of peptides and proteins either associated or not associated with amyloid disease implies that amyloid cytotoxicity arises from shared characteristics of the supramolecular structure of the aggregates rather than from any specific feature of the amino acid sequences of their parent soluble polypeptides (Bucciantini et al., 2004; Demuro et al., 2005). This concept appears in contrast with the properties of functional proteins, whose native structures and biological functions are specifically determined by their amino acid sequences. It can be concluded that neither the structure nor the functional properties (notably cytotoxicity) of protein conformational states other than the native one are directly determined, as in the latter, by the specific interactions among side-chains (reviewed in Chiti and Dobson, 2006).

In spite of the increasing number of techniques used to get information on oligomer structural features and growth (see below), the experimental information accumulated in the past years on these issues is still remarkably poor. Several models have been proposed to describe mechanistically the self-organization into oligomeric assemblies of misfolded monomeric peptides/proteins. According to the widely accepted model known as "nucleated conformational conversion" (Carulla et al., 2009; Petty and Decatur, 2005), a group of unstable misfolded monomers in solution coalesce generating relatively disordered "molten" or "metastable" oligomers whose constituting monomers undergo extensive structural reorganization which templates new incoming misfolded monomers. Such a reorganization and monomer recruitment results in increasingly structured oligomers and higher order assemblies eventually culminating with the appearance of mature fibrils (Cerdà-Costa et al., 2007). Although this model has been supported by experimental and theoretical observations (Plakoutsi et al., 2005; Serio et al., 2000; Bader et al., 2006), an in depth experimentally based description at the molecular level of the phenomenon, particularly of its earliest steps, remains largely elusive.

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In most of the investigated systems, experimental and theoretical observations support a generic "two step" mechanism of oligomer growth that appears highly dependent on the degree of hydrophobicity and the extent of exposed hydrophobic surface of the misfolded monomers. Hydrophobicity favors monomer coalescence into initially disordered oligomers, whose subsequent conformational reorganization gives rise to increasingly ordered species that sequester more efficiently a progressively more compact hydrophobic core (Plakoutsi et al., 2005; Cheon et al., 2007; Carulla et al., 2009; Petty and Decatur, 2005; Cerdà-Costa et al., 2007; Serio et al., 2000; Bader et al., 2006; Nguyen et al., 2007). In case of insufficient monomer hydrophobicity, the first coalescence step can be skipped and the monomers can organize directly into ordered oligomers where secondary interactions such as hydrogen bonds predominate (Cheon et al., 2007). An example of this "one step" mechanism can be provided by aggregation from natively folded proteins [Chiti and Dobson, 2009], in which the degree of exposure of hydrophobic residues in the aggregating monomers is likely to be low. This view suggests that the intermolecular hydrophobic interactions, together with protein concentration and temperature, are among the major determinants of the rate of the hydrophobic collapse of the misfolded monomers into different types of oligomers. It also supports the idea that the sequence of appearance of disordered–ordered, or only ordered, oligomers depends on the balance between the rapidly forming intermolecular hydrophobic interactions and the slower exchange of the directional hydrogen bonds into the assembling oligomers (Bader et al., 2006). Finally, it provides a theoretical frame to the widely accepted ideas that aggregation is a generic property of virtually every polypeptide chain and that amyloids grown from structurally different peptides and proteins share a basic structural model.

The importance of the relative contributions of the hydrophobic forces and the hydrogen bonds rationalizes the findings that different solution conditions favoring or disfavoring hydrogen bonding can promote the growth of different aggregates (fibrillar or non-fibrillar) with similar β-sheet content, yet with distinct morphological features (Calamai et al., 2005). Therefore, it provides a molecular basis to the polymorphism of amyloid oligomers grown from the same peptide/protein under different solution conditions or in the presence of differing destabilizing agents (temperature, pressure, urea, co-solvents, ionic strength, and others). The importance of oligomer polymorphism is increasingly recognized, explaining several observations, such as the propagation of prion strain infectivity and other protein polymorphisms (Petkova et al., 2005; Calamai et al., 2005; Chien et al., 2004; Jones and Surewicz, 2005), the variable cytotoxicity of amyloids grown from the same peptides/proteins at different conditions (Kayed et al., 2009; Chafekar et al., 2008; Deshpande et al., 2006; Iijima et al., 2008), the appearance of in-path or off-path intermediates of fibril growth (Poirier et al., 2002; Gosal et al., 2005; Iijima et al., 2008; Dusa et al., 2006), and the structural heterogeneity of amyloid fibrils and their precursors grown from the same peptide/protein under different environmental conditions (Gosal et al., 2005; Mayer-Luheman et al., 2006; Goldsbury et al., 2005) (see Par. 9).

6. Several newly introduced techniques can be used to study the structure of amyloid oligomers

As pointed out above, in spite of the considerable information currently available on the cytotoxicity of amyloid pre-fibrillar aggregates, the heterogeneity, remarkable instability and intrinsically disordered nature of these species makes it very difficult to get solid data on their conformational features. An important contribution to unravel this point has came from the use of conformational antibodies raised against amyloid fibrils and their precursors that are able to recognize specific structural features in different forms of these assemblies and hence to discriminate among them. In 2002 a seminal study reported the generation of an antibody recognizing a generic conformational epitope in amyloid fibril, thus highlighting for the first time the presence of a shared conformation in fibrils grown from different peptides and proteins (O'Nuallain and Wetzel, 2002). This knowledge was implemented and substantiated by subsequent reports describing the generation of antibodies able to specifically recognize amyloid oligomers grown from differing peptides and proteins instead than mature fibrils (Kayed et al., 2003) or to cross-react with either amyloid pores and with pores formed by pore-forming proteins (Yoshiike et al., 2007). Another recent report deals with the generation of an anti-Aβ amyloid fibril antibody that recognizes a shared, sequence-independent, conformational epitope present in amyloid fibrils grown from different peptides (Kayed et al., 2007). This antibody also recognizes soluble Aβ fibrillar oligomers but not apparently similar pre fibrillar oligomers, indicating the existence of structurally distinct families of amyloid oligomers with potentially different cytotoxicities (Par. 9). These data suggest the existence of at least two alternative aggregation nuclei for amyloid fibrils grown from Aβ and, possibly, other peptides: one type evolving into mature fibrils directly but only after extensive structural reorganization, and another type, possibly the true fibril precursor, growing into increasingly sized aggregates by addition of monomers that acquire a new structure when incorporated into the oligomer. Finally, a recent study reports the generation of conformation-dependent monoclonal antibodies able to distinguish between different subsets of replicating strains of conformers in the same population of pre-fibrillar Aβ oligomers displaying different size distributions. These data indicate that Aβ oligomer structure varies with size and confirm the existence of structural polymorphisms in amyloid oligomers (Kayed et al., 2010) (Par. 9).

In general, the use of conformational antibodies recognizing specifically amyloid oligomers, pre-fibrillar assemblies or mature fibrils has provided a valuable tool to distinguish these distinct entities, clearly showing the existence of differences in their structural features (Glabe, 2004; Mamikonyan et al., 2007; Kayed and Glabe, 2006). It has also allowed to definitively demonstrate (i) that the same protein/peptide can generate oligomeric and pre-fibrillar assemblies apparently undistinguishable, yet with differences in their fine structural details (Kayed et al., 2007; Kumar and Udgaonkar, 2009), (ii) that, conversely, structural similarities are present in comparable assemblies grown from different peptides and proteins (Yoshiike et al., 2007; Kayed et al., 2007; Glabe, 2004); (iii) that immunological tools such as monoclonal and polyclonal antibodies, nanobodies, or others can be of key importance to study amyloid polymorphism. Finally, it has provided knowledge showing that the same protein/peptide at the onset of aggregation can generate oligomeric and pre-fibrillar assemblies with distinctly different structural features (Kayed et al., 2007, 2010; Kumar and Udgaonkar, 2009); accordingly, monoclonal conformational antibodies provide a tool to investigate the structural modifications underlying the hierarchical growth of amyloid fibrils as well as oligomer structural heterogeneity.

Besides the above described immunological tools, *in silico* molecular dynamics simulations (Berryman et al., 2011) and traditional high resolution imaging techniques are also of great value to get information on the structural features and polymorphism of fibril nuclei, oligomers and other amyloid assemblies (reviewed in Dobson, 2003). These include electron and atomic force microscopy (Dobson, 2003), X-ray crystallography and other recently introduced biophysical tools, such as single-molecule spectroscopic (notably fluorescence) techniques (Orte et al., 2008;

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Calamai and Pavone, 2011; Kamibnski-Schierle et al., 2011), ESI/MS (Smith et al., 2006a), SAXS in solution (Giehm et al., 2011; Oliveira et al., 2009) and ss-NMR ((Mustata et al., 2009), reviewed in Langkilde and Vestergaard, 2009). For example, a recent SAXS study of α-synuclein fibrillation in solution has revealed the presence of a wreath-shaped oligomer made of around 16 monomers with a central pore. The oligomer appears to behave as an in-path fibril building block by a stacking process and displays intrinsic cytotoxicity most likely through membrane permeabilization (Oliveira et al., 2009).

Overall, the data obtained by these immunological and biophysical techniques indicate that pre-fibrillar aggregates of different proteins and peptides share some basic structural features that are different from those displayed by the folded monomers or their fibrillar counterparts. These features can include the different exposure to the aqueous environment of hydrophobic patches normally buried into the compact structure of the monomeric parent protein following its unfolding. For example, extensive investigation on alpha-synuclein has shown that its transiently formed oligomeric species are rich in beta-sheet, expose hydrophobic clusters and display a partially folded structure (Uversky et al., 2001; Dusa et al., 2006). On this aspect, chemical modifications and, more generally, altered environmental conditions can play a pivotal role in aggregate nucleation and growth; in fact, they can not only promote/hinder protein misfolding and aggregation but also affect the conformational features of the aggregation nuclei thus targeting them to organize into fibril precursors and mature fibrils with different structures, stabilities and cytotoxicities (Chen and Kokholyan, 2005; Danzer et al., 2007; Bravo et al., 2008; Bolognesi et al., 2010). The conformational features of amyloid oligomers besides explaining their intrinsic instability also provide clues to explain their tendency, and that of other unstable pre-fibrillar aggregates, to interact inappropriately with cellular components and, accordingly, their toxic potential.

7. Amyloid fibrils can be toxic either directly or as a source of toxic species in tissue

As reported above, presently the most toxic species amongst amyloids are considered the early oligomers and other pre-fibrillar aggregates whereas amyloid fibrils are taken as by far less cytotoxic or even inert reservoirs of toxic species and, as such, with protective significance for the cell. However, in some cases, direct fibril cytotoxicity (Gharibyan et al., 2007; Novitskaya et al., 2006; Bucciantini et al., 2012), or cytotoxicity associated with fibril assembly and growth on lipid membranes (Engel et al., 2008), has been reported, supporting the idea that any oversimplification of this theme can be misleading.

Even though the amyloid cascade hypothesis remains the first choice to explain AD pathogenesis, it is possible that the effective importance of non-fibrillar precursors of amyloid as key players of cell/tissue impairment *in vivo* has been overemphasized and that the scenario is much more complex *in vivo*. Actually, a recent re-evaluation of the amyloid cascade hypothesis proposes that three key events are likely to occur in sequence in the aged brain developing sporadic AD: (i) an initiating injury (including a number of different noxious stimuli favoring Aβ production and extracellular Aβ accumulation as amyloid deposits and toxic oligomers); (ii) the resulting inflammatory response triggered by the microglia and (iii) the subsequent functional changes of the brain cells (Herrup, 2010). This new view integrates the amyloid hypothesis cascade into an amyloid deposition cycle where the amyloid plaque pathology, stemming from extracellular Aβ accumulation, by reinforcing microglial activation is strictly correlated with, but distinct from, the fundamental steps of AD pathogenesis mentioned above. Extracellular Aβ accumulation can also depend on specific neuronal functional features, as shown by recent data indicating that in APP transgenic mice the regional vulnerability to Aβ deposition is modulated by the level of neuronal activity (Bero et al., 2011).

In addition to trigger a noxious inflammatory response in tissue, amyloid fibrils can affect cell viability also as providers of toxic species. That fibrillar deposits can leak toxic species *in vitro* and *in vivo* is supported by several recent investigations. A first study concerns the positive effect of sulfated polysaccharides on Aβ42 assembly into highly stable, thin and harmless amyloid fibrils with very reduced hydrophobic exposure and minimal tendency to spontaneously disassemble (Bravo et al., 2008). The conclusions of such study agree with the general idea that polysaccharides promote the formation of innocuous forms of amyloid oligomers and fibrils possibly through the formation of glycated derivatives; the cross-links in the latter could stabilize oligomers and fibrils by making them more rigid and stable and hence less prone to interact with cell membranes (oligomers) or to leak oligomers (fibrils) (DeGroot, 2004). A second study reports that Aβ fibrils grown *in vitro* are destabilized by the interaction with vesicles made from biologically relevant lipids, including brain lipid extracts. The authors found that, under these conditions, the fibrils were disassembled into heterogeneous populations of neurotoxic oligomers and protofibrils; these appeared very similar to those preceding fibril appearance in the forward aggregation process and were internalized by exposed neurons (Martins et al., 2008). Another investigation, carried out by an array tomography approach, imaged a sharp reduction of dendritic spine density in neuronal cells surrounded by amyloid plaques in tissue slices taken from AD transgenic mice. In this study, the authors have shown that such a reduction depends strictly on the gradient of Aβ oligomers irradiating from the fibrillar plaques deposited in tissue, strongly suggesting that the latter can be a source of toxic species (Koffie et al., 2009). These and other evidences agree with the idea that mature fibrils can be not so stable as usually believed and can be disassembled under suitable mild conditions. They also support the suggestion to consider with care the information obtained by chromatographic techniques, electrophoresis and AFM on the structure and molecular mass of the amyloids, particularly oligomers, present in a sample; in fact, the AFM, chromatographic or electrophoresis support, or the detergent micelles could disassemble into smaller oligomers large aggregates present in the original sample (Hepler et al., 2006). Recent data on solubilization of beta2-microglobulin fibrils into toxic oligomers by a number of tetracyclines (Giorgetti et al., 2011) further reinforce the idea that amyloid fibrils, under a panel of different conditions, can be partially disassembled or, at least, can leak toxic oligomers. In other words, the effect of surfaces, previously known to favor protein aggregation into oligomers and mature fibrils (Stefani, 2007) in some cases can result in the opposite, inducing fibril disassembly.

In conclusion, the data indicating both direct and indirect fibril toxicity imply that amyloid plaques in tissue should not be considered always protective as recruiters of toxic assemblies arising from peptide/protein misfolding; rather, they could also be potential sources of toxicity. Actually, recent data suggest that fibrils of the same protein/peptide grown from structurally different oligomers or deposited under differing conditions, and hence with variable structures and stabilities, can display different cytotoxicities possibly resulting from their differing abilities to leak toxic oligomers. These data concern monomer/oligomer recycling within amyloid fibrils both *in vitro* (Carulla et al., 2005) and *in vivo* (Shankar et al., 2009), the different stabilities and biophysical properties of fibrils grown from the same monomer at different conditions (Smith et al., 2006b) and the apparent leakage

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of toxic oligomers around fibrillar deposits *in vivo* (Koffie et al., 2009). Accordingly, spontaneous fibril decomposition or fragmentation in tissue can be seen as a possible important determinant of amyloid cytotoxicity.

8. Biological surfaces are primary sites of amyloid interaction and toxicity

Besides recruiting protein monomers favoring their misfolding and aggregation, surfaces, notably cell membranes, can also bind actively the unstable oligomeric assemblies preceding the appearance of mature amyloid fibrils. The importance of the relation between membrane lipid composition and the ability of early amyloid aggregates to bind to, and to disassemble, lipid membranes has been extensively investigated (Par. 3). As pointed out above, many studies highlight the important role played by anionic surfaces and by membranes containing anionic phospholipids as sites not only of protein/peptide misfolding, but also of oligomer interaction; in fact, clusters of negative charges, together with the strong electrostatic field therein, can be sites of preferential interaction with pre-fibrillar aggregates resulting in phospholipid bilayer or cell membrane destabilization (Arispe et al., 1993; Mirzabekov et al., 1996; Lin et al., 1997; Kourie, 1999; Volles and Lansbury, 2001; Hirakura and Kagan, 2001; Kourie and Henry, 2002; Ding et al., 2002; Hirakura et al., 2002). These effects modify membrane permeability and impair the function of specific membrane-bound proteins and signaling pathways (Hou et al., 2005; Mattson, 1999). The role of cholesterol and gangliosides as modulators not only of Abeta peptide generation and aggregation but also of oligomer interaction with the membrane and internalization has been extensively studied as well (Par. 3). Recently, it has been reported that pre-fibrillar aggregates supplemented to the culture media display reduced interaction with the cells, internalization and cytotoxicity upon enriching in cholesterol the cell membrane whereas opposite effects were found in cholesterol-depleted cells (Crameri et al., 2006; Cecchi et al., 2007, 2008). Although requiring more extensive research, these data support the idea that, in general, a higher membrane rigidity following increased cholesterol content can hinder aggregate interaction with the cell membranes and internalization thus improving membrane resistance against disassembly. On turn, oligomer interaction can by itself provide toxicity by altering membrane fluidity (Hou et al., 2005) and integrity, with possible phospholipid pull-out from the bilayer.

Amyloid interaction with the cell membrane raises another question debated since long time: are there on the cell membrane specific receptors for amyloids responsible for the amyloid–membrane interaction? The surface of the cell membrane is crowded of protein molecules. It has been estimated that the average plasma membrane surface is around 2000 μm^2 with a density of membrane proteins averaging about 20,000 molecules/μm^2, accounting for a total 40×10^6 protein molecules per cell surface (Simons and Ehahalt, 2002). It is therefore conceivable that amyloid oligomers may interact more or less specifically with some of the membrane proteins they contact.

In the past, several cell surface proteins have been considered as possible candidate receptors of Aβ aggregates. These receptors could be specific for the shared cross-beta fold rather than for any peculiar structural feature of the Aβ peptides although, in some cases, they could also be monomer-specific, as in the Aβ-APP, Aβ-TNFR1 or Aβ-PrP interactions proposed to be at the origin of Aβ cytotoxicity (Shadek et al., 2006; He et al., 2004; Laurén et al., 2009). Since 1996, the receptor for advanced glycation end products (RAGE) has been proposed as a major candidate as amyloid receptor (Yan et al., 1996). RAGE is increased in systemic amyloidoses, is able to interact with amyloid assemblies made from serum amyloid A, amylin and prion-derived peptides (Yan et al., 2000) and appears involved in Alzheimer's and Creutzfeldt-Jacob diseases (Yan et al., 1998; Sasaki et al., 2002). By competing for ligand binding with cell-surface RAGE, its plasma soluble form, sRAGE, might trap circulating ligands preventing their interaction with cell surface receptors. Actually, sRAGE appears protective against cytotoxicity of transthyretin aggregates (Monteiro et al., 2006) and its high plasma levels are associated with a reduced risk of several diseases including AD. Increasing plasma sRAGE is therefore considered a promising therapeutic target potentially preventing vascular damage and neurodegeneration (Geroldi et al., 2006).

In addition to RAGE, several cell surface proteins, including voltage-gated (Hou et al., 2007) or ligand-gated calcium channels such as the glutamate NMDA and AMPA receptors (Hsieh et al., 2006; De Felice et al., 2007; Pellistri et al., 2008) have also been considered as possible receptors or specific interaction sites for amyloids. In addition, tissue-type plasminogen activator (tPA) has been proposed as a multiligand specific for the cross-β structure (Kranenburg et al., 2002). Finally, increasing evidence suggests that additional neuronal binding sites, including anionic lipid clusters (see above), could be involved in the interaction with the plasma membrane of amyloid aggregates made from different peptides and proteins (Lacor et al., 2004). Such an idea is supported by the finding that any rise of the content of negatively charged lipids results in increased channel formation by amyloids in synthetic lipid bilayers (Jayakumar et al., 2004).

The presence of specific effects mediated by the preferential, or even specific, interaction with membrane proteins could, at least in part, explain the variable vulnerability to amyloids of different cell types (Cecchi et al., 2005). However, in spite of these and other data on specific interaction sites for amyloids, the tendency of early amyloid aggregates to interact with synthetic lipid membranes supports the idea that the interaction can be non-specific but, possibly, modulated by the membrane lipid content (Par. 3), such an interaction can also, by itself, impair cell viability by altering membrane structure and permeability.

The "channel hypothesis" of amyloid toxicity, proposed since 1993, states that toxic amyloid aggregates form non-specific pore-like channels in the membranes of the exposed cells (Arispe et al., 1993, reviewed in Kourie and Henry, 2002). This proposal is now supported by studies carried out both on synthetic phospholipid bilayers and on cell membranes showing that the function of specific membrane proteins is impaired by the interaction with misfolded species or their oligomers (Hou et al., 2005; Mattson, 1999, reviewed in Kourie and Henry, 2002). For example, the size-dependent permeabilization of artificial vesicles by protofibrillar α-synuclein suggests that vesicle permeabilization may occur mainly as a result of a specific membrane perturbation *via* the formation of pores at least 2.5 nm in diameter coexisting with fibrils, raising the possibility that, at the conditions found in the cytoplasm, these "pores" may be stable enough to be the true pathogenic species in Parkinson's disease (Lashuel et al., 2002). The ability of most amyloidogenic peptides and proteins to form "pores" in their aggregation path supports the idea that membrane permeabilization can result even *in vivo* from the presence of such species and be the key trigger of the sufferance and death of exposed cells (reviewed in Caughey and Lansbury, 2003). Overall, the channel hypothesis and the data showing that protein aggregation at, and oligomer interaction with, the cell membrane can disassemble membrane structural organization destroying its selective permeability (see below), further confirm the key importance, for amyloid cytotoxicity, of the early cell membrane perturbation by amyloids.

The presence of toxic aggregates inside or outside the cells, together with their interaction with cell membranes can impair a

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number of functions ultimately leading to cell death by apoptosis or, less frequently, by necrosis (Ceru et al., 2008; Chung et al., 2003b; Watt et al., 1994; Morishima et al., 2001; Velez-Pardo et al., 2001; Ross, 2002). This is true even for aggregates formed from proteins not associated with amyloid disease, featuring cytotoxicity as a generic property of all amyloid aggregates possibly arising from their shared cross-beta structure (Bucciantini et al., 2004; Sirangelo et al., 2004, reviewed in Stefani and Dobson, 2003; Chiti and Dobson, 2006). In most cases, initial perturbations of fundamental cellular conditions such as redox status and free Ca^{2+} levels appear to underlie the impairment of cell function induced by the aggregates (Kayed et al., 2003; Ceru et al., 2008; Demuro et al., 2005; Azimov et al., 2001; Zhu et al., 2000; Kourie, 2001; Butterfield et al., 2001; Milhavet and Lehmann, 2002).

In general, in diseased tissue oxidative stress results pruimarily from the inflammatory response to the presence of amyloid deposits. Intracellular oxidative stress in cells exposed to toxic aggregates has also been related to some form of destabilization of cell membranes resulting in the loss not only of selective permeability but also of appropriate regulation of membrane proteins such as specific enzymes, receptors and ion pumps (Hou et al., 2005; Mattson, 1999, reviewed in Kourie and Henry, 2002). Actually, oxidative stress has been considered, at least in part, a consequence of Ca^{2+} entry into cells following non-specific membrane permeabilization by pre-fibrillar aggregates. The latter can result from structural modifications of the membrane following the interaction with the aggregates or their monomers (see above), from membrane lipid peroxidation or from chemical modification of membrane ion pumps (Butterfield et al., 2001; Milhavet and Lehmann, 2002; Hyun et al., 2002; Varadarajan et al., 2000, reviewed in Stefani and Dobson, 2003). Ca^{2+} ingress can also be the result of oligomer interaction with specific Ca^{2+} channels (see Par. 8). The increased levels of intracellular free Ca^{2+} can stimulate the oxidative metabolism providing the ATP needed to support the activity of membrane ion pumps involved in clearing the excess Ca^{2+}. The resulting ROS elevation could, in turn, oxidize membrane pumps and their regulatory proteins resulting in further free Ca^{2+} increase (Squier, 2001) and in uncontrolled ingress of Ca^{2+} into, and release of pro-apoptotic signals from, the mitochondria. Such a chain of events could explain the relation between ROS, increase of intracellular free Ca^{2+}, mitochondrial damage and apoptosis described in cells exposed to toxic amyloid aggregates (Bucciantini et al., 2004; Varadarajan et al., 2000; Kawahara et al., 2000; Kawahara, 2004; reviewed in Stefani and Dobson, 2003). Data on the biochemical features possibly accounting for the different vulnerability of varying cell types exposed to the same toxic pre-fibrillar aggregates highlight significant correlations between cell resistance, cholesterol content, total antioxidant capacity and Ca^{2+}-ATPase activity (Cecchi et al., 2005). Finally, as reported above, modifications of the structure and permeability of the mitochondrial, lysosomal and ER membranes induced by internalized β-amyloid can contribute in several ways to amyloid cytotoxicity, including further deregulation of intracellular Ca^{2+} levels (Ferreiro et al., 2004, 2006; Aleardi et al., 2005; Caspersen et al., 2005) (Fig. 2).

9. Oligomer/fibril polymorphism underlies different amyloid interaction with the cell membrane and toxicity

Even though, in general, pre-fibrillar amyloid aggregates display generic cytotoxicity, the work carried out in the last few years increasingly supports the idea that amyloid assemblies even of the same protein/peptide carrying differences in their basic supramolecular organization and biophysical properties can display different cytotoxicities. Accordingly, the latter can no more be considered directly associated to the shared basic structure of amyloids, as previously stated (Bucciantini et al., 2002; Kayed et al., 2003), rather, it appears to result from the specific arrangements of the constituting misfolded monomers and, accordingly, from their different conformational and biophysical properties.

The ability of differing conditions to generate structurally different oligomers endowed with variable cytotoxicity appears of particular importance for what the role of amyloid oligomers as key players of cell/tissue impairment is concerned. In most cases, as reported above (Par. 8), amyloid cytotoxicity appears primarily associated to the ability of amyloid assemblies to interact with, and to permeabilize, cell membranes, particularly the plasma membrane, and is therefore restricted to the unstable pre-fibrillar assemblies [reviewed in Stefani, 2007]. In addition, the data on amyloid fibril stability and ability to leak toxic species suggest that structurally different fibrils of the same protein/peptide grown under variable conditions from structurally different oligomers can display variable cytotoxicity or protection against the appearance of toxic oligomers (Par. 7). Either behavior could be associated to a different fibril stability, and hence to the relative frequency of spontaneous, lipid-, or ligand-induced fibril breakage (Xue et al., 2009). In fact, less stable and more flexible fibrils are expected to undergo increased rate of rupture, resulting either in increased leakage of toxic oligomers even *in vivo* (Koffie et al., 2009; Martins et al., 2008) and/or in a higher number of fibril fragments, and hence of free ends at which misfolded monomers can bind before undergoing oligomerization into more toxic assemblies. More information on a wider population of fibrils grown under different conditions from a variety of peptides and proteins is needed to explore the generality of these considerations. Nevertheless, an increasing number of coherent data depict amyloid deposits in tissue no more in terms of inert remnants of toxic species; more convincingly, together with misfolded monomers and toxic oligomers, they appear key components of complex equilibria and, as such, as possible reservoirs of toxicity (Carulla et al., 2005), providing additional clues to explain the molecular basis of amyloid toxicity in tissue.

Oligomer (and mature fibril) heterogeneity and polymorphism are undoubtedly key issues in view of the fact that these species can affect directly or indirectly the load of toxic amyloids. As reported above (Par. 5, 7), many data indicate that, at different conditions, the same peptide/protein can start its aggregation path by generating oligomers with different conformational features eventually resulting in polymorphic mature fibrils (Kumar and Udgaonkar, 2009; Lee et al., 2007; Meinhardt et al., 2009); the same can be true for the aggregation under similar conditions of proteins/peptides carrying specific amino acid substitutions or chemical modifications modifying the physicochemical properties of their polypeptide chains (Chen and Kokholyan, 2005). Besides the environmental conditions, specific structural features of the monomer can determine the way it aggregates under suitable conditions and the toxicity of its aggregates. These conformational peculiarities are not necessarily associated with the presence of specific amino acid substitutions but in some cases can be much more subtle and can be traced back to the folding/aggregation landscape of a protein/peptide, where alternative conformations with similar free energies can be populated. For example, recently it has been reported that after release from the cell membrane upon APP processing, Aβ42 can exist into two different conformations; a harmless, normally prevalent, physiological conformation with a turn at positions 25–26 unable to aggregate by itself, and a second, less populated conformation, with a turn at positions 22–23, that aggregates into toxic oligomers (Masuda et al., 2009).

A number of recently reported studies have investigated the structure–toxicity relation of amyloids indirectly, for example by establishing a link between the stability and the ability to impair

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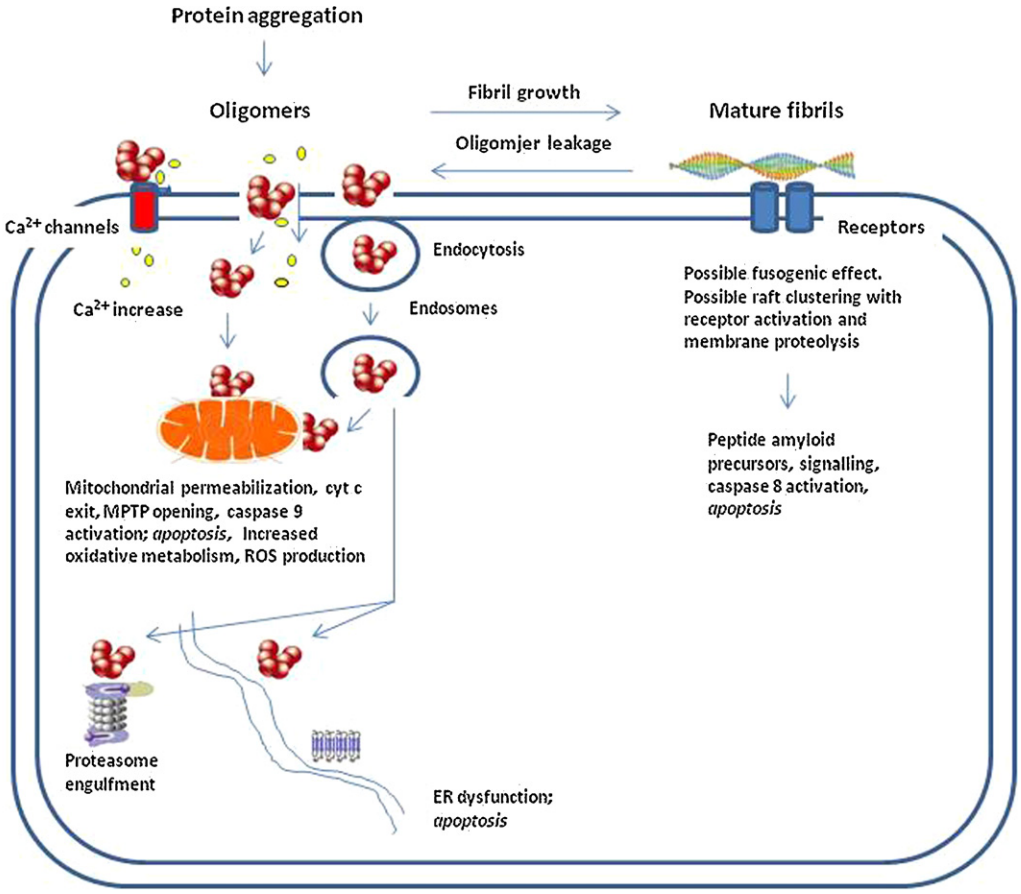

Fig. 2. Schematic cartoon where various possible mechanisms of penetration of amyloid assemblies inside the cell and their consequences to cell function and viability are depicted. Amyloid oligomers as soon as they are generated in the extracellular space or arise from mature fibril leakage can penetrate inside the cell with different mechanisms (passive diffusion across the lipid bilayer, endocytosis) and, upon entry, impair cell viability in various ways. The physical interaction of the oligomers with the plasma membrane can also occur at the level of specific interaction sites such as ligand-gated (Sasaki et al., 2002; Monteiro et al., 2006; Geroldi et al., 2006) or voltage-gated (Yan et al., 1998) calcium channels, RAGE (Simons and Ehahalt, 2002; Shadek et al., 2006; He et al., 2004; Laurén et al., 2009), tissue plasminogen activator (Hou et al., 2007) and possibly others modifying their function. Oligomer–bilayer interaction can, by itself, disrupt or disassemble the bilayer with loss of selective permeability and free ingress of external Ca^{2+} (Campioni et al., 2010). The latter can activate the membrane and ER ATPases as well as the oxidative metabolism providing the ATP needed to booster the ATPases themselves, resulting in ROS increase and oxidative stress thus enhancing intracellular protein misfolding and impairing the activity of the cellular ATPases (Butterfield et al., 2001). The internalized oligomers can also interact with the ER and the mitochondria membrane favoring MPTP opening, cyt c release and caspase 12 activation, respectively, triggering intrinsic apoptosis. Finally, unfolded proteins and their oligomers can engulf the proteasome with loss of functionality and further increase of the intracellular load of misfolded/unfolded polypeptides. Mature fibrils usually do not interact heavily with the plasma membrane. However a number of data support the idea that mature fibrils in some cases do interact with the cell membrane, possibly at the GM1 level (Novitskaya et al., 2006), displaying fusogenic effects, inducing raft clustering and activation of specific receptors (Sasaki et al., 2002; Monteiro et al., 2006; Geroldi et al., 2006) or signaling pathways (Ding et al., 2002), resulting in extrinsic apoptosis (Novitskaya et al., 2006; Bucciantini et al., 2012). Other mechanisms of fibril/oligomer interaction with the cell membrane may also occur; moreover, in tissue these effects can be made much more complex by the intervention of other factors such as the build-up of a detrimental inflammatory response to the presence of amyloid fibrils with secretion of toxic cytokines and others.

cell viability of amyloids grown from the same peptide/protein at different conditions and hence displaying subtle structural differences. One of these studies has shown that cytotoxicity can be significantly related to the extent of hydrophobic exposure, that can vary in oligomers of the same protein grown at different conditions (Bolognesi et al., 2010). Another study has shown that Aβ fibrils grown normally or under agitation, although apparently similar in morphology, display different stabilities to guanidinium hydrochloride and significantly different toxicities, the less stable being more cytotoxic; moreover, intermediates of fibril growth arising at different aggregation conditions displayed significantly different structural rearrangements (Lee et al., 2007). Considering that many data support the idea that Aβ interaction with the cell membrane implies peptide conformational changes and is important in determining Aβ toxicity (Bokvist et al., 2004), it could be proposed that the toxicity of any Aβ species can, at least in part, be related to its ability to change structure on, or within, the cell membrane and hence, ultimately, to its flexibility. Similar conclusions have been drawn in a more recent research on the relation between different conformations of huntingtin exon-1 with an expanded polyglutamine stretch and their relative toxicities both to cultured cells and in animal tissue. At different temperatures (37 °C or 4 °C) the polypeptide aggregated *in vitro* into differing conformations, a more flexible and highly cytotoxic one, with exposed β-sheets and loop-turns, or a more rigid and substantially less toxic conformation, with extended and buried β-sheet (Nekooki-Machida et al., 2009). The authors subsequently extended these findings to mice models of Huntington disease by extracting amyloid fibrils from diseased and non-diseased brain regions. They found that huntingtin fibrils extracted from diseased brain areas showed the same structural features as those displayed by the toxic conformation generated *in vitro*; conversely, fibrils extracted from unaffected brain regions, such as the hippocampus, were more rigid and substantially harmless, similarly to *in vitro* grown fibrils with the less cytotoxic conformation (Nekooki-Machida et al., 2009). These data suggest that the same protein in different brain areas experiences differing conditions that modulate its stability and aggregation pathway eventually resulting into

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mature fibrils with different structural, physical and biological properties. Finally, it has been reported that mild detergent treatment releases soluble 30–50 nm-sized annular alpha-synuclein oligomers from glial cytoplasmic inclusions purified from brain tissue of people affected by multiple system atrophy, showing that in this α-synucleinopathy the pathological aggregates can be a source of annular alpha-synuclein species. In contrast to pathological alpha-synuclein, recombinant alpha-synuclein yielded only spherical oligomers after detergent treatment, indicating a greater propensity of the pathological protein to form stable annular oligomers (Pountney et al., 2005).

Our most recent results further confirm and extend the generality of these considerations providing clues on the structural features of amyloid oligomers and their relation to toxicity. We previously reported that HypF-N, a bacterial protein not associated with any amyloid disease, aggregates into pre-fibrillar and fibrillar amyloid assemblies undistinguishable from those associated with amyloid diseases and endowed with the same cytotoxicity (Bucciantini et al., 2002, 2004). Very recently, we have found that, at different destabilizing conditions (presence of trifluoroacetic acid or trifluoroethanol), HypF-N misfolds and generates morphologically similar oligomers, yet with different structural features in terms of stability, exposure of hydrophobic surface, compactness and flexibility. We also found that the less stable and compact oligomers grew into mature fibrils whereas the more stable and compact ones never organized into mature fibrils but eventually assembled into curvy protofibrils with no further evolution (Campioni et al., 2010). The two types of oligomers displayed differing cytotoxicities and abilities to interact with, to permeabilize, and to cross the cell membrane, the more stable and compact, as well as less flexible and hydrophobic, assemblies being substantially non-toxic. Subsequent mapping by fluorescence spectroscopy of the regions of the polypeptide chain involved in the intermolecular interactions in either type of oligomer showed that (i) these regions closely corresponded to the most hydrophobic ones; (ii) the main conformational difference between the two types of oligomers was the degree of burial of hydrophobic residues and, as a consequence, the density of packing and flexibility as well as the extent of the solvent-exposed hydrophobic area (Campioni et al., 2010; Zampagni et al., 2011). These data establish a direct link between general structural features of pre-fibrillar oligomers and the ability of the latter to grow into distinct, stable amyloid assemblies; they also provide clues on the relation between oligomer conformational features and their ability to stick to, to disassemble and to permeabilize the cell membrane impairing cell viability, further highlighting the importance of the concept of amyloid polymorphism.

Finally, in addition to the oligomer conformational features and biophysical properties, the structural and biochemical properties of the cell membranes also play an important role in modulating the cytotoxic potential of amyloid oligomers, as pointed out in Par. 3 and 8. Actually, a large number of studies appeared in the last ten years have provided convincing data indicating that the ability of amyloids to grow on, to interact with, and to permeabilize cell membranes depends strictly on the membrane biophysical properties (Par. 3). The importance, for amyloid cytotoxicity, of the relation between oligomer (hydrophobic exposure, rigidity) and membrane (curvature, compactness, fluidity, hydrophobicity, density of charge) biophysical features must be better assessed. To do this, very recently we have implemented the data on the different types of HypF-N oligomers mentioned above by investigating the effect on the oligomer structure–toxicity relation of the cell membrane biophysical properties, stemming from its lipid composition. In particular, we checked the ability of either HypF-N oligomer (toxic or non-toxic) to bind to, to permeabilize, and to cross the plasma membrane of the same cell line with normal, reduced or increased content of either cholesterol or GM1, or of both. We found that increasing membrane cholesterol made safe the oligomers toxic to untreated cells by hindering their interaction with the phospholipid bilayer, whereas in cells with decreased membrane cholesterol the oligomers non-toxic to untreated cells became able to stick with, and to penetrate within,

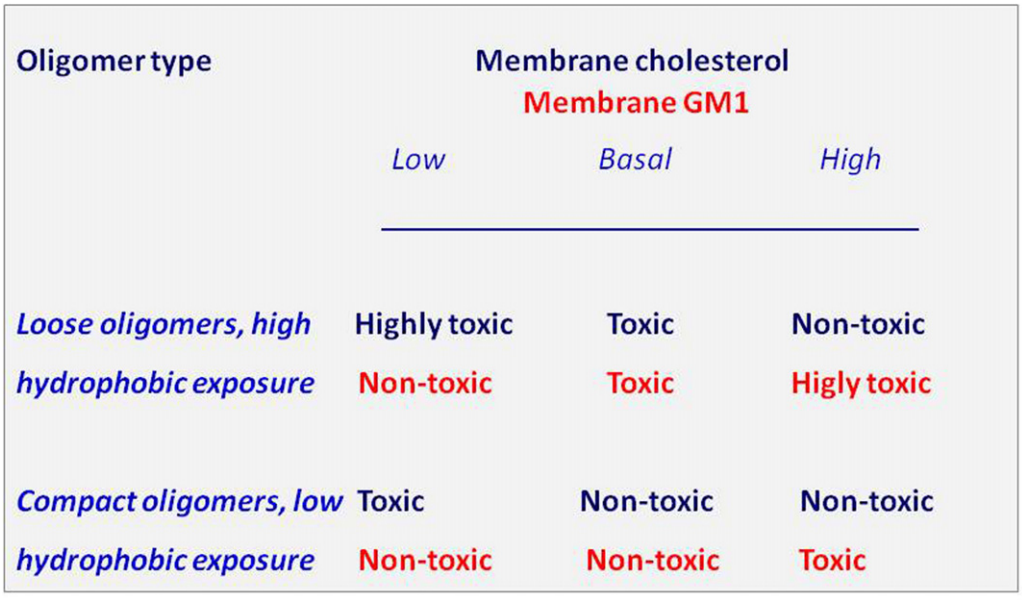

Fig. 3. Oligomers with different physicochemical features display variable cytotoxicity. However, the latter also depends on the biophysical properties of the cell membrane where oligomer interaction occurs resulting from the membrane biochemical features and lipid content. Accordingly, the concept of oligomer cytotoxicity starting at the level of the cell membrane must be considered as relative, arising from the interplay of both oligomer and membrane biophysics.

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the bilayer impairing cell viability; opposite effects were found following modulation of the content of membrane GM1 (Evangelisti et al., 2012) (Fig. 3). These and our previously published results (Campioni et al., 2010; Zampagni et al., 2011) can lead to propose that the notion of oligomer cytotoxicity should be reconsidered; in fact, the latter could be the net result of the interplay between oligomer and membrane structural features determining the mode and the extent of oligomer–membrane interaction and the severity of the resulting membrane structural perturbations. Besides establishing a more complex link between oligomer/membrane structural features and the resulting cytotoxicity, these data also provide a rationale contributing to explain the different vulnerability to the same amyloids of different cell types, either cultured or in tissue (Cecchi et al., 2005; Capetillo-Zarate et al., 2006) as well as the increased vulnerability to tissue amyloid deposition in aged people.

Taken together, these results can tentatively be generalized, leading to propose that the conformational and structural features, at least in terms of exposure of hydrophobic surface and flexibility, of amyloid aggregates grown from proteins/peptides involved in amyloid diseases can remarkably affect the way and the extent they interact with the cell membrane and the resulting consequences in terms of membrane structural and functional perturbations. Conversely, any modification of membrane lipid composition and biophysical properties in cells of different types, in different functional states or of different age, possibly resulting from altered lipid metabolism, can affect profoundly the ability of the membrane itself to interact with a given type of amyloid, and must therefore be considered a major contributor to cytotoxicity.

10. Concluding remarks

The data reported in the last few years have made increasingly evident that amyloid fibrils and their precursors grown from structurally different monomers of the same peptide/protein or from monomers misfolded at different environmental conditions can display differing structural features. Such a conformational polymorphism appears of great importance in determining the cytotoxic potential of amyloids. On this line of evidence, it is increasingly recognized that amyloid fibrils, previously considered as harmless reservoirs of toxic oligomers, in some cases can be a source of toxic species to exposed cells depending on their structural and stability properties stemming from the environmental conditions under which they were grown and deposited. Mature fibrils can leak toxic oligomers following their fragmentation by thermal motion mechanical shearing or interaction with disassembling surfaces or molecules; conversely, it can also be considered that the increased number of free ends in fragmented fibrils can, in principle, recruit more efficiently misfolded monomers hindering their assembly into toxic oligomers. This view still assigns to the oligomers the role of direct cytotoxic entities; however, also in this case, structural polymorphism can play a key role in modulating the ability of these assemblies to interact with cell membranes and hence their toxic effects to exposed cells. Unfortunately, at variance with mature fibrils, getting information on the structural features of fibril precursors, in particular the early oligomeric assemblies, is made challenging by the heterogeneous, transient, unstable and highly flexible nature of the latter. Nevertheless, new experimental approaches are starting to fill the gap, providing information on the oligomer structure–toxicity relation. Finally, the importance of the biophysical features of synthetic or biological membranes in determining the way amyloids can grow on, or interact with, them is being increasingly taken into consideration.

Overall, all these points can be considered as details of a highly incomplete, yet extremely complex, picture, where amyloid polymorphism and the cell membrane biochemical and biophysical features both contribute, though differently in different *in vitro* systems and tissues, to the overall cytotoxicity of these assemblies. Such a view leads to consider amyloid oligomer cytotoxicity as the result of a complex interplay between oligomer and membrane biophysical features and hence as a relative concept rather than an inherent property. Besides reconciling the alternative views of amyloid fibril toxicity *versus* amyloid fibril safety, these data provide new clues to explain the molecular determinants of sporadic amyloid diseases, the variable susceptibility of different cell types to amyloid cytotoxicity and the lack of any direct relation between amyloid load and severity of the clinical signs (Dickson, 1995). We are still at the beginning in the way that, hopefully, will lead to unravel the oligomer mystery, however, the present intense research warrants that in a near future we will be able to get solid knowledge in this field, opening new scenarios that will be of great value for rational drug design to treat efficiently amyloid diseases.

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