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The amyloid-cell membrane system. The interplay between the biophysical features of oligomers/fibrils and cell membrane defines amyloid toxicity

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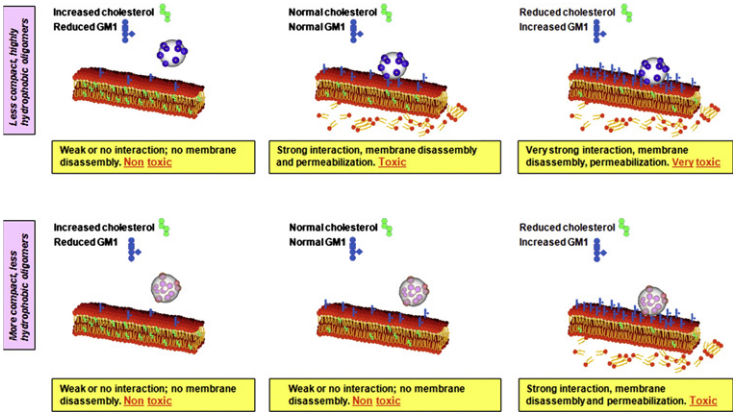
HIGHLIGHTS

- Amyloid aggregation and aggregate cytotoxicity are generic properties of any polypeptide chain.
- A close relation between amyloid biophysical features and cytotoxicity does exist.
- Biological surfaces are important inductors of protein misfolding/aggregation and sites of amyloid interaction.
- A close relation between cell membrane biophysical features and amyloid cytotoxicity does exist.
- Amyloid cytotoxicity appears as an emerging property depending on the biophysical features of the amyloid-membrane system.

GRAPHICAL ABSTRACT



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ABSTRACT

Amyloid cytotoxicity, structure and polymorphisms are themes of increasing importance. Present knowledge considers any peptide/protein able to undergo misfolding and aggregation generating intrinsically cytotoxic amyloids. It also describes growth and structure of amyloid fibrils and their possible disassembly, whereas reduced information is available on oligomer structure. Recent research has highlighted the importance of the environmental conditions as determinants of the amyloid polymorphisms and cytotoxicity. Another body of evidence describes chemical or biological surfaces as key sites of protein misfolding and aggregation or of interaction with amyloids and the resulting biochemical modifications inducing cell functional/viability impairment. In particular, the membrane lipid composition appears to modulate cell response to toxic amyloids, thus contributing to explain the variable vulnerability to the same amyloids of different cell types. Finally, a recent view describes amyloid toxicity as an emerging property dependent on a complex interplay between the biophysical features of early aggregates and the interacting cell membranes taken as a whole system.

1. Introduction

A broad range of human diseases arises from the failure of a specific peptide or protein to adopt, or remain in, its native functional

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conformational state. These pathological conditions are generally referred to as protein misfolding diseases. The largest group of misfolding diseases is represented by amyloidoses [1], that are characterized by the presence of deposits of fibrillar aggregates found as intracellular inclusions or extracellular plaques whose main constituent is a specific peptide or protein, different in the varying disorders [2]. These diseases include a range of sporadic, familial or transmissible degenerative conditions, some of which affect the brain and the central nervous system (e.g. Alzheimer's and Creutzfeldt–Jakob diseases), while others involve peripheral tissues and organs such as the liver, heart and spleen (e.g. systemic amyloidoses and type II diabetes) [3]. In addition, there are other diseases (Parkinson's and Huntington's diseases) characterized by the presence of intracellular, rather than extracellular, deposits localized in the cytoplasm, or less frequently in the nucleus, in the form of specific inclusions known as aggresomes, Lewi bodies, or others. The various peptides and proteins associated with amyloid diseases have no obvious similarities in size, amino acid composition, sequence or structure. The features of the soluble forms of the proteins involved in the amyloidoses are varied, ranging from intact globular proteins to largely unstructured peptide molecules, but the aggregated forms have many common characteristics [4]. In particular, the amyloid fibrils into which they convert are very similar in their morphology and internal structure, sharing a common β sheet-rich motif [3]. Such structure confers to the amyloid fibril specific biophysical features and a variety of tinctorial properties, notably staining with thioflavin T (ThT) and Congo red (CR) and is considered the main structural hallmark of the amyloid aggregates [1].

Until the end of 1990s the data available and the genetics of amyloid diseases supported a quite general consensus that the amyloid fibrils were the main toxic species in amyloid plaques, even though no mechanistic data supporting fibril cytotoxicity had been clearly reported. Such a scenario provided a theoretical frame to understand the molecular basis of amyloid diseases and stimulated the exploration of therapeutic approaches mainly focused at hindering amyloid fibril growth and deposition. However, at the end of the 1990s the attention shifted to the cytotoxicity of amyloid fibril precursors, notably amyloid oligomers and protofibrils [5]. Indeed, the severity of cognitive impairment in Alzheimer's disease (AD), the most severe form of senile dementia associated with the presence, in the brain parenchyma, of amyloid-like deposits of the Aβ peptides, appears to correlate with the levels of aggregates of low-molecular-weight species of Aβ rather than with the amount of fibrillar deposits [6]. The appearance of pre-fibrillar aggregates in tissues precedes the expression of the clinical phenotype thus explaining the lack of relationship found in most cases between extent of amyloid deposits and severity of the clinical symptoms [7]. In addition, transgenic mice show deficits in cognitive impairment, cell function, and synaptic plasticity before the accumulation of significant quantities of amyloid plaques [8]. At the present, the pivotal role of amyloid oligomers as key players of amyloid cytotoxicity is widely recognized and the amyloid cascade hypothesis, initially stated to explain AD, has subsequently been extended to cover other neurodegenerative diseases with amyloid deposits [9]. 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, β2-microglobulin, transthyretin and others [10–18]. A further step forward was done in 2002, when data were reported suggesting that cytotoxicity is a generic property of amyloid oligomers, which is associated with a shared "toxic" fold [19,20]. These data have led to propose that the pre-fibrillar assemblies share basic structural features that, at least in most cases, seem to underlie common biochemical mechanisms of cytotoxicity. This new view shifted the target of pharmacological research aimed at finding molecules useful to prevent cell/tissue impairment in amyloid diseases from counteracting fibril growth to hindering the appearance of amyloid oligomers.

The toxicity of early oligomers appears to result from their intrinsic ability to impair fundamental cellular processes most often upon their interaction with cell membranes and subsequent disassembly of the lipid bilayer. It has been hypothesized that pre-fibrillar aggregates impair cell function because they expose on their surface an array of groups that are normally hidden in globular proteins or dispersed in unfolded peptides or proteins. The exposed regions can be rich in hydrophobic groups able to stick onto, and to penetrate inside, the cell membrane. Actually, pre-fibrillar assemblies have been shown to interact with synthetic phospholipid bilayers and with cell membranes, possibly destabilizing them and impairing the function of specific membrane-bound proteins [21–23]. The data on aggregate interaction with the cell membrane underscore a key role of the resulting free Ca^{2+} level alterations with subsequent intracellular redox status modifications, suggesting a mechanism of cell death possibly shared among pre-fibrillar aggregates of most peptides and proteins [19,21,23–25].

On the other hand, peptides and proteins can interact with, and be actively recruited by, biological membranes, thus modifying their conformational states which result in non-native, aggregation-prone conformations [26]. Such a view has led to the proposal that surfaces can catalyze amyloid aggregate nucleation and growth by a mechanism that can be different from that observed in the bulk solution (reviewed in [27,28]) (see below). Moreover, the biochemical and biophysical features of the cell membrane can affect the conformation, distribution and proteolytic processing of membrane proteins involved in neurodegenerative conditions such as AD or prion diseases. In addition, the protein/peptide interaction with the cell surface, particularly with cholesterol and ganglioside-rich areas such as lipid rafts, is considered an important requirement for cytotoxicity (reviewed in [28–30]). This review will focus the role of biological surfaces, notably phospholipid bilayers, as key players of protein destabilization and aggregation as well as of aggregate recruitment. The importance of the interplay between membrane and oligomer physicochemical features modulating amyloid cytotoxicity in the membrane–oligomer complex will be also discussed.

2. Structural features of amyloid aggregates

The observation that proteins associated with amyloid diseases, thought displaying in their soluble native state a very different nature, can generate similar fibrillar forms, encouraged the proposal that there are strong relationships between the intrinsic structure of the amyloid fibrils and in the mechanism by which they are formed [31]. Actually, the ability of polypeptide chains to form amyloid structures is not restricted to the relatively small number of proteins associated with recognized clinical disorders, but it now seems to be a generic feature of polypeptide chains [31,32]. The core structure of the fibrils seems to be stabilized primarily by interactions, particularly hydrogen bonds, involving the polypeptide main chain. Because the main chain is common to all polypeptides, this observation explains why fibrils formed from polypeptides of very different sequence bear significant similarities [4,32]. The fibrils can be imaged in vitro using transmission electron microscopy (TEM) or atomic force microscopy (AFM). By these techniques it has been shown that amyloid fibrils usually consist of a number (typically 2–6) of protofilaments, each about 2–5 nm in diameter, that are often twisted around each other to form supercoiled rope-like structures typically 7–13 nm in width [4,33] or that associate laterally to form long ribbons that are 2–5 nm thick and up to 30 nm wide [34,35]. Circular dichroism, Fourier transform infra-red spectroscopy and solid-state NMR and X-ray fiber diffraction data have shown that in each individual protofilament the protein or peptide molecules are arranged so that the polypeptide chain forms β-strands that stack in register and run perpendicular to the long axis of the fibril to generate what is described as a cross-β structure [4]. Usually, each strand of in register β-sheets makes its full complement of hydrogen bonds with the strands above and below it in the fibril. However, it has been recently reported an out of register amyloid fibril structure, in which each antiparallel pair of strands is out of register with neighboring pairs by six

residues, leaving dangling hydrogen bonds [36]. Protofibrillar intermediates are structurally closest to the mature fibrils. They are thought to represent late-stage intermediates and can be distinguished from oligomers by their elongated, linear shape [37]. However, protofibrils lack the very high order and periodic symmetry of mature fibrils, are curvilinear, thinner (usually less than 10 nm in diameter) and shorter (usually below 400 nm in length) than the latter.

The generic amyloid structure contrasts strongly with the highly unique globular structures of most natural protein, where the interactions associated with the very specific packing of the side chains seem to ignore the main-chain preferences [32,38]. In a globular protein, the polypeptide main chain and the hydrophobic side chains are largely hidden within the folded structure. Only when they become exposed, for example upon partial unfolding in mildly denaturing conditions (such as low pH) or fragmentation (by proteolysis), the conversion into amyloid fibrils is possible. 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 [39,40], and a theoretical frame of these modifications has been provided by molecular dynamics simulation studies [41].

3. Amyloid growth proceeds through several steps

The body of data arisen from these investigation supports the involvement of several more-or-less defined steps in amyloid fibril growth. Often, transient, unstable, roundish or tubular particles 2.5–5.0 nm in diameter generally enriched in β-structure, often called "amorphous aggregates" are present at the onset of the aggregation process, as shown by TEM and AFM images [16,40–42]. More recently, small-angle X-ray scattering (SAXS), which allows assessing the oligomeric state of proteins and protein complexes has provided another valuable tool to get low-resolution images of early aggregates preceding mature fibril assembly [43,44]. These species are characterized as protein/peptide oligomeric assemblies that frequently associate to each other into bead-like chains or small annular rings ("doughnuts" or "pores"). The latter often appear to be precursors of highly organized amyloids such as large closed rings, ribbons [16,23,40,42] or longer protofilaments eventually generating mature fibrils (reviewed in [45]). These data testify that amyloid oligomers are highly polymorphic states, i.e. that oligomers grown from a specific polypeptide chain can vary in structure [37,46]. Finally, many data, such as the intrinsic fluorescence and those obtained with the fluorescent dye 1-anilino-8-naphthalene sulfonate (ANS) clearly indicate that oligomeric intermediates derived from Aβ, lysozyme and several other polypeptide chains display solvent-accessible hydrophobic surface, thus explaining their intrinsic instability and low persistence.

Recently, the atomic structure of unusual oligomeric species obtained from αB crystallin has been described by X-ray diffraction [47]. These toxic oligomers, named cylindrin, are cylindrically shaped β barrel formed from antiparallel, extended protein strands with the cylinder filled with packed side chains. When unrolled into a β-sheet, each antiparallel pair of strands in the cylindrin sheet is out of register, similarly to out of register amyloid fibrils. This means that a cylindrin unrolled into a sheet would not be an in register structure, ready to bond with an identical sheet to form the steric zipper spine of an amyloid fiber. The transition from cylindrin to steric zipper involves breaking of hydrogen bonds and re-registration of the strands into an in-register structure [47].

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 and variously structured oligomers with variable hydrophobic exposure and subsequently in oligomer reorganization into increasingly more complex, ordered and stable β-sheet-rich assemblies.

4. Biophysics of protein aggregation

As reported above, the intrinsic tendency to aggregate into amyloid assemblies of proteins/peptides is only marginally related to the specific amino acid sequence; rather, it is a general property of the peptide backbone arising from the intrinsic tendency of the latter to self-organize into ordered polymeric assemblies. The latter are stabilized by inter-molecular hydrogen bonds between parallel or anti-parallel β-strands [1,3,19,48] resulting in ordered arrangement (cross-β structure) which characterizes the amyloid fold (reviewed in [33]). However, in spite of the key importance of the main chain contacts, the side chain sequence is also important for amyloid fibril growth, as it determines the environmental conditions favoring aggregation. This view considers peptides and proteins as evolutionarily-selected polymers endowed with the intrinsic property to self-organize into higher order assemblies and has led to the concepts of the possible amyloid origin of protein folds [49] and of the amylome as the universe of proteins that are capable to form amyloid-like fibrils [50]. It also suggests that protein folding and protein aggregation, though distinct processes, are indeed in competition to each other and that the environmental conditions dictate which one is favored for a given polypeptide chain. On this basis, extensive studies have been carried out in vitro to investigate the nature of the transition between natively folded states and soluble aggregate-precursor states, and between the latter and mature amyloid fibrils and the factors affecting all of these [51]. Recent data indicate that these dangerous aggregation-prone states, although similar to the native conformation, display altered surface charge distribution, alternative β-sheet topologies and increased solvent exposure of hydrophobic surfaces and of aggregation-prone regions of the sequence [52]. However, under normal physiological conditions, specific energy barriers prevent the protein structural ensemble from populating those dangerous aggregation-prone states allowing the protein to undergo functional dynamics while remaining soluble and without a significant risk of misfolding and aggregation into non-functional and potentially toxic species [52].

That protein folding and aggregation can be competing processes is confirmed by several theoretical and experimental evidences. For example, the choice between folding and aggregation can be modulated by the high intra/extracellular concentration of macromolecules (macromolecular crowding). In fact, the excluded volume effects can affect thermodynamically the conformational states of proteins favoring, to a different extent for any given protein, compact structures, including both natively folded and aggregated states [53]. Protein folding and aggregation are also affected similarly by several physicochemical parameters of the polypeptide chain including propensity to gain secondary structure, net charge and relative hydrophobicity [54]. The importance of these properties for protein folding and aggregation is supported by the structural adaptations found in the natively unfolded (or unstructured) proteins (NUPs), whose unfolded state is maintained by specific physicochemical adaptations such as a low mean hydrophobicity and a high net charge that differentiate them from proteins undergoing normal folding [55].

5. The formation of aggregation nuclei is needed for aggregation to occur

From the above considerations it results that any polypeptide chain, in principle endowed with the intrinsic property to aggregate, can effectively undergo folding or aggregation depending on its structural and physicochemical features and the environmental and medium conditions [56]. Protein aggregation starts with the more or less rapid appearance of aggregation nuclei, whose growth is considered the

rate-limiting step of the process. However, reduced knowledge is presently available on the conformational states available to an aggregating polypeptide chain and on the structural features at the atomic level of the oligomeric assemblies arising in the early aggregation steps. Actually, it can be expected that, due to their broad heterogeneity, some of the unstable, highly dynamic early oligomeric states endowed with comparable free energies corresponding to energy minima in the aggregation side of the energy landscape of protein folding and aggregation are poorly defined [57]. On the contrary, the energy minima of the stable higher order species (protofibrils, protofilaments and mature fibrils) can be much more structurally defined; in the case of mature fibrils and their structural variants grown at different conditions [58], these minima can be deeper than those of the natively folded monomers due to the reduced molecular dynamics and the consistency of the ordered structure of the fibril core. Fibril stability stems also from the physical basis of the nucleation-dependent polymerization mechanism of fibril polymerization, which recalls that occurring in crystal growth, which represents a key difference with respect to protein folding (reviewed in [59]).

6. Many factors affect protein choice between folding and aggregation

A protein can be shifted from folding to aggregation by enhancing the factors that promote more consistently the latter over the former. This can be done in several ways, for example by reducing protein stability or peptide solubility following modification of the environmental and medium conditions or of protein chemical properties (mutations, truncations, chemical modifications), or even by merely increasing peptide/protein concentration (see above). The ensuing aggregate nucleation results when the concentration of the nucleation precursors exceeds a critical threshold (reviewed in [1]). This is further supported by recent data on the physiological significance of amyloid in the pituitary, whose secretory granules store several peptide hormones in an aggregated form, whose growth is most likely favored by the high local hormone concentration [60]. Protein aggregation can be favored by mild destabilizing conditions including a moderate shift of temperature or pH or the presence of reduced amounts of denaturing agents or of organic co-solvents. The latter modify the dielectric constant of the medium, increasing the stability of the secondary, and reducing the stability of the tertiary, interactions in the folded protein [61–63] resulting in partial unfolding/misfolding. The so-generated unstable structures can bear similarities to folding intermediates [54,64,65] or to some of the many near-native conformations in dynamic equilibrium to each other and with the natively folded protein that represent the conformationally heterogeneous population of the molecules of a folded protein in dynamic equilibrium in solution.

Most often, protein concentration, protein structure and medium conditions are among the factors affecting the height of the energetic barrier to amyloid aggregate nucleation and hence its kinetics, whereas the subsequent thermodynamically and kinetically favored extension of the nuclei proceeds until the completion of fibril growth. In some cases spherical oligomers and other pre-fibrillar forms, including curvy protofibrils, can be formed instead of aggregation nuclei, apparently resulting from a nucleation-independent path in the absence of any lag phase [66–69]; however, it remains poorly defined the difference between oligomers and aggregation nuclei and often it is not clear whether oligomers are on-pathway intermediates growing by direct binding of previously or subsequently unfolded monomers, or off-pathway dead-end reversible intermediates [67,70–72]. For example, β2-microglobulin and other proteins can exist in different aggregation states; some of these are off-pathway products (oligomers and beaded protofibrils) arising from the polymerization of partially folded species retaining significant native structure [61,67,73,74] whereas others are different from the oligomers appearing in the path of fibril growth and involve extensive structural rearrangement [69].

That the conformational states of a protein/peptide undergoing the onset of aggregation can maintain significant native structure is also supported by data showing that in some cases a protein can aggregate from monomeric or oligomeric states where the natively folded structure is largely maintained before undergoing structural rearrangements into amyloids. Ordered fibrillar polymerization keeping the native fold at least in the initial steps is shown by several proteins, including the serpins (reviewed in [75]), transthyretin [76], T7 endonuclease I [77], and p13suc1 [78]. A similar mechanism could also work in the growth of native-like fibrils of the yeast prion Ure2p [79] and in the first step of aggregation of acylphosphatase from *Sulfolobus solfataricus* [80]. Apart serpins, in all these cases marked modifications of the secondary, in addition to the tertiary structure are found in the final fibrillar products.

7. Only limited information is available on oligomer structural features

In spite of the increasing number of techniques used to get information on oligomer structural features and growth, much must still be learnt on these issues. The large body of data of the last twelve years has led to propose several models to describe mechanistically the way misfolded monomeric peptides/proteins self-organize into oligomeric assemblies. The widely accepted "nucleated conformational conversion" model [81,82] envisages the coalescence of a group of unstable misfolded monomers in solution, which produces relatively disordered "molten" or "metastable" oligomers where the monomers undergo subsequent extensive structural reorganization; the latter, usually are steadily enriched in β-structure giving rise to increasingly ordered species that sequester more efficiently a progressively more compact hydrophobic core; the process results in increasingly structured oligomers and higher order assemblies eventually organizing into mature fibrils [83]. This model, though supported by experimental and theoretical observations [41,80,84–86], still lacks an in depth experimentally-based description of the process at the molecular level, particularly of its earliest steps.

The generic "two step" mechanism of oligomer growth mentioned above is supported by experimental and theoretical observations that highlight its strict dependence on the overall hydrophobicity, and particularly on the hydrophobic exposed surface of the misfolded monomers. However, when the latter is inadequate, the system can skip the first coalescence step and the monomers can organize directly into ordered oligomers strongly enriched in hydrogen bond-stabilized secondary interactions [41]. Such a "one step" mechanism can be found in the aggregation from natively folded proteins mentioned above, in which the degree of exposure of hydrophobic residues in the aggregating monomers is expected to be low.

This view highlights the importance of the intermolecular hydrophobic interactions as major determinants of the rate of the hydrophobic collapse of the misfolded monomers into different types of oligomers. It also suggests that the appearance of "disordered–ordered" or "only ordered" oligomers results from some balance, into the assembling oligomers, of the relative contributions of different forces comprising primarily the slower exchange of the more ordered directional hydrogen bonds and the rapidly forming less ordered intermolecular hydrophobic interactions [84]. The importance of the balance of the two types of forces has recently been shown to be at the basis of the choice of a polypeptide chain between folding to native state or misfolding to the competing amyloid structures. In fact, the minima of the protein free energy landscape for folding and misfolding tend to be dominated by hydrophobic and by hydrogen bonding interactions, respectively [87]. Accordingly, the interactions at the basis of the competition between protein folding and misfolding are primarily determined by the hydrophobic interactions between side-chains (folded states) or by the backbone intermolecular interactions through hydrogen bonding (amyloid fibrils). It also rationalizes the findings

that different solution conditions favoring or disfavoring hydrogen bonding can promote the growth of morphologically distinct fibrillar or non-fibrillar aggregates yet with similar β-sheet content [88]; more generally, it provides a molecular scenario to explain the polymorphism of amyloid assemblies grown from the same peptide/protein under different environmental conditions (temperature, pH, ionic strength) or in the presence of differing destabilizing agents (pressure, urea, co-solvents and others) (see later).

8. Surfaces can favor protein aggregation

Most of the data on protein aggregation stem from in vitro experiments where the peptide/protein is made to aggregate by modifying any of the aforementioned parameters. However, the contribution of surfaces, both synthetical and biological, another important factor acting in vivo together with macromolecular crowding (see above), has been most often neglected. Surfaces, play a key role among the factors potentially favoring or disfavoring protein aggregation. Polypeptide chains are synthesized, fold and operate in a chemically very complex intra- or extracellular environment, where they are in close contact with other molecules, macromolecules and biological surfaces (membranes, macromolecular assemblies). These components can, in some cases, favor (protein chaperones) or induce (as it is the case of the molecules specifically interacting with NUPs, see above) correct folding; however, heavily charged or hydrophobic biological surfaces, notably nucleic acids, glycosaminoglycans and lipid membranes, can also affect the conformation of the interacting proteins by modifying the contacts stabilizing the native fold of the polypeptide chain thus populating secondary structure-rich aberrant states [26,89,90].

In the case of phospholipid bilayers, protein–lipid interaction results in protein structural alterations and local modifications of the lipid arrangement with possible lipid extraction and membrane disruption [91–93]. Depending on their biophysical properties, surfaces can also actively recruit protein molecules increasing local concentration of their misfolded derivatives, that stay in close proximity to each other in a two-dimensional environment. Both unfolding and local concentration effects can result in enhanced tendency of proteins/peptides to undergo aggregation. Actually, for monomer concentrations above 1 nM, adsorption at a two-dimensional surface reduces considerably the average distance among molecules with respect to that in the three-dimensional bulk solution; this can favor monomer–monomer interactions with further structural modification, aggregate nucleation and insertion into the lipid bilayer [94–101] (reviewed in [102]), eventually leading to membrane disorganization [90,91]. Growth of pre-fibrillar assemblies on nanoparticles [103] anionic surfaces such as mica, fatty acid and SDS micelles, and anionic phospholipid vesicles [100,104,105], synthetic phospholipid bilayers [94–99,106] and cell membranes [22,107] has been reported. In the latter case, the resulting alteration of the organization of membrane lipids results in derangement of selective permeability and impairment of the function of specific membrane-bound proteins and signaling pathways [108–111]. Moreover, data have been reported suggesting that, at least in some cases, the aggregate growth process itself on the membrane rather than the oligomers per se can be the true trigger of cell impairment [112].

9. A surface provides an environment with defined physicochemical properties

The misfolding and aggregation promoting effects of a surface and the details of its interaction with monomers/oligomers depend on several properties both of the protein and the surface. The protein properties include the chemical and biophysical features of the monomer (thermodynamic stability, mean hydrophobicity, net charge, propensity to secondary structure), its folded or unfolded state and the way it interacts with the surface. The surface physicochemical properties include electrostatic potential, hydrophobicity (reviewed in [28,112]) and, in the case of lipid membranes, density of lipid packing, curvature, compactness, rigidity or fluidity. The two-dimensional environment of a surface possesses physicochemical properties that can be very different from those of the bulk aqueous phase where usually a protein/peptide is dissolved. For example, the strong electrostatic field or the non-polar environment of heavily charged or hydrophobic surfaces, respectively, can alter the native fold of a protein leading it to expose on the surface regions that normally are hindered from exposure to the solvent being associated with each other through electrostatic or hydrophobic interactions [113]. This view agrees with experimental data showing that surfaces can catalyze the formation of amyloid aggregates by a mechanism substantially different from that occurring in the bulk solution (reviewed in [28,112,113]).

When it is adsorbed at a surface, a protein/peptide experiences two phases with different physicochemical properties; accordingly, for several/many protein residues it becomes energetically favorable breaking native interactions to establish interactions with surface-exposed functional groups that stabilize non-natively folded states. For example, hydrophobic or charged surfaces may induce local or more extensive unfolding where hydrophobic groups normally buried into the compactly folded native state become able to interact with hydrophobic clusters exposed on the interacting surface without any energy penalty (reviewed in [28,113]). These effects may also be physiologic, as it is the case of chaperone-assisted protein folding, target-induced folding of NUPs and reversible unfolding/refolding of specific proteins when they physiologically translocate across a membrane [114]. In most cases, the interaction of a misfolded or unfolded species with a lipid membrane is likely to occur via a two-step mechanism. The first step involves the electrostatic interaction of the positively charged residues with negatively charged or polar lipid head groups with structural alteration; the second step occurs with the insertion of hydrophobic regions of the protein/peptide inside the bilayer (reviewed in [27]) whose hydrophobic interior favors loosening of the tertiary interactions while strengthening the secondary ones resulting in enhanced tendency of proteins and peptides to aggregate, as it has been shown for the prion protein, the Aβ peptides and amylin [26,90,92,115].

As stated above, the surface electrostatic potential may play an important role in destabilizing protein fold. Indeed, several reports highlight the key role of either anionic surfaces and anionic phospholipid-rich membranes in triggering protein/peptide fibrillization [104,116]. Negatively charged membrane surfaces also appear to efficiently induce the organization of β-sheet structures, thus acting as conformational catalysts for amyloids [106,117]. This is the case of phosphatidylserine (PS)-containing liposomes, whose behavior as inductors of amyloid aggregation in vitro of a variety of proteins is widely demonstrated [92,118]. Anionic phospholipid-rich membranes have also been proposed to interact with amyloid aggregates possibly by recognizing a shared fold [106]. The exposure of negative charge can also explain the effect of membrane gangliosides, particularly GM1, as mediators of monomer misfolding and oligomer recruitment to the cell membrane resulting in cytotoxicity (see below).

As pointed out above, protein aggregation can also be enhanced by biological surfaces other than membranes. Several data indicate that monomer binding to the collagen triple helix either in vivo and in vitro favors β2-microglobulin aggregation and that β2-microglobulin susceptibility to aggregate is modulated by fluctuations of binding affinity and hence of its concentration in the proximity of collagen fibers [119–121]. Glycosaminoglycans can also provide extracellular surface suitable to promote amyloid aggregation, as in the case of gelsolin [122] and acylphosphatase [123]. Finally, protein misfolding and amyloid aggregation can also be accelerated by anionic clusters such as those provided by nucleic acids, SDS and phospholipid micelles [104–106,124].

10. The biophysical features of lipid bilayers modulate protein destabilization and aggregation

As stated above, lipid membrane-favored amyloid aggregation depends on the membrane biophysical features and hence primarily on its lipid composition. On this aspect, cholesterol and gangliosides appear to play key roles as determinants of membrane rigidity and electrostatic potential, respectively [29,30]. In particular, several reports indicate that the cholesterol content affects membrane physical features such as fluidity, density of lipid packing and dielectric properties, hindering both aggregate recruitment at the cell membrane and membrane permeabilization [125,126]. In this regard, the ability of Aβ peptides to insert into the membrane seems to depend on the membrane cholesterol:phospholipid ratio. In synthetic, low cholesterol dimyristoylphosphatidylcholine (DMPC) vesicles, Aβ preferentially localizes at the membrane surface exhibiting aggregation-prone β-structure; however, in the same vesicles containing 33% cholesterol, the C-terminal tail of the peptide inserts into the membrane with an altered, β-helix rich, structure that hinders peptide aggregation [127]. These data support the idea that the content in cholesterol affects the way Aβ peptides interact with, and insert into, the phospholipid bilayer, modulating aggregation into fibrillar assemblies. It has been also reported that the interaction of pre-fibrillar aggregates with the cell membrane is impaired when the membrane is enriched in cholesterol (reviewed in [29]). Moreover, a higher membrane rigidity following increased cholesterol can be protective against any perturbation of membrane integrity and cell demise following aggregate growth at, or interaction with, the cell membrane [126,128,129]. Since Aβ toxicity is associated with the aggregation state of the peptide, many studies have investigated the cholesterol–Aβ toxicity relation. Some studies have shown that decreasing cholesterol levels can be protective and increasing cholesterol level can make the cells vulnerable to Aβ [130–136]. On the contrary, a number of studies have reported that increasing cholesterol levels can protect the cells against Aβ toxicity, whereas low membrane cholesterol results in cell vulnerability to Aβ peptide [90,125,126,128,137,138]. These results, taken together, reveal that Aβ–cholesterol interactions are rather complex and subtle changes in the levels and/or distribution of cholesterol in various cell compartments may produce contrasting effects on Aβ toxicity, possibly due to its influence on the state of peptide aggregation, generation or degradation [139]. Another study reports that increased cholesterol levels alter the structure, dynamics, and surface chemistry of the lipid bilayer, resulting in increased bilayer thickness, hydrophobic chain order, surface hydrophobicity, and in decreased lipid mobility. Inclusion of cholesterol makes this binding process more energetically favorable, significantly promoting the binding of Aβ to the lipid bilayer. In particular, it has been suggested that charged residues act as anchors to establish the initial binding of Aβ to phosphate headgroups of the bilayer driven by electrostatic interactions, which further facilitates hydrophobic residues to reside on the bilayer. Once hydrophobic residues especially from the C-terminus are locked on the bilayer, the interactions among charged residues, lipid bilayer, and calcium ions are optimized to provide additional attractive forces to stabilize Aβ adsorbed on or inserted into the lipid bilayers [140].

11. Lipid rafts: key sites favoring amyloid aggregation at, and aggregate interaction with, the cell membrane

Lipid rafts, cholesterol-, sphingolipid- and ganglioside-rich ordered membrane microdomains freely floating through the more fluid lipid bilayer, have been proposed to function as platforms where neurotoxic oligomers of proteins and peptides are assembled [141]. Indeed, lipid rafts appear directly involved in prion protein stabilization and in the pathological conversion of the cellular (PrP^{c}) to the scrapie (PrP^{sc}) form [142–144]. Therefore, it has been proposed that soluble Aβ peptide and prion protein aggregation can be raft-associated processes [145,146] and that any alteration of cholesterol (as well as sphingolipid) homeostasis can be a shared primary cause of a number of neurodegenerative diseases [146,147]. Cholesterol can also facilitate Aβ aggregation through the structural modification of other lipid raft components including gangliosides [148–150]. In particular, a recent study using reconstituted membranes has shown that cholesterol can either facilitate or inhibit the interaction of Aβ peptides with lipid rafts through fine-tuning of the ganglioside conformation [151]. This reinforces the notion that Aβ binding and aggregation within the neuronal lipid raft domains is most likely mediated by multiple players rather than any single component [139]. It has been suggested that the interaction between ganglioside sialic acid and Aβ induces a conformational rearrangement of the peptide chain from an α-helix-rich to a β-sheet-rich structure, leading to the generation of a GM1/Aβ complex which acts as an endogenous seed to promote amyloid oligomerization and subsequent fibril formation [148,152–155]. Indeed, it has been recently suggested that low-polarity environments provided by GM1 clusters play an important role in the acceleration of fibrillization [156]. Increased Aβ40 retention onto, or into, acidic phospholipid membranes has also been reported, resulting in different outcomes. The peptide anchored via its C-terminal tail, presumably in its native, partially β-helical structure, remained monomeric; conversely, upon peptide interaction at the membrane surface, peptide aggregation was accelerated [92], indicating that a delicate balance between two different mechanisms of peptide–membrane interaction could possibly be involved in AD pathophysiology. Electrostatic interactions may also play a pivotal role in mediating Aβ–GM1 interactions with an attractive force at pH 5.5, where Aβ is positively charged and GM1 is negatively charged and a repulsive strength at pH 7.2 where both molecules are negatively charged [157,158].

12. Amyloid polymorphisms and cytotoxicity

The data reported in the past few years have considerably improved the knowledge of the relationship between structure and toxicity of the amyloid aggregates, even though the specific nature of the pathogenic species, and their ability to damage cells are the subject of intense debate [1,3]. The different assemblies grown in the fibrillization path differ greatly not only in their cytotoxic potential, but also in the cellular mechanisms and functions they interfere with, that appear to be specific for the aggregation state. Indeed, small Aβ42 oligomers found in the brains of AD people impair long-term potentiation [18,159] and raise endoplasmic reticulum stress [160], whereas the neuroinflammatory response in AD brain appears more specifically associated to the presence of fibrillar Aβ [161]. Moreover, at least in the case of the Aβ peptides, cytotoxicity appears to be mediated by an aggregation state-specific uptake; in fact, oligomer internalization by endocytosis into lysosomes appears associated with oligomer toxicity, whereas harmless amyloid fibrils are not internalized [162]. Actually, although in some cases mature amyloid fibrils can impair directly cell viability [163,164], most often the oligomeric assemblies transiently arising in the path of fibrillization of several peptides and proteins are the main or even the sole cytotoxic species [5,18,165–167]. Further support for a toxic role of oligomers has been provided by the use of oligomer-specific conformational antibodies or sera, which are able to block these oligomer-dependent cellular effects [20,168]. Physical fragmentation of preformed fibrils into small-sized fragments, which resemble fibrillation intermediates, exacerbated the effects of fibrils on cells and has further promoted the concept of smaller species being more effective as toxic agents [37,169]. The relative instability with the higher tendency to release oligomers, makes the out-of register fibrils more toxic to mammalian cells, with respect to in-register fibrils [36] (see above).

The latter finding can be comprised in an increasing body of data indicating that at different conditions, both in vitro and in tissue, the same peptide/protein can populate variously misfolded species

which generate oligomers with different conformational features eventually resulting in differently organized mature fibrils [170–173]. Oligomer (and mature fibril) heterogeneity and polymorphisms are key issues considering that these species can affect directly or indirectly the load of toxic amyloids in tissue. Specific structural features of the natively folded monomers can also determine the way they misfold and aggregate under suitable conditions and the toxicity of their aggregates [174]; the aggregation under similar conditions of proteins/peptides carrying specific amino acid substitutions or chemical alterations modifying their physicochemical properties [175] is a well known example. However, in some cases the conformational peculiarities of an aggregating peptide/protein can be much more subtle (reviewed in [176]). For example, after release from the cell membrane upon APP processing, Aβ42 can exist in two different conformations; of these, the normally prevalent, harmless physiological conformation with a turn at positions 25–26 does not aggregate by itself, whereas the second, less populated, conformation, with a turn at positions 22–23, aggregates into toxic oligomers [177]. A more recent example with Aβ42 highlights the sequential growth, at low monomer concentration, of two similarly sized oligomers with different toxicity and hydrophobic exposure whereas higher monomer concentrations resulted in rapid fibril growth [178]. Another recent example of amyloid polymorphs with different cytotoxicity regards oligomers with different size, structure, hydrophobicity and compactness grown during the aggregation of the prion-determining region of yeast Sup35, whose different cytotoxicity was directly related to the extent of these properties [179]. That hydrophobic exposure is a key determinant of oligomer instability and cytotoxicity is supported by a recent report where the close relation between the extent of hydrophobic exposure and amyloid oligomer cytotoxicity is clearly depicted [180].

13. Different oligomers of the same protein can display variable cytotoxicity

However, although increasing results indicate that protein oligomer toxicity is determined by a well-defined set of parameters such as size and hydrophobic exposure, it is important to emphasize that toxicity does not reside in one or a limited number of oligomeric forms of a given protein [181]. Different oligomers grown from the same protein have been shown to affect cell viability to some degree. The general emerging picture is that oligomers cannot be described as a finite number of protein structures, each identified by a defined set of parameters. Rather, they are best described as a number of conformational ensembles, each containing an indefinite number of different assemblies that vary in secondary and tertiary structure, compactness, shape, size, number of monomers and hydrophobic exposure. As aggregation proceeds the increase in size, stability, compactness, β sheet regularity and hydrophobic burial reduce dynamical fluctuations, hydrophobic exposure and thus oligomer toxicity [181]. Actually extensive work has convincingly demonstrated that aggregate size is a critical determinant of toxicity [178]. However, conformational difference between amyloid oligomers of similar size and dissimilar toxicity confirms hydrophobic exposure on the aggregate surface as another important determinant of oligomer toxicity [46,178,179,182]. In fact, the exposed flexible hydrophobic surface mediates aberrant interactions with plasma membrane and other proteins, resulting in cell functional impairment and oligomer sequestration.

14. Aβ peptides and HypF-N are two examples confirming that oligomer biophysical features affect cytotoxicity

In the case of the Aβ peptides, the most studied model, there is growing awareness that Aβ interaction with the cell membrane implies, among others, peptide conformational changes and is important to Aβ toxicity [153,171]; accordingly, the aggregation and toxicity of any Aβ species could, at least in part, be related to its ability to change structure on, or within, the cell membrane and hence, ultimately, to its flexibility and molecular dynamics (see also above). This idea is supported by a recent study on the relation between the differing conformations at two different temperatures of amyloid aggregates of the poly(Q)-expanded peptide encoded by the huntingtin exon-1 and their relative toxicity both to cultured cells and in tissue [183]. The conclusions suggest that in distinct brain areas the same protein can experience different environmental conditions that modulate its stability favoring aggregation into fibrils with differing physical and biological properties and, possibly, stability. That mature fibrils with different structural features can display different stability and hence ability to leak toxic oligomers [184–188] is further demonstrated by another study carried out on glial inclusions purified by mild detergent treatment from brain tissue of people affected by multiple system atrophy. Soluble 30–50 nm-sized annular α-synuclein oligomers were released from these inclusions, whereas the aggregates of the recombinant protein yielded only spherical oligomers [189]. These and other data, besides suggesting the existence of different structural features in either aggregated form, also indicate that pathological protein aggregates in tissue can be a source of toxic species [16] (see also later). Recently, a study based on the use of novel amyloid dyes such as luminescent conjugated polythiophene probes has been carried out; these dyes are useful to investigate the supramolecular structure of amyloids by highlighting particular conformations of protein aggregates both in vitro and in vivo. The data reported in such study confirm that conformational differences do exist in different prion strains [190] and that conformationally distinct Aβ deposits do occur in brain tissue of mice models of Aβ deposition [191].

Our results on HypF-N, a bacterial protein not associated with amyloid disease, confirm and extend the above considerations. At different destabilizing conditions (acidic pH or reduced medium polarity), HypF-N misfolds generating two types of β-structure enriched amyloid oligomers morphologically similar, yet with differing stabilities and structural features. These oligomers were carefully characterized in terms of the burial of hydrophobic residues, density of packing, flexibility and extent of exposed hydrophobic surface by using rationally designed mutants of the monomeric protein. Even in this case, the more hydrophobic, less stable oligomers (arising in a reduced polarity medium in the presence of TFE) grew into mature fibrils whereas the less hydrophobic, more stable ones (grown in an acidic medium in the presence of TFA) eventually assembled into stable off-path curvy protofibrils with no further evolution. The two types of oligomers displayed differing cytotoxicities and abilities to interact with, to permeabilize, and to cross the plasma membrane of exposed cells both in culture and in tissue, toxicity and ability to trigger apoptosis being associated exclusively to the less compact, less stable and more hydrophobic assemblies [46,192–194]. These data highlight some structural and biophysical properties of amyloid oligomers generated at different conditions and establish a direct link between the latter and oligomer ability to grow into distinct, stable amyloid assemblies; they also provide clues on the relation between oligomer physicochemical properties and cytotoxicity coming from their ability to interact with the cell membrane (see later). When taken together with other data mentioned above [178,179], these results can tentatively be generalized suggesting that the conformational (molecular dynamics), biophysical (exposure of hydrophobic or charged surface, flexibility) and stability (compactness) properties of amyloid assemblies are major determinants of cytotoxicity, affecting the way and the extent amyloids interact with membranes and other cell components.

Oligomer polymorphism explains the propagation of prion strain infectivity and other protein polymorphisms [59,88,195,196], the appearance of both in-path and off-path intermediates of fibril growth [16,197] and the reported structural heterogeneity [67,198,199] and variable cytotoxicity [162,174,197] of amyloid fibrils and their precursors grown at different conditions from the same peptide/protein. The latter issue appears of particular importance since, as discussed in the next section, in most cases pre-fibrillar aggregate cytotoxicity appears primarily associated to their ability to interact with the

cell membranes, particularly the plasma membrane, with ensuing derangement of selective permeability and/or interference with signaling pathways (reviewed in [28]).

15. Mechanisms of amyloid cytotoxicity

As reported above, biological, notably lipid, surfaces can induce protein/peptide misfolding and aggregation and recruit actively amyloid oligomers and fibrils. Accordingly they can be expected to be directly involved in amyloid toxicity. Actually, increasing evidence indicates that the toxicity of amyloid oligomers is directly correlated with their shared ability to disrupt the membrane barrier function. In this context, three main mechanisms of membrane mediated toxicity have been proposed for Aβ, which include carpeting of the peptide on one leaflet of the membrane surface, resulting in an asymmetric pressure between the two leaflets and the leakage of small molecules [158,200]. This model is believed to be of minor physiological relevance for amyloid diseases because it has been demonstrated to cause similar membrane damage for both human IAPP and non-amyloidogenic mouse IAPP [201]. The carpet model was also proposed to explain the exponential leakage kinetics and absence of a lag phase in human IAPP- and mouse IAPP-induced large unilamellar vesicle permeation [201]. The second proposed model is based on the detergent-like effects of amyloid-forming peptides on lipid membranes. This mechanism of permeation is proposed to occur through membrane association of amyloid-forming peptides in the form of micelle-like structures [202]. The electrostatically driven initial interaction is followed by alignment of the oligomers so that the hydrophilic surface faces the phospholipid head groups, resulting in membrane disintegration by disruption of the bilayer curvature [202]. The detergent effect results from the surfactant-like properties associated with the amphiphilic peptides causing a reduction in membrane surface tension, where it forms a hole by lipid removal from the bilayer, either in the outer leaflet, which results in membrane thinning, or in both leaflets, which results in holes [200]. The formation of stable pores and ion channels is the third model proposed for amyloid-induced toxicity. Initially, it was reported that Aβ exhibits ion-channel activity in planar lipid bilayers implying that Aβ toxicity is based on an ion-channel mechanism that causes membrane depolarization, Ca^{2+} leakage, and disruption of ionic homeostasis [23,94,203,204]. Channel activity was subsequently reported for a number of other amyloidogenic proteins, including IAPP [97], α-synuclein [205,206], polyglutamine [207] and prion-derived peptides [208]. Thus, the toxicity of these proteins may be related to their shared capabilities to form channels or pores in membranes and to enable unregulated ion leakage, in analogy to pore-forming toxins [204,209].

The mechanism of toxicity associated with amyloid oligomers may not be exclusively related to a single mechanism such as pore formation, detergent-like or carpeting effects but more likely a collection of these mechanisms. This conclusion is consistent with the fact that a disruption of Ca^{2+}-ion homeostasis is a characteristic feature of several neurodegenerative diseases, including AD and PD [210]. Recently, it has been proposed that amyloid oligomers may act at two steps, separated in time, a first, very rapid step, where Ca^{2+} increases due to glutamate receptor stimulation by the oligomers, followed by a second, delayed step, where oligomers permeabilize non specifically the cell membrane, possibly via the formation of amyloid pores [211]. An increased intracellular Ca^{2+} level affects a range of Ca^{2+}-sensitive enzymes, causing synaptic degeneration and cell death. In neuronal cells, Ca^{2+} perturbations are characteristically associated with excitotoxicity, a pathological process that is usually caused by neurotransmitter overstimulation [212].

Oligomers may interact with monolayers or bilayers formed from pure lipids, and they are able to induce the release of dye molecules that are engulfed within the lumen of liposomes in vitro [113,209]. The tendency of amyloid oligomers to interact with synthetic lipid membranes supports the idea that the interaction can be non-specific but, possibly, modulated by the membrane lipid content, particularly in terms of membrane cholesterol and ganglioside content (see above). Actually, the biochemical and biophysical features of the cell membrane can affect the conformation, distribution and proteolytic processing of membrane proteins involved in neurodegenerative conditions such as AD or prion disease; it can also modulate oligomer–cell membrane interaction, most often a key step of cytotoxicity (see above). However, in spite of the different proposed mechanisms, it is not clear whether the oligomer–membrane interaction is generic or it is a targeted process involving specific membrane receptors. In this context several cell surface proteins have been considered as possible candidate receptors of Aβ oligomers, including APP [213], TNFR1 (tumor-necrosis factor receptor-1) [214], the receptor for advanced glycation end products (RAGE) [215], the non-infectious form of the prion protein (PrP^c) [216], voltage-gated [217] or ligand-gated calcium channels such as the glutamate N-methyl D-aspartate (NMDA) receptors and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor [211,218].

16. Aggregate interaction with the plasma membrane impairs ion and redox homeostasis in exposed cells

The presence of toxic aggregates inside or outside the cells, together with their interaction with cell membranes can impair a number of functions ultimately leading to cell death by apoptosis or less frequently, by necrosis [24,219–223]. In most cases, initial perturbations of fundamental cellular conditions such as redox status and free Ca^{2+} levels underlie cell function impairment induced by the aggregates [100,208,219,224–226]. In general, in diseased tissue oxidative stress results primarily 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 of selective permeability and of appropriate regulation of membrane proteins such as specific enzymes, receptors and ion pumps [108,109,115]. In addition, Aβ aggregates directly induce ROS production, protein oxidation and lipid peroxidation leading to cell degeneration [3,227–229]. Oxidative stress can also result from oligomer-induced mitochondrial deregulation, as in AD, PD and several other neurodegenerative diseases [230,231]. Aβ peptide impairs mitochondrial functions in cultured cells and binds to mitochondrial proteins, including cyclophilin D [232]. This peptidyl-prolyl *cis*/*trans* isomerase is a key component of the mitochondrial permeability transition pore, and its interaction with Aβ induces profound mitochondrial degenerations with numerous cellular consequences, many of which can be associated with Ca^{2+} leakage to the cytoplasm, reduced ATP levels and increased ROS production [232,233]. Actually, oxidative stress has been considered, at least in part, a consequence of Ca^{2+} entry into cells. 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 [223]. Finally, as reported above, modifications of the structure and permeability of the mitochondrial, lysosomal and ER membranes induced by internalized Aβ can contribute in several ways to amyloid cytotoxicity, including further deregulation of intracellular Ca^{2+} levels [234–236]. Although neuronal loss is a key neuropathological feature of AD [237,238], increasing evidence argues that such cell death may not constitute the primary insult provoked by Aβ. Rather, the peptide may initially affect the ordered function of neuronal networks and synaptic plasticity [239,240]. According to this view, oligomers induce a remodeling of the synaptic contacts of neuronal circuits.

17. The amyloid-cell membrane system

The information provided above, suggests convincingly that the membrane biophysical features arising primarily from their lipid

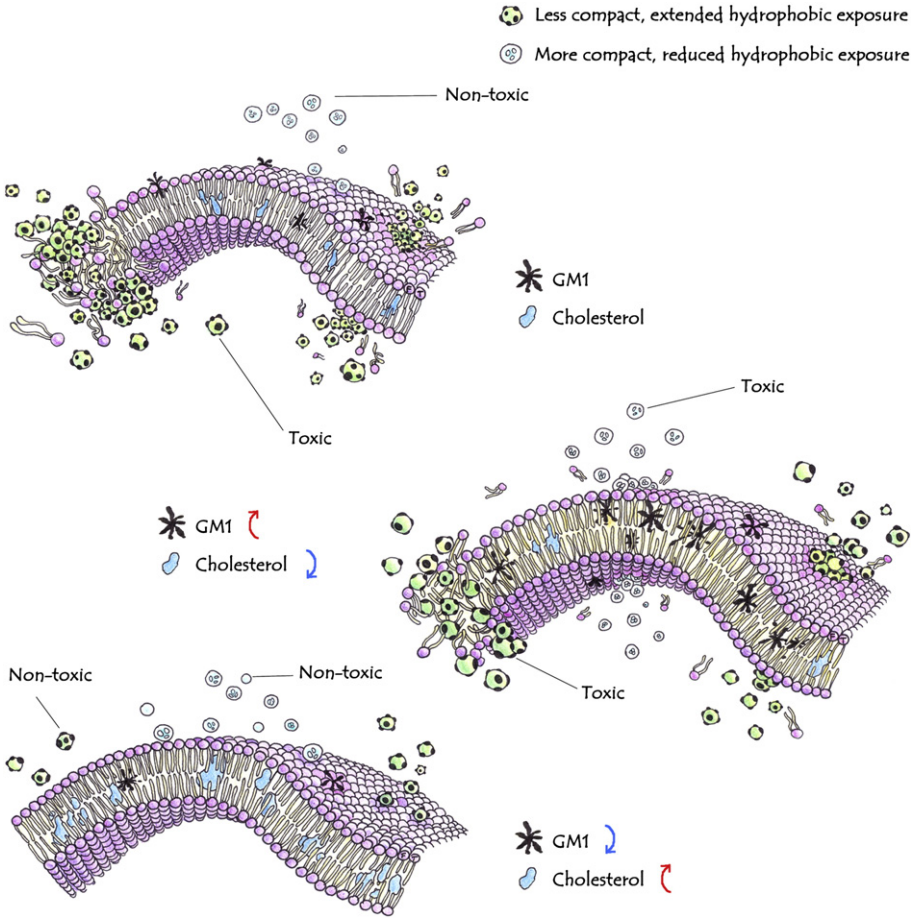

Fig. 1. Membrane lipid composition is a key determinant of the relative toxicity of amyloid oligomers. Oligomers that are non-toxic to normal cells become toxic when the content of cholesterol is decreased or that of GM1 is increased. Conversely, oligomers that are toxic to normal cells become non-toxic when the levels of cholesterol in the cell membrane are increased or those of GM1 are decreased.

composition play a key role in modulating protein/peptide aggregation and aggregate cytotoxicity. In particular, as indicated above, cholesterol and gangliosides, key components enriched in lipid rafts, play pivotal roles in modulating membrane protein processing as well as protein aggregation at the membrane level and the interaction of pre-formed aggregates with the cell membrane.

In summary, up until now, two distinct lines of evidence are increasingly supported experimentally. The first indicates that amyloid pre-fibrillar aggregates, particularly oligomers, grown at different conditions and hence displaying differing structural and biophysical properties can affect variously cell viability or be completely harmless in particular by displaying different abilities to interact with, and to permeabilize, the cell membrane; the second shows clearly that the lipid composition of the cell membrane affects heavily several processes: proteolytic processing of membrane proteins with the production of aggregating peptides; protein/peptide misfolding/aggregation; aggregate interaction with the membrane itself with the ensuing membrane damage and cell functional and viability impairment.

Taken together, these two bodies of data suggest that there should be a reciprocal effect of both the aggregate and the membrane biophysical features resulting in the final outcome in terms of cell sufferance and death. Our most recent data on the effect of the membrane lipid composition and physicochemical features in modulating the relative cytotoxicity of the two types of HypF-N oligomers with different biophysical features and cytotoxicities described above [46] show that cytotoxicity is the result of a complex interplay between oligomer and membrane biophysical and biochemical features (Fig. 1). Overall, our data support the concept that cytotoxicity is not a property inherent to a specific type of aggregate; rather, it emerges from the characteristics of the oligomer membrane complex as a whole. In fact, we found that in cultured cells either normal, enriched or depleted in membrane cholesterol or GM1 exposed to either type of HypF-N oligomers, the extent of cytotoxicity of any oligomer was strongly modulated by the lipid composition and biophysical features of the interacting cell plasma membrane. In particular, increasing membrane cholesterol made safe the oligomers toxic to untreated cells whereas in cells with decreased membrane cholesterol the oligomers non-toxic to untreated cells became cytotoxic. Opposite effects were found following the modulation of the content of membrane GM1; finally, increasing both cholesterol and GM1 resulted in a net effect that was substantially related to the GM1 (not cholesterol) content, suggesting that this lipid plays a pivotal role [192]. 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 [223,241,242].

18. Conclusions

The data reported in the last few years have made it 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, both in vitro and in tissue can display differing structural and biophysical features. Such a

conformational polymorphism appears to be of great importance in determining the effective cytotoxic potential of amyloids. On this line of evidence, it is increasingly recognized that amyloid fibrils, previously considered as harmless sinks of toxic oligomers can indeed be a source of cytotoxic species to exposed cells, depending on their structural and stability properties and the conditions of the environment where they are grown and deposited. Actually, increasing information supports the idea that mature fibrils can indeed be a source of toxic oligomers following their fragmentation by thermal motion or by interaction with disassembling surfaces or molecules. This view still assigns to the pre-fibrillar aggregates, notably oligomers, the role of direct cytotoxic entities, however this behavior, rather than being absolute, depends on their structural polymorphism. In particular, the latter can play a key role in modulating the ability of these species to interact with cell membranes and, hence, their cytotoxicity.

Unfortunately, at variance with mature fibrils, obtaining information on the structural features of fibril precursors is made challenging by their heterogeneous, transient, unstable and highly flexible nature. Nevertheless, new experimental approaches and techniques are starting to provide more accurate information on the structural features of these assemblies and on the structure-toxicity link. Finally, an increasing body of data support definitely the importance of the lipid composition and the ensuing biophysical features of synthetic or biological membranes as key determinants affecting the in situ generation of amyloid precursors and the way amyloids can grow on, or interact with, them. Overall, all these points can be considered as details of a highly incomplete, yet remarkably complex picture, where amyloid polymorphism and the cell membrane biochemical and biophysical features both contribute to the net cytotoxicity of these assemblies, although the way these factors affect each other can be different in differing systems.

Such a view leads to consider cytotoxicity as a relative concept rather than an inherent property of amyloid oligomers or, in other words, the net result which emerges from a complex interplay between self-reinforcing or opposite structural and biophysical features of both the oligomer and the cell membrane that must not be taken separately but considered as a whole system. 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 relationship between amyloid load and the severity of the clinical signs [243]. We are still at the beginning of the way to unravel the oligomer world. However, the present intense research warrants that in the near future we will be able to obtain solid knowledge in this field that will be of great value for new approaches and rational drug design against oligomer cytotoxicity.

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