The Key Role of Membranes in Amyloid Formation from a Biophysical Perspective

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Abstract: Even though our knowledge of how proteins misfold and aggregate is deeper nowadays, the mechanisms driving this process are still poorly understood. Among the factors involved, membranes should be taken into account. Indeed, convincing evidence suggests that membranes may influence protein folding, misfolding and aggregation. In fact, membrane lipid composition of different cellular types may attenuate or intensify the environmental pressure over protein folding equilibrium. In the present review the aim is to make an up-to-date analysis of the membrane influence on protein aggregation from a biophysical point of view in order to provide useful tools for researchers from other fields. In particular, we discuss how membranes can alter protein environment, e.g. increasing local protein concentration, lowering pH and dielectric constant, allowing accessibility to the hydrophobic milieu and promoting surface crowding, all of which will lead to protein aggregation. In addition, we review the role that specific lipids may exert on protein aggregation and finally we analyse the possible implication of membrane-related oxidative stress on amyloidogenesis.

Keywords: Membranes, amyloids, proteins, neurodegenerative diseases, oxidative stress, alpha-synuclein, amyloid-beta, GAPDH.

INTRODUCTION

According to a well-established paradigm, protein folding depends nearly exclusively on its amino acid sequence (genetic factor) and its environment. The nascent proteins are released *in vivo* in a crowding environment that continuously changes in response to extracelullar stimulus, thus modifications in the intracellular composition are more probable than amino acid mutations. Within the living cell, membranes are qualitatively and quantitatively strong environment modulators and the role as a protein structure-determinant factor was proposed in the last decade [1]. However, the membrane effect in the protein folding/unfolding equilibrium is complex and depends on the membrane composition and dynamics as well as protein structure features [2-3]. Furthermore, the cellular response to unbalanced oxidative stress can also change membrane composition [4-5], which may attenuate or intensify the environment pressure over protein folding.

The protein folding problem has been a challenging issue shrouded in mystery for decades. However, the problem has become more relevant nowadays since one specific type of filamentous protein aggregates has drawn particular interest due to its involvement in the pathogenesis of the so-called conformational diseases. Nowadays, there is a general consensus on the fact that the cytotoxic effect of aggregated proteins is responsible for cell death in protein deposition pathologies like Alzheimer's (AD), Parkinson's (PD) and prion diseases, several systemic amyloidoses and type 2 diabetes diseases.

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Among the factors associated with protein misfolding in neurodegenerative diseases, oxidative stress is one of the most closely scrutinized [6-8]. A growing body of evidence indicates that oxidatively modified lipids promote amyloid formation [9-11]. Cellular membranes in human brain contains relatively high concentrations of a variety of polyunsaturated fatty acids (PUFA) [12-13] which are highly susceptible to react with reactive oxygen and nitrogen species (ROS and RNS, respectively). In this way, a rational relationship between membrane composition, oxidative stress and amyloid fibrils formation could be established.

Considering that the understanding of the amyloidogenic process involves inter-disciplinary efforts, the aim of the present review is to provide useful tools from a biophysical point of view to researchers from other fields to analyze the current knowledge of the membrane influence on protein folding, misfolding and aggregation processes.

PROTEIN-MEMBRANE INTERACTION CAN MODIFY THE PROTEIN FOLDING EQUILIBRIUM

With few exceptions [14], the relevant biological form in proteins is characterized by a unique well-defined structure called folded or native state (*N*). However, a protein solution can be considered a collection of different conformational states undergoing a very rapid exchange where the native state is the most highly populated, while the unfolded state is a heterogeneous population of conformations (*U*), spanning a wide range of protein conformations where no molecule is like to another, nor like itself from one moment to another ($U_{1,2,...n}$). To reach the *N* state, any newly synthesized protein

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may pass through structural intermediates which represent on-pathway 'stepping stones' toward N [15-16]. Misfolded, molten globule or random coil could be defined as sub-sets of the U state [17]. At physiological conditions, the equilibrium constant of the $U \rightleftharpoons N$ transition is $K_{eq} = [N]/[U] > 1$, which implies that folding is a spontaneous process [18] and N, the most populated state, occupies a minimal energy state Fig. (**1**). However, the folded state is generally only 5 to 10 kcal/mol more stable than the unfolded one [18] and therefore the N state is frequently considered as metastable. For this reason, small changes in intrinsic and extrinsic variables, *e.g.* mutations; pH, additives, or temperature can disturb the natural folding equilibrium.

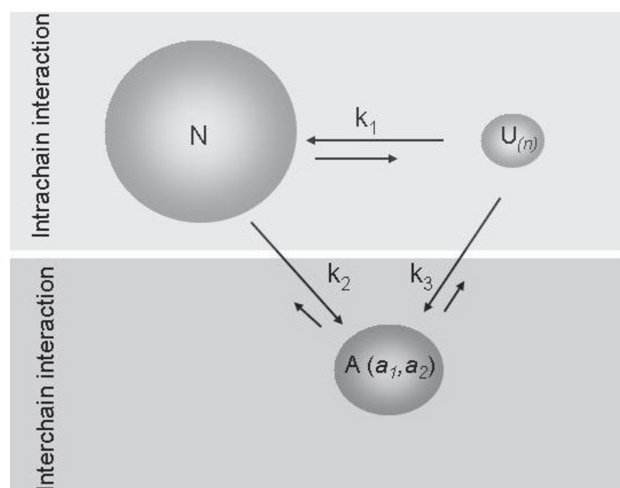

Fig. (1). Schematic representation of protein folding equilibrium. N is the protein population in native, U in unfolded and A in aggregated states. The different aggregation states a_1 (amorphous) and a_2 (amyloid) are also indicated. The circle surfaces are only indicative of the different population sizes but they do not represent any real system.

Considering the $U \rightleftharpoons N$ equilibrium to the cellular crowded environment where the protein concentration is high due to the confined cytoplasm and membrane space, spontaneous folding is not an absolute certainty even for RNAse A, as Anfinsen and Haber described in 1961 [19]. In fact, the heterologous expression of proteins in bacteria and other hosts often gives low yields of native protein given that failure in the protein folding process. In fact, the accumulation of protein insoluble states seems to be a common phenomenon in living cells. At present, the formation of proteins in an aggregated state (A) is considered as a process that completes the folding reaction, since the intermolecular interactions are present in a physiological medium. The protein aggregates can range from amorphous structures (a_1) without any order to highly structured fibrils (a_2), arising by distinct aggregation pathways [14].

Even for proteins that successfully fold, small fluctuations in the intracellular medium together with the metastable nature of the native state can shift the $N \rightleftharpoons U$ equilibrium to protein-misfolding and aggregation. It is important to note that neither human nor animal pathology has been associated to the presence of amorphous protein aggregates. On the contrary, the presence of the a_2 or 'amyloid fibril aggregates' have been considered hallmarks of more than fifty clinical entities called amyloid disorders although since recent years it is claimed that the oligomeric prefibrillar aggregates are presumably more cytotoxic than the mature fibrils [20].

In this scenario, synthetic and natural membranes have shown the ability to shift the protein folding equilibrium with different structural features towards the aggregation state (A) [21-25]. This fact strongly suggests that a general mechanism based on membrane physicochemical properties must be involved. On the other hand, the Singer–Nicholson model of fluid-mosaic membrane has been improved considering the membranes as patchy, with segregated regions of structure and function, and lipid regions varying in thickness and composition, and with a crowding limit exposure of lipid to the adjacent aqueous regions [26]. Thus, the differences in the topological and reological properties of the membrane can also play a role in protein solubilization and its folding. It has been proposed that protein aggregation induced by membranes involves a two-stage mechanism, which includes the insertion and reorganization of the proteins into the membrane, followed of side-to-side helix associations to induce the protein folding and oligomer formation [27-28].

Proteins that reach the membrane interface sense a different environment than those in the bulk aqueous media. In membranes, the protein-intermolecular interactions become more relevant and the activation energy for the $N \rightarrow A$ and/or $N \rightarrow U \rightarrow A$ processes can be lowered. In fact, the polypeptide chain in the presence of membrane can undergo: *i*) local protein concentration increment, *ii*) lower pH and dielectric constant, *iii*) hydrophobic milieu accessibility to, and *iv*) surface crowding. The main features of each effect are:

i) Increment of the Local Protein Concentration

The aqueous phase immediately adjacent to the membrane undergoes the effect of the membrane electrostatic potential. The Gouy-Chapman (GC) theory for the electrostatic potentials and ion distributions near charged membranes could be used to predict the solute concentration at the membrane interface. Moreover, GC theory has been successfully applied to predict the adsorption of charged peptides, hormones, or drugs at the membrane interface [29-30] Fig. (**2A**). Recently, the GC theory has been validated by molecular dynamics simulations (MD) showing a remarkable agreement [31] Fig. (**2B**). According to the GC theory, at physiological boundary conditions, the concentration of proteins at the lipid membrane surface are around one order of magnitude higher than their concentration in the bulk aqueous medium [32].

The membrane electrostatic potential depends on the presence of charged lipids which are extremely frequent in biological membranes. The main effect of the lipid charge is the accumulation of proteins or peptides in the vicinity of the membrane which, of course, increases the protein /peptide binding to the bilayers [33-35]. However, this crowding effect is essential in the $\boldsymbol{N \rightarrow A}$ folding process since some proteins like acylphosphatase from *Sulfolobus solfataricus* and Ure2p from yeast can reach the fibrillar state retaining their native structure [36-37]. In this process the membrane-

recruitment activity is essential since only when the polypeptide chain exceeds a certain concentration known as the critical concentration, the aggregates begin to appear.

ii) Low pH

Once a protein/peptide is adsorbed and concentrated in the membrane interface, a new scenario is offered to the polypeptide chain. The pH values in regions close to the membrane surface are frequently 1-2 units lower than in the bulk solution [38-39]. Decreased pH at the interface influences the protein conformation, stability and capability to form aggregates. In fact, for many globular proteins the formation of a non-native partially unfolded conformation was proposed [40-43]. In the tightly packed conformation of the *N* state, the constraints of the tertiary structure prevent the conformational rearrangements to reach the interchain β-sheet-structure.

iii) Hydrophobic Milieu

The dielectric constant value drops from 80 to 2 from the bulk to the hydrophobic membrane matrix. Thus, adsorbed peptide can relocate the nonpolar amino acid side chains with a significant free energy reduction. The accessibility to the hydrophobic milieu is considered the major driving force for membrane-induced protein unfolding [44-45]. Moreover, membrane lipids also compete for the same hydrophobic interactions, which were the stabilizing factors in the native protein structure in aqueous solution [46]. Since lipids as a solvent is unable to compete with the functional groups to form H-bonds, new hydrogen bonds between amino acid side chains could be induced in a hydrophobic milieu. In addition, changes in membrane tension due to, for instance, swelling by osmotic pressure, increase the interfacial membrane tension with a concomitant exposition of more hydrophobic surface which strongly enhances the amphipilic protein membrane partition [47] .

iv) Surface Crowding Effect

The cell cytoplasm is a very crowded environment with a protein concentration of about 200–300 $g.L^{-1}$. A large fraction of the volume in a cell is not available to other macromolecules. This is described by the term "excluded volume" which is estimated to be at least 20–30% the total volume [48-49]. The environment of the cell cytoplasm is therefore very different from that of dilute solutions, where most biochemical studies are carried out [50]. In "crowded" solutions, the volume exclusion affects protein folding equilibrium enhancing the tendency of diluted as well as concentrated proteins to self-associate [50].

As mentioned before, the pictorial view of the membrane can be described as a two-dimensional and irregular crowding surface (S) [26] and its influence on the protein folding equilibrium may be analysed by constructing a simple thermodynamic cycle as shown in Fig (**3**). The thermodynamic characterization of the change in a folding equilibrium of a protein in native state (*N*) after its adsorption to a membrane surface (S) could be obtained calculating the standard Helmholtz free energy (*F*) changes by $\Delta F_{Na} = -RT \ln K_{Na}$, if the $Nf + S \rightleftharpoons Na$ equilibrium is considered. *Nf* and *Na* represent the protein free in the bulk solution and adsorbed to the membrane surface respectively.

Of course, in a limited surface, the previously adsorbed ligands can influence the adsorption of extra ligands because they generate an excluded volume that could push the system to a new equilibrium in which a conformational change of the protein could occur in order to lower the free energy (ΔF_{Ua}) of the system by $\Delta F_{Ua} = -RT \ln K_{Ua}$, if the $Na \rightleftharpoons Ua$ equilibrium is considered. *Ua* is the unfolded protein adsorbed to the membrane surface.

The crowded surface forces align and adjust the conformation of polypeptide chain according to the new physicochemical environment. A cross α-sheet structure allows an efficient alignment of polypeptide chains with a strong di-

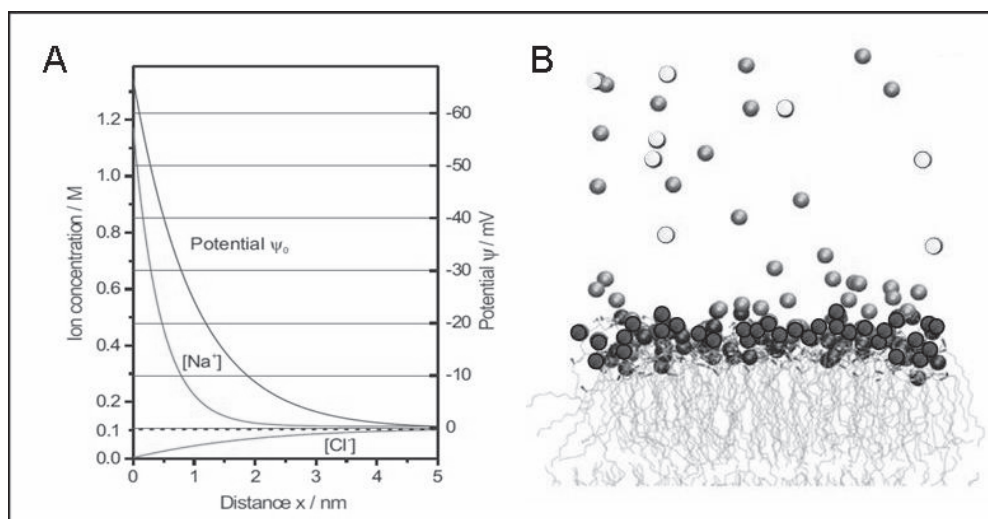

Fig. (2). **(a)** Surface membrane potential calculated from the Gouy-Chapman theory with a ψ_0 = -60 mV and ionic density profiles for a 0.1 M monovalent electrolyte near a membrane surface of charge density σ = -0.0621 cm^{-2} (1 electronic charge per 2.6 nm^2). **(b)** A snapshot of the MD simulations. Lipids are displayed with carbonyl and hydroxyl groups highlighted with thick bonds and phosphorus atoms as medium dark spheres. Adsorbed and diffuse Na^+ ions are displayed as dark spheres, respectively, and C_l- are displayed as open spheres in the bulk water. The lower leaflet is only partially shown. From [31].

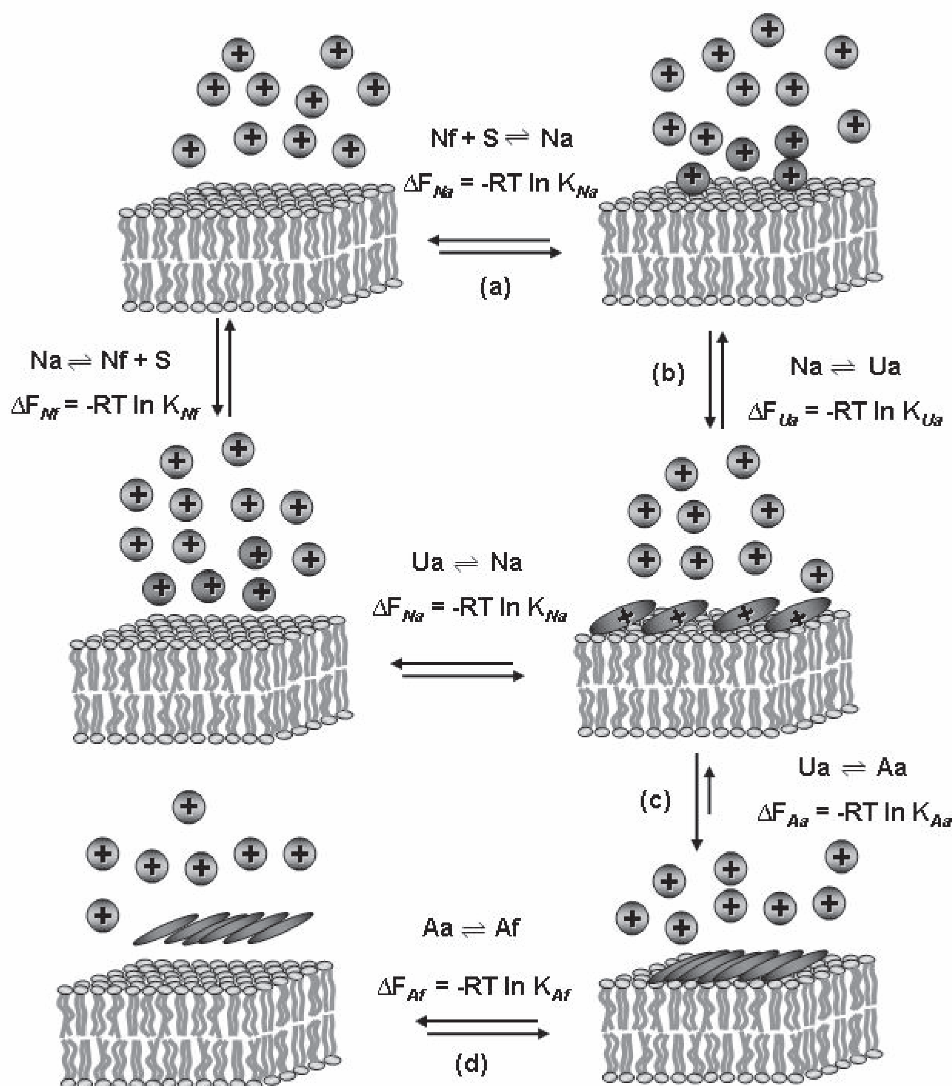

Fig. (3). Thermodynamic cycles illustrating coupling between free energy of transfer of proteins from the bulk solution to a membrane interface **(a),** interface conformational change **(b)**, association in interface **(c),** and desorption from membrane **(d)**.

pole moment. Driven by charge-dipole interactions, the oligomerization can progress easily [51]. The cross α-sheet then can evolve into the thermodynamically more stable cross beta-sheet [52]. In fact, α-sheets have been proposed to be an intermediate of the amyloid formation in some proteins [53-54]. Then, the formed oligomer could be desorbed from the membrane and a new equilibrium between the adsorbed and the desorbed oligomers could be obtained, with $\Delta F_{Af} = -RT \ln K_{Af}$, if the $Aa \rightleftharpoons Af$ equilibrium is considered. *Aa* and *Af* are the aggregated protein adsorbed and free in the bulk respectively.

Even though the influence of the crowding effect on membrane-mediated aggregation has been described years ago [55], it has rarely been taken into account experimentally, probably due to the difficulty of quantifying its effects [56-57]. However, using the model peptide LAH(4) Aisenbrey *et al.*, have demonstrated a correlation between the peptide concentration in the membrane and the topology of this membrane-associated polypeptide. They described that high surface concentration of the peptide shifts the molecule conformations, reducing the occupied surface area per molecule. In a cellular context, the crowding-dependent conformational change might provide a molecular switch for a cell to sense and control its membrane occupancy [58].

MEMBRANE HETEROGENEITY IS DETERMINANT IN MEMBRANE-INDUCED PROTEIN AGGREGATION: ROLE OF SPECIFIC COMPONENTS

The general view of membrane architecture strongly suggests that the Singer-Nicolson fluid mosaic model needs to be updated since experimental data have shown that membranes are patchy, with segregated regions of structure and function. Moreover, lipid regions may vary in thickness and composition [26]. Furthermore, there are different kinds of microdomains in the membrane, which can be divided in transient microdomains, also called “rafts”, which are unstable and more stable lipid-ordered microdomains[26, 59]. A large body of evidence has shown that *in vivo* amyloid fibril

formation might be related to the presence of these stable and specialized microdomains in the plasma membrane. These microdomains are normally composed by glycosphingolipids, cholesterol and sphingomyelin, which are more ordered structures than the surrounding membrane because the hydrophobic chains of lipids in the microdomains are more saturated and therefore they are tightly packed [60-61]. It has been proposed that microdomains may be involved in key cellular processes such as cell adhesion and signal transduction [62-63]. Notwithstanding, there is always some controversy around microdomains and lipid rafts since there is no conclusive evidence of their existence in plasma membranes. The main reason for that is the impossibility of studying them in a direct way in living organisms [64] allegedly because of their size. However, new technologies are being developed that will allow us to overcome this limitation [65-67]. It is important to note that co-existence of pure liquid crystalline and gel-like domains in plasma membranes of MDCK cells was proposed after a careful analysis of Generalized Polarization using the fluorescent probe Laurdan, in the pioneering work performed by Mamdouh *et al.* [68].

Microdomains undoubtedly participate in amyloid-induced neurotoxicity. Indeed, the depletion of any of these lipid components leads to a reduced neurotoxicity. In this regard, Wang *et al.* showed that decreasing cholesterol contents protected somehow cells from amyloid-beta peptide (Aβ) toxicity [69]. In the same trend, this group also demonstrated that cholesterol or ganglioside removal significantly reduced calcitonin neurotoxicity [70]. Moreover, Nicholson and Ferreira suggested that mature hippocampal neurons were more susceptible to Aβ amyloid than young neurons because they have increased cholesterol content in membranes [71].

Interestingly, Gellermann *et al.* were able to demonstrate by high-performance thin layer chromatography that microdomains are part of extracellular amyloid fibrils in several types of amyloidosis [72]. Indeed, it has been suggested that microdomains function as platforms where neurotoxic oligomers of proteins are assembled [73-75]. These data identify microdomains as key mediators of membrane oxidative damage as a result of their ability to recruit 42-residue form of Aβ (Aβ42) aggregates to the cell surface [76].

Even though the effect of isolated lipid molecules on fibril formation might be different from the effect observed in complex lipid assemblies, the study of individual lipids is definitely important to understand the contribution of each lipid in biological membranes. In recent years, an increasing body of evidence points out the crucial role that specific lipids play in the aggregation of amyloidogenic proteins. The importance of choline-bearing phospholipids, cholesterol, GM1 ganglioside, and certain anionic phospholipids will be discussed in this section.

A common feature of proteins that are able to oligomerize in the presence of membranes is their relatively high isoelectric point [24]. In fact, most of them are positively charged at physiological pH. Therefore, the binding of these proteins to negatively charged membranes may neutralize the cationic charges of proteins thus allowing protein-protein interactions which in turn would lead to oligomerization as described above. Furthermore, it is well known that membrane charge changes with aging, i.e.: the brains of aged rats have elevated levels of phosphatidic acid, [5] and the ratio of anionic to zwitterionic phospholipids increases in the brains of PD patients [77]. Actually, the acidic phospholipid:total phospholipid ratio needed for protein fibril formation strongly depends on the protein under study. For instance, Human Islet Amyloid Polypeptide (IAPP) requires a 30% of dioleoyl phosphatidylglycerol (DOPG) in order to oligomerize [78]. However, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) needs less negative charges for starting protein cluster formation. In fact, it takes only 10% of dioleoyl phosphatidic acid (DOPA) for triggering protein misfolding. Moreover, amyloidogenesis is accelerated by increasing anionic phosholipid content [25]. The structure of the anionic phospholipid would not be very important. Indeed, the mere presence of a negative charge would be sufficient for the pro- aggregation effect. Taking into account GC theory that states that pH close to the membrane is lower than in the bulk (see above), proteins might display more net positive charges in close contact with the bilayer thus enhancing electrostatic interactions with negative phospholipid. In this regard, Hou *et al.* showed in an elegant paper that both phosphatidylglycerol (PG) and phosphatidylserine (PS) were equally able to bind and trigger transthyretin fibril formation [79]. α-Synuclein (AS) is another protein that binds and forms fibres upon contact with anionic membranes. However, there is some controversy in this field. On one hand, AS was shown to bind palmitoyl-oleoyl phosphatidylglycerol (POPG) liposomes with a concomitant reduction in vesicle size. A nucleation centre was constituted where more AS could be recruited [80]. On the other hand, Zhu *et al.* showed that fibril formation was abrogated upon binding to membrane vesicles. However, this inhibition strongly depended on vesicle size as well as on lipid:protein ratio [81]. Auluck *et al.* recently proposed a possible model that may explain both findings: once AS binds membrane surfaces, it has two plausible fates: the induction of 2 α-helices that allows the protein to stay associated to the surface with no aggregation, or alternatively AS dimer formation (in the case of certain mutant forms of AS or if the protein-lipid ratio is rather high) which ultimately leads to protein oligomerization [82].

Even though negative charges in membranes are undoubtedly important for protein aggregation, other factors play important roles as well. For instance, Devanathan *et al.* showed that sphingomyelin promotes Aβ aggregation on the membrane surface perhaps due to a certain affinity of the peptide for this sphingolipid [83]. In fact, membranes prepared from dioleoyl phosphatidylcholine (DOPC) did not promote any aggregation in spite of sharing the same choline moiety. However, these authors did not discuss the possible involvement of sphingomyelin-induced ordered domains as the cause of Aβ aggregation. In this regard, the addition of dipalmitoyl phosphatidylcholine (DPPC), a saturated PC, to palmitoyl-oleoyl phosphatidylcholine (POPC) liposomes did induce aggregation of amylin and prion protein [78, 84]. Furthermore, DPPC was also able to trigger Aβ aggregation by stimulating peptide binding to membranes thus increasing peptide-peptide interactions [85]. Although rigid lipid domains seem to be a necessary condition for protein aggrega-

tion, it is definitely not sufficient for all the proteins. Actually, calcitonin showed a strong dependence on the lipid nature for aggregating in the presence of lipid vesicles even in gel phase. Indeed, neither DPPC nor dioleoyl phosphatidylserine (DOPS), an anionic lipid, were able to induce amyloid formation from calcitonin. On the other hand, aggregation was more likely the result of the affinity of this protein for cholesterol and ganglioside domains [86]. Most of the reports dealing with sphingomyelin and Aβ consider this phospholipid alongside cholesterol and ganglioside GM1 as key components of microdomains [87-88].

Another very important naturally-occurring mammalian lipid involved in protein aggregation is cholesterol. Cho *et al.* demonstrated by time-lapse atomic force microscopy that cholesterol induced the formation of 200- to 500-nm protein clusters of amylin that can act as seeds for protein aggregation. These clusters were larger but fewer than in cholesterol-depleted membranes [89]. In the same trend, even though transthyretin needs electrostatic forces to bind lipid vesicles, an event that is associated to the cytotoxicity induced by amyloidogenic transthyretin, addition of cholesterol markedly enhances the amount of high-affinity binding of an amyloidogenic mutant (L55P) transthyretin [79]. Also, it was reported that cholesterol, at concentrations above 30%, can inhibit aggregation of both amyloidogenic immunoglobulin light-chain variable-domain [90] as well as in AD Aβ peptide [91]. In the latter case, fibril inhibition was achieved by promoting peptide insertion into the membrane. However, the authors used Aβ 1-40 instead of Aβ42, which is considered the peptide involved in AD [92]. Even though there is no explanation for the lack of aggregation at high cholesterol content for the amyloidogenic light-chain variable-domain, one can speculate that a similar mechanism can take place.

Cholesterol was shown to facilitate the insertion of Aβ into the membrane thus promoting its aggregation [83]. It is important to note that aggregated peptides in sphingomyelin- and cholesterol-containing membranes presumably forced sphingomyelin molecules out from the bilayer. It was proposed by Devanathan *et al.* that cholesterol might be incorporated into these Aβ clusters.

There is an enormous body of evidence pointing out that Aβ binds to ganglioside GM1 (see below), but increasing cholesterol content in membranes can accelerate the binding of Aβ to GM1 by cholesterol-induced clustering of GM1 in membranes [62]. Furthermore, Hayashi *et al.* showed that human Apolipoprotein E4 knock-in mice have increased cholesterol content which can definitely intensify the conformational alteration of Aβ upon its binding to GM1 and therefore act as a seed for peptide aggregation [93].

Interestingly, Rymer and Good showed that synthetic human prion peptides with beta-sheet and amyloid structures were able to bind cholesterol-rich membranes, which in turn can lead to their toxicity. Moreover, these peptides displayed low affinity for cholesterol-depleted membranes [94].

Finally, there are several reports pointing out to the apparently specific binding of Aβ peptide to ganglioside GM1. For instance, Nakazawa *et al.* were able to show a strong interaction of Aβ with liposomes by ^{31}P NMR [95]. In the same trend, Mandal and Pettegrew also showed the interaction by multidimensional NMR studies. In the latter study, the authors compared the asialo GM1 with the GT1b, a trisialo ganglioside that could not bind Aβ, confirming the specific interaction between GM1 and Aβ [96]. Both studies worked with Aβ 1-40 instead of Aβ42, which is the most relevant peptide derived from APP in AD [92, 97]. Interestingly, Aβ can interact with pure GM1 micelles and form fibrils upon binding with this ganglioside. Okada *et al.* suggested that dying neurons might release GM1 which in turn would trigger Aβ aggregation *in vivo* in AD [98]. In strike contrast to Mandal and Pettergrew's findings, Okada *et al.* showed that GT1b also facilitated the aggregation, strongly suggesting that this ganglioside is also able to bind Aβ peptides, although the GT1b effect was weaker than that of GM1 [98]. The same group demonstrated later that even though Aβ can form fibrils in buffer, they are unable to interact with membranes, because they have a small surface hydrophobicity. In contrast, Aβ readily aggregated in the presence of GM1 containing-liposomes. These fibrils were much more toxic [99]. In the same trend, Kakio *et al.* showed by Fourier transform infrared spectroscopic as well as fluorescence measurements that Aβ aggregates formed in buffer and in membranes have different structure, the protein aggregates in buffer having no affinity for membranes [100].

GM1 is the well-known receptor for cholera toxin [101]. Based on this fact, it was possible to demonstrate a partial co-localization of Aβ42 with GM1-rich domains in cells by using a fluorescently labeled cholera toxin [102-103]. It is important to note that although most of the work on amyloidogenesis related to membranes were carried out with Aβ, other proteins such as prion and amylin can also bind GM1. In fact, these proteins displayed an enhanced activity on liposomes in the presence of GM1, the aggregation of lipid vesicles being the result of this interaction [104]. Another report stated that GM1 was involved in AS-membrane interaction. Actually, the presence of GM1 in small unilamellar vesicles inhibited AS aggregation in striking contrast to what was described for Aβ [105].

MEMBRANE PERTURBATION AND AGGREGATED PROTEIN CYTOTOXICITY

The ability of amyloid aggregates to bind the cell membrane and induce disruption of lipid bilayer structure was extensively correlated with their cytotoxicity [106]. However, the mature amyloid fibrils appear to be substantially less toxic than the soluble oligomeric pre-fibrillar aggregates. The toxicity of these early aggregates depends on their intrinsic ability to interact with cellular membranes, suggesting that the fibrillar inclusions may have a protective role [20].The membrane activity of aggregated peptides/proteins has been well documented for Aβ [107-112], AS [113], IAPP [114], Stefin B [115-116] or human prion amyloidogenic fragment PrP [117-118].

One of the most popular mechanisms proposed to justify the toxicity of the amyloid aggregates is the ‘amyloid pores’ or ‘amyloid channels’ theory, which has received extensive experimental support. However, another mechanism different from the amyloid pore has been proposed recently for the GAPDH:acidic membrane interaction. GAPDH is a multifunctional enzyme extensively studied because of its relevant

Fig. (4). Model of GAPDH interaction with a PC/PS (8:1) bilayer in the orientation of minimum free energy. The electrostatic properties of the system in 20 mM salt solution, pH 7.40, were calculated by using DelPhi software. From [122].

role in the cell energy production. However, current evidence reveals that this enzyme is actually a multifunctional protein, displaying a number of diverse cellular functions unrelated to glycolysis [119]. GAPDH was also involved in neurodegenerative diseases since immunofluorescence analysis revealed the presence of GAPDH in Lewy bodies (LB) in PD [120] and in amyloid plaques [121]. The ability of acidic membranes to trigger the GAPDH amyloid fibril formation was also reported [24]. The availability of GAPDH structural data allowed the use of biocomputational techniques to study the protein:membrane structure. The orientation by which a protein is immobilized on the membrane surface was calculated by using the Poisson- Boltzman finite difference (FDPB) methodology and *in silico* alanine scanning mutagenesis [122]. The presence of a huge electropositive crevice in the GAPDH surface facing the membrane was evidenced when protein–membrane complex reaches its minimum ΔG_{el} Fig (**4**). The relationship between curved protein surfaces and bilayer packing alteration was strongly suggested by molecular dynamics simulations of the GAPDH:membrane system. In fact, in the nanosecond timescale the tracking effect of the GAPDH positive crevice on the membrane phospholipids was evidenced by a significant increment in the phospholipid order parameter and bilayer thickness. By using spectroscopic techniques, the membrane damage induced by GAPDH binding was evidenced by changes in membrane permeability and membrane fusion of liposomes in the presence of the enzyme [122]. These changes were demonstrated in the protein:membrane interaction region forming microdomains with different biophysical properties. The boundaries of these microdomais showed phospholipid packing defects that could be responsible for the permeability changes observed after protein:membrane interaction.

Moreover, biophysical analysis carried out in the timescale of seconds has demonstrated the sequence of conformational changes which destabilizes the GAPDH tetramer triggering the aggregation Fig. (**5**). The combination of techniques covering different time-scales is nowadays essential to understand the membrane-induced amyloid formation, and the GAPDH model of fibrillation could be extended to other multimeric amyloidogenic proteins like transthyretin.

OXIDATIVE STRESS AND AMYLOIDGENESIS

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated extra- and intracellularly by various mechanisms. Cellular generation of ROS is initiated by the reduction of molecular oxygen O_2 to anion superoxide $O_2^{\bullet-}$ by the mitochondria in the respiratory chain. The process is thermodynamically very favored, but fortunately it is spin forbidden, reducing largely the reaction rate (kinetic control), and thus allowing aerobic life to exist. However, oxidative stress occurs due to an imbalance in the oxidant and antioxidant levels. As depicted in Fig. (**6**), there are several chemical and enzymatic reactions that convert $O_2^{\bullet-}$ in other ROS and also RNS under physiological conditions. It is interesting to note that neutral and ionic, or radical and non-radical reactive species can be formed, allowing a wide range of reactivity in several cell environments.

Based on several laboratory and clinical studies, it seems that these oxidant species are the major intermediary risk factors that initiate and promote oxidative stress in cellular aging [123] and in the pathogenesis of neurodegenerative

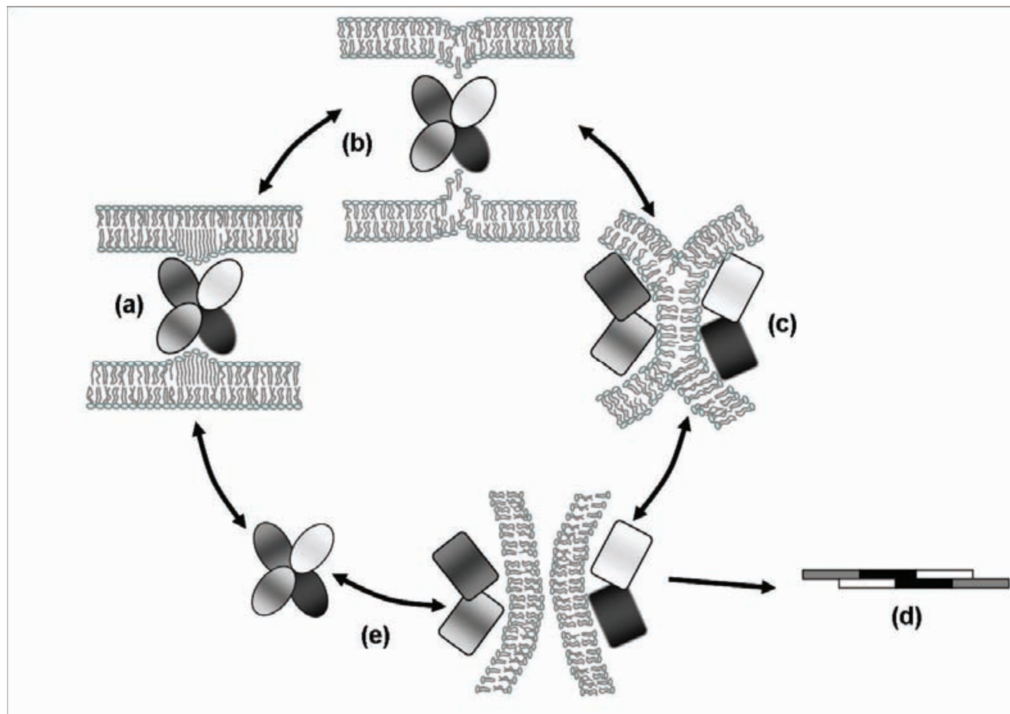

Fig. (5). The putative pathway of GAPDH amyloid fibril formation in the presence of acidic membranes based on biocomputational and spectroscopic data. The tetrameric GAPDH interaction with acidic membranes induces bilayer destabilization (**a**). The tracking effect of the GAPDH positive crevice could form a membrane hemifusion pore together with GAPDH conformational change and tetramer dissociation (**b** and **c**). The unfolded monomers could self aggregate to achieve the amyloid fibrils (**d**) or re-associate in a tetrameric globular state (**e**).

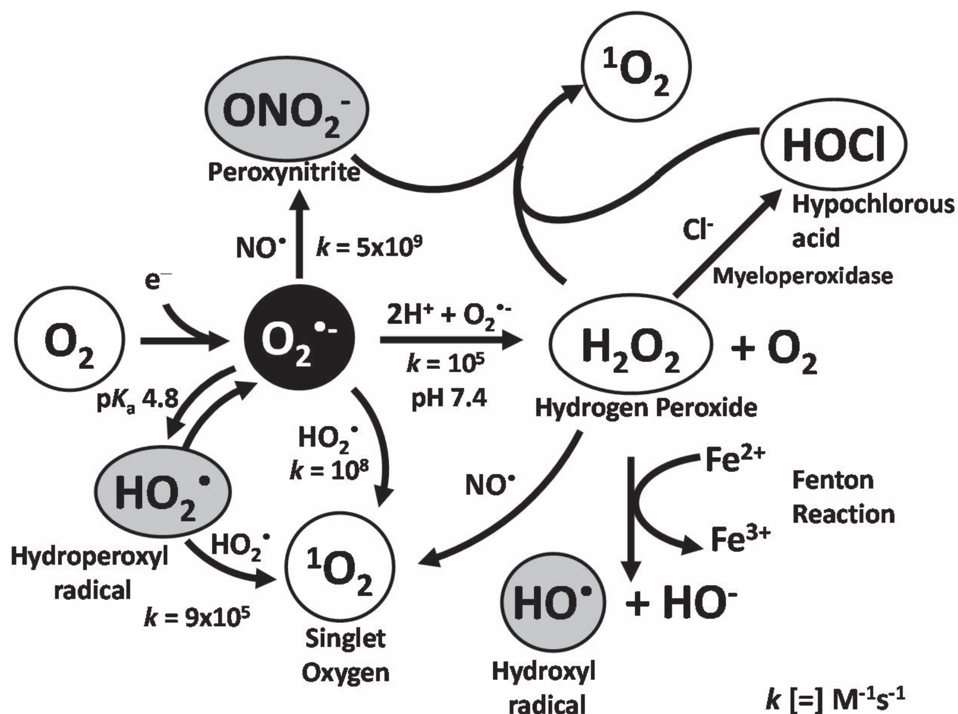

Fig. (6). Schematic representation of the chemical and enzymatic processes leading to the formation of oxidant species in biological media. Bimolecular rate constants k are in $M^{-1}s^{-1}$.

diseases [6-8, 124-126]. Oxidants can damage a plethora of cellular macromolecular targets, including proteins, lipids, carbohydrates, DNA and RNA. In addition to the known antioxidant defense mechanisms, e.g. activation of antioxidant enzymes, oxidative stress resistance depends on the composition of cellular membranes. Increased oxidative

stress induces modification of the membrane properties, such as fluidity that can redistribute membrane-associated molecules and proteins. Nevertheless, it is still difficult to define a chemical mechanism linked to a specific oxidative process in neuropathologies. In an effort to define such mechanisms, several works have been performed to describe the effect of oxidative changes in proteins, nucleic acids, and lipids in neuropathologies.

Among amyloidogenic proteins, the neurotoxic Aβ contributes to oxidative damage in AD by inducing lipid peroxidation, which in turn generates downstream additional cytosolic free radicals and ROS, leading to mitochondrial and cytoskeletal compromise, depletion of ATP, and ultimate apoptosis. The effect of Aβ42 inducing oxidative stress and neurotoxicity has been reviewed by Butterfield [127]. Addition of Aβ42 to neurons leads to increased protein carbonyls and decreased cell survival compared to controls [125, 128], and it has been demonstrated that lipid peroxidation is increased in AD brain as assessed by increased levels of thiobarbituric acid reactive substances (TBARS), isoprostanes and neuroprostanes, 4-hydroxy-2-nonenal (HNE), and acrolein [129]. Furthermore, the addition of Aβ42 to synaptosomal membranes was shown to lead to the formation of HNE or isoprostanes, both products of lipid peroxidation [130-131].

Alternatively, Aβ42 was found to accelerate oxidative lipid damage caused by physiological concentrations of ascorbate and submicromolar concentrations of Cu(II) ion [132]. Under these conditions, Aβ42 was aggregated; however it was non fibrilar, a repeated characteristic of oxidized amyloidogenic proteins. Ascorbate and copper produced H_2O_2, but Aβ42 reduced H_2O_2 concentrations, and its ability to accelerate oxidative damage was not affected by catalase. Lipids could be oxidized by H_2O_2 and Cu(II) in the absence of ascorbate, but only at significantly higher concentrations, and Aβ42 inhibited this reaction. These results indicate that the ability of Aβ42 to promote oxidative damage is more potent and more likely to be manifest *in vivo* than its ability to inhibit oxidative damage. In conjunction with prior results demonstrating that oxidatively damaged membranes cause Aβ42 to misfold and form fibrils, suggesting a specific chemical mechanism linking Aβ42-promoted oxidative lipid damage and amyloid fibril formation [132]. This hypothesis has been revised by using polarized attenuated total internal reflection infrared FT-IR spectroscopy for characterizing the conformation, orientation, and rate of accumulation of Aβ on lipid membranes under oxidative stress conditions [133]. Oxidative damage, induced by a mixture of ascorbate and cupric ions, causes Aβ to form amyloid fibrils and accumulate on the membrane with their long axis oriented parallel to the membrane surface. The ability of oxidatively damaged lipids to promote pathological β-structure in Aβ proteins, coupled with the ability of Aβ proteins to promote oxidative damage, suggested that a positive feedback mechanism may be responsible for producing amyloid fibrils [133]. The positive feedback mechanism involving either the oxidation of amyloidogenic proteins or membrane components is one by which oxidative stress may be linked to an enhanced fibril formation. Such a mechanism may be initiated by an acute oxidative stress, a deficiency of antioxidant mechanisms in the brain, and/or Aβ overproduction due to the mutations known to cause familial AD [134].

Acute oxidative stress effect on specialized membrane binding proteins plays an important role in neurodegenerative diseases. It has been suggested that Aβ protein promotes the copper-mediated generation of HNE from polyunsaturated lipids, and in turn, HNE covalently modifies the His residue side chains of Aβ [135]. Thus, HNE-modified Aβ have an increased affinity for lipid membranes and an increased tendency to aggregate into amyloid fibrils [11]. Thus, the pro-oxidant activity of Aβ leads to its own covalent modification and to accelerated amyloidogenesis. These results illustrate how lipid membranes may be involved in the pathological misfolding of Aβ, supporting the positive chemical feedback mechanism linked with oxidative stress [133-135]. In addition, Butterfield *et al.* [136], have shown that in AD patients' brain the neuropolypeptide h3 (NPPH3) is a specifically oxidized protein. NPPH3 is a phosphatidylethanolamine binding protein (PEBP) playing a relevant role in maintaining phospholipid asymmetry, which is important in order to maintain the structure and function of membranes [137]. The oxidation of NPPH3 produces the loss of PEBP activity that may lead to loss of membrane asymmetry. This, in turn, may initiate apoptosis and consequently cell death. Furthermore, *in vitro* models of AD such as neuronal cells and synaptosomes, treated with Aβ42 and HNE were shown to increase the levels of all the oxidative stress parameters [138]. This enzyme also regulates the levels of choline acetyltransferase, an enzyme that is reported to have decreased activity in AD brain [139], and this could be related to the reported cognitive decline in AD.

The intermediacy of radical species in cells, and in particular their role in membranes, has been proved by the effect of Vitamin E and numerous other antioxidants, which inhibit Aβ-induced lipid peroxidation [140-143]. In fact, it has been proposed that the antioxidant mixture of Vitamin E (which prevents *de novo* membrane oxidative damage), folate (which maintains levels of the endogenous antioxidant glutathione) and acetyl-carnitine (which prevents Aβ-induced mitochondrial damage and ATP depletion) provides superior protection than each agent alone [143], indicating that the oxidative-induced amyloid formation and membrane damage involves both intracellular and membrane located oxidative stress.

The presynaptic protein AS, which is associated with PD, is found in both soluble cytosolic and membrane-bound forms [144]. In PD, AS is known to be the major component of LB [145], but the reason why AS is deposited into LB is not clear. It has been suggested that oxidative-damaged AS is more prone to aggregation and therefore it is selectively deposited into the LB [146]. Although the function of AS is unknown, several observations suggested that its association with membranes is important. In fact, it is supposed to exert toxic effects by inducing the formation of membrane pores [147-148]. Recently, the binding of AS to membranes using the multiparametric fluorescence response of excited state intermolecular proton transfer (ESIPT) dyes has been described by Shvadchak *et al.* [149]. It has been shown that AS strongly binds to both anionic phospholipid vesicles and cell membranes through α-helix formation in its N-terminal re-

peat region whereas cytosolic AS is disordered (see above) [113, 150, 151]. The binding depends on the mutant variants of AS [150]. However, contrary to the interaction with artificial membranes, the interaction with biological membranes is rapidly reversible and is not driven by electrostatic attraction [113].

Protein partition between cytosol and membrane has an influence on the oxidative processes of AS depending on the localization of the oxidant. The nitrosative stress of AS in 1,2-dilauryl-sn-glycero-3-phosphatidylcholine (DLPC) liposomes induced by the radical $NO_2^{\bullet}$ did not cause a significant difference on the amount of aggregated and nitrated AS in the aqueous phase. However, when the DLPC lipid phase was examined under these conditions, there was an increase in aggregated and nitrated AS in the lipid phase, indicating that hydrophobic $NO_2^{\bullet}$ reacts with membrane-bound AS more efficiently [152]. Thus, the hydrophobic membrane matrix provides a focal point of AS aggregation and nitration. The presence of unsaturated fatty acids initially abrogated the nitration and aggregation of AS by $NO_2^{\bullet}$. However, upon prolonged incubation of AS in presence of unsaturated fatty acid, protein aggregation was shown to be enhanced [152]. This process can be a consequence of the oxidative modification of lysine residues of AS during the lipid-peroxidative damage that greatly accelerates the aggregation of membrane-bound AS [153].

Several observations suggested that the nitration of tyrosine residues of AS under pathological conditions facilitates the formation of LB, as observed in immunoelectron microscopy studies [154]. Nitration of AS is observed in all tyrosine residues (Tyr39, Tyr125, Tyr133 and Tyr136), contributing to the peroxynitrite-induced aggregation of AS [155]. Nitrated AS is more resistant to proteolysis and prone to aggregation, and has reduced lipid binding tendency and solubility in the cells [156]. In contrast with hydrophobic RNS-mediated aggregation and nitration of AS, the DLPC membrane aggregation of AS was unaffected by the hydrophilic $CO_3^{\bullet -}$, even in the presence of membrane radical scavengers [152].

The effect of AS on lipid oxidation in membranes containing phospholipids with unsaturated fatty acids was also analyzed [157]. The results showed that monomeric AS efficiently prevented lipid oxidation, whereas fibrillar AS had no such effect, suggesting that the prevention of unsaturated lipid oxidation by AS requires its binding to the lipid membrane. The antioxidant functions of AS is attributed to its easy oxidation via the formation of methionine sulfoxide, as shown by mass spectrometry [158]. These findings suggest that the inhibition of lipid oxidation by AS may be a physiological function of the protein. Furthermore, the *in vitro* oxidation of the methionine residue of AS to methionine sulfoxide by hydrogen peroxide favors the formation of porous oligomeric species with rounded or "donuts-like" shapes instead of the growth of elongated fibrils [157-158], which are believed to induce more membrane damage and cell death. The same result was found by singlet oxygen mediated oxidation of the methionine residues of AS in buffer solution, where the aggregation of AS-oxidized lead to the formation of well-shaped oligomeric pores as proven by atomic force microscopy (AFM) [159].

Finally, the role of oxidative stress on GAPDH and its relationship with neurodegerative disease was recently reviewed [160]. This abundant oxidoreductase plays a role in glucose metabolism, and recently has been related with cell death is triggered by oxidative stress [161]. It was shown that under nonreducing *in vitro* conditions, oxidants induced oligomerization and insoluble aggregation of GAPDH via the formation of intermolecular disulfide bonds through the four Cys residues. Studies with Cys mutants of GAPDH showed that the oxidation of the active site Cys149 is crucial for disulfide-bonded aggregation. The oxidation also caused conformational changes in GAPDH concomitant with an increase in β-sheet content; leading to the formation of amyloid-like fibrils. Additionally, continuous exposure of GAPDH-overexpressing HeLa cells to oxidants produced both detergent-insoluble and thioflavin-S-positive aggregates of GAPDH, which were associated with oxidative stress-induced cell death. Thus, the enzyme oxidative stress induces amyloid-like aggregation of GAPDH via aberrant disulfide bonds of the active site cysteine, and the formation of such abnormal aggregates promotes cell death [161]. Under mild oxidative stress conditions, GAPDH showed an unusual binding to erythrocytes from patients with autosomal dominant polycystic kidney disease (ADPKD), that it was associated with an abnormal membrane organization [162].

In agreement with the results observed in several reports, the role of oxidative stress on neurodegenerative diseases seems to be rather complex in the cellular context, since there are many molecular targets that can trigger the misfolding of the protein after oxidative modification. Originally, the cause of neurodegenerative diseases was associated with the 'amyloid hypothesis', in which the aberrant protein fibrillar aggregation compromises cellular functionality, ultimately leading to neurodegeneration [163]. However, oligomeric intermediates of the aggregation pathway are currently considered as the more likely reason for cellular toxicity [164] dysfunction and death, rather than the large amyloid fibrillar aggregates, which instead may function as a protective sequestration mechanism [165] . In this framework, oxidative and nitrosative stresses can contribute to both the protein and membrane modifications, either in a selectively or non-selectively fashion, increasing the formation of toxic oligomeric and fibril species, Fig. (**7**). In principle, the oxidative or nitrosative challenge can be initiated in several ways, degrading membrane components (lipids and hydrophobic molecules) leading to structural membrane modifications, which in turn favors the aggregation of the native protein forming a vicious oxidative-amyloidogenic circle (positive feedback mechanism, Fig. (**7a**). Another approach is based on specific modification of the amyloidgenic proteins by ROS and RNS, inducing the formation of toxic oligomeric and/or fibril aggregates, which in turn interact with the membrane modifying its structure and properties with the sequential cellular misfuction and death, Fig. (**7b**). In any case, both general scenarios are favored by the drastic unbalance of the antioxidant cell mechanism.

GENERAL CONCLUSIONS

Membranes are frequently considered as non-specific catalysts of the protein aggregation reaction. In fact, membranes can efficiently alter the protein folding equilibrium

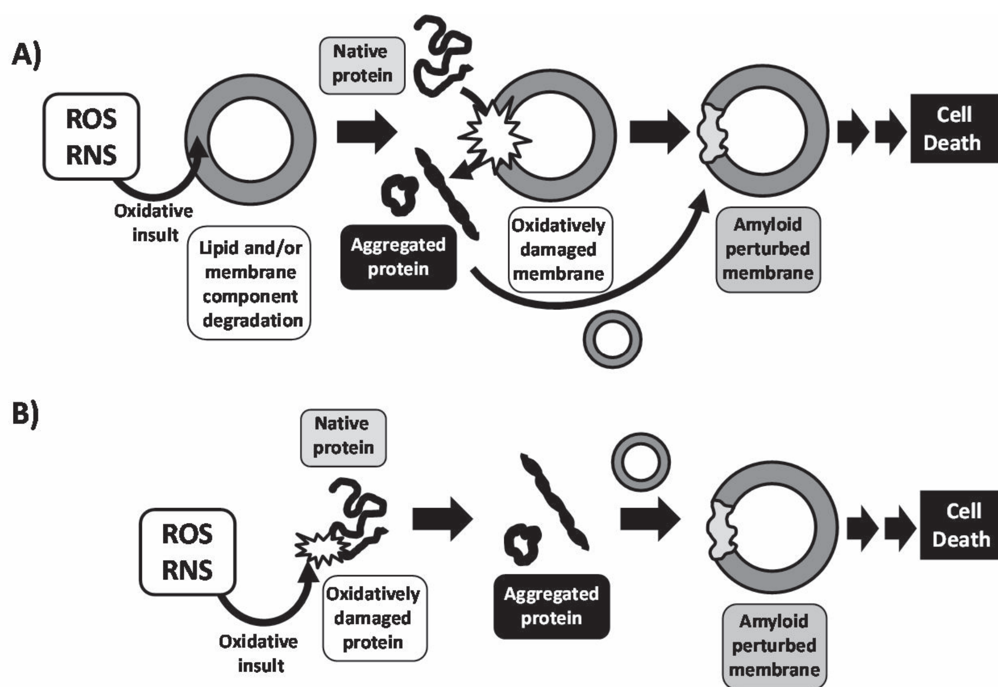

Fig. (7). Two general scenarios for the role of oxidative stress in the formation of amyloid protein aggregates: **A**) the oxidative challenge is specific on membrane components, e.g. PUFA. The modified membrane is more prone to induce aggregation of native protein leading to a vicious circle of oxidation-aggregation resulting in amyloid-modified membrane. **B**) In this case, the oxidative stress is specific on the amyloidogenic protein that triggers the enhanced aggregation process leading also to the amyloid-modified membrane.

due to their biophysical properties as well as their domain architectures and the ability to undergo oxidative and nitrosative changes as discussed above. Strictly speaking, catalysts must be recovered unchanged at the end of the process in order to be recycled. Nevertheless, a large body of experimental data has shown that after triggering the conformational changes leading to the aggregation state, some biophysical parameters of the membranes also change. Even though membranes can not be considered catalysts in protein misfolding and aggregation processes, they play an essential role. At the present time, there is an active search for a common molecular mechanism in the area of neurodegenerative disease, and thus the role for cellular membranes as a common target in the neurotoxic action of oligomeric and/or fibrillar structures have become relevant. However, the biophysical mechanism by which soluble oligomers or fibrils associate with membranes could follow different pathways. Moreover, not only the specific components are important for aggregation, but modification of membrane components and properties could be essential to understand and regulate the process. In this way, the obvious question is if oxidation or response to stress conditions could change the membrane pressure on protein folding. In this way, could amyloidosis be considered as a membrane-associated pathology? The present review aims to provide a theoretical base as well as an up-to-date survey of the current knowledge on the essential role that membranes may have in amyloidosis.

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