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Review

Amyloid-Mediated Mechanisms of Membrane Disruption

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Abstract: Protein aggregation and amyloid formation are pathogenic events underlying the development of an increasingly large number of human diseases named "proteinopathies". Abnormal accumulation in affected tissues of amyloid β (Aβ) peptide, islet amyloid polypeptide (IAPP), and the prion protein, to mention a few, are involved in the occurrence of Alzheimer's (AD), type 2 diabetes mellitus (T2DM) and prion diseases, respectively. Many reports suggest that the toxic properties of amyloid aggregates are correlated with their ability to damage cell membranes. However, the molecular mechanisms causing toxic amyloid/membrane interactions are still far to be completely elucidated. This review aims at describing the mutual relationships linking abnormal protein conformational transition and self-assembly into amyloid aggregates with membrane damage. A cross-correlated analysis of all these closely intertwined factors is thought to provide valuable insights for a comprehensive molecular description of amyloid diseases and, in turn, the design of effective therapies.

Keywords: amylin; lipid bilayer; protein aggregation; misfolding

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1. Introduction

In the first decade of the last century, Alois Alzheimer described the presence of "amyloid" plaques and neurofibrillary tangles (NFTs) post-mortem in the brain of a woman suffering from cognitive decline and memory loss [1]. Historically, this is the first scientific article which documents a case of a neurological disorder that was later generally identified as Alzheimer's disease (AD). Nearly 90 years after that groundbreaking report, protein aggregates that form amyloids [2] and NFTs [3] present in AD brains have been thoroughly described and are universally considered as diagnostic traits of this fatal neurological disorder [4]. Indisputably, attention devoted to "amyloid" aggregation by peptides and proteins has considerably augmented over the last two decades, transforming it from a fascinating phenomenon associated only to a small number of proteins into a major topic involving many disciplines ranging from biophysics and chemistry to medicine. The mounting interest in this field is surely associated with the growing number of illnesses related to amyloid formation [5]. Protein misfolding and amyloid aggregation are common pathogenic mechanisms of more than 40 progressive diseases, termed protein conformational diseases (PCDs) or proteinopathies, which are the most detrimental in terms of social and health care costs in civilized countries [6]. These disorders include AD, type II diabetes mellitus (T2DM) and prion diseases (see Table 1) [7].

AD is a fatal, neurodegenerative disorder characterized by a progressive cognitive decline and memory loss. It is likely the most widespread form of dementia especially among the elderly people: 60–70% of all diagnosed cases of dementia are associated with AD [8] and 32% of people 85 years old and older have Alzheimer's disease [9]. T2DM is a metabolic disease associated with hyperglycemia and insulin resistance. It is a globally occurring disease associated with aging, a sedentary lifestyle and obesity. As of now, the number of patients suffering from T2DM diabetes worldwide is estimated to be 463 million,

and it is expected to grow to 700 million in less than 20 years [10]. Transmissible Spongiform Encephalopathies (TSEs) are infectious, fatal neurodegenerative disorders sharing many pathological aspects with Alzheimer's disease [11,12]. In the late 1990s, occurrences of Bovine Spongiform Encephalopathies (BSE), also known as "mad cow disease", created great concern regarding the safety of food supply and considerably affected the farm animal industry [13,14]. Chronic wasting disease (CWD) infected about 15% of deer and elk in the U.S. and Canada [15,16]. Since the early 1990s, nearly 140 patients in the U.K. have died from variant Creutzfeldt–Jakob disease (vCJD), a human variant of TSE contracted from bovine contaminated food [17–19]. For each disease, there is a specific associated protein that may aggregate in a single organ or in many different tissues (systemic amyloid diseases). The first group includes AD and prion disease, in which amyloid-β (Aβ), or prion protein deposits, respectively, accumulate in the brain, or T2DM, which is associated with the accumulation of IAPP in the pancreas. Conversely, in systemic amyloidosis, protein aggregates may involve numerous organs as the heart or liver [7]. Amyloid aggregates are usually found in the same cellular districts where the protein is produced, but they may be also found extracellularly [7]. Although the majority of PCD cases are sporadic, in some cases, a heritable mutation causing an aberrant misfolding and aggregation of the protein may be at the root of a familial proteinopathy [20]. The molecular mechanisms causing amyloid toxicity to the cells have not yet been fully elucidated, but many reports suggest that anomalous amyloid/membrane interactions may play a crucial role in toxic protein aggregation by catalyzing the conversion of disordered peptides into β-sheet rich conformations [21].

Table 1. Proteins involved in the formation of amyloid deposits and related diseases.

Protein	Disease
Amyloid-β peptide (Aβ)	Alzheimer's disease
Islet amyloid polypeptide (IAPP)	Type 2 diabetes mellitus
α-Synuclein (αs)	Parkinson's disease
Prion protein (PrP)	Prion diseases
Transthyretin (TTR)	Senile systemic amyloidosis
Serum amyloid A (SAA)	AA amyloidosis

In this review, we will focus on three different proteinopathies, i.e., AD, T2DM and prion diseases, paying major attention to the role played by amyloid/membrane interaction in the underlying pathogenic events leading to the development of the diseases.

2. Aβ Peptides

The two main isoforms of Aβ peptides are Aβ1-40 and Aβ1-42 [22]. The 40-residue peptide, Aβ1–40, represents the most common Aβ isoform in the brain [23], while the 42-residue one, Aβ1–42, is more toxic, and its abnormal levels are typical of certain forms of AD (Figure 1, down) [24].

Aβ peptides originate, in vivo, from larger amyloid precursor proteins (APPs) which are integral membrane glycoproteins of 695, 714, 751, and 770 amino acids [2,25–28]. The amyloidogenic Aβ peptide encompasses 28 residues of the extracellular and 11–15 residues of the APPs transmembrane domain. Proteolytic processing of APP may occur in neuronal cells, according to two different pathways, i.e., non-amyloidogenic and amyloidogenic. In the amyloidogenic pathway, APP is initially cleaved by a β-secretase (β-site APP-cleaving transmembrane aspartic protease, BACE 1) with the consequent release of a soluble extracellular domain (sAPPβ), and an intracellular segment that is further cleaved by the γ-secretase to form Aβ peptides and the APP intracellular domain [29,30]. Cleavage of APP by β- and γ-secretase occurs preferentially in cholesterol-enriched lipid rafts [31,32]. In the non-amyloidogenic pathway, α-secretase, another membrane enzyme, cleaves APP between amino acids 16 and 17 of the Aβ peptide, thus preventing Aβ peptides generation. This cleavage produces a neurotrophic and neuroprotective soluble peptide [33]. The

activity of α-secretases occurs mostly in non-raft domains [34]. Aβ, when present at low concentrations, plays a key physiological role in the central nervous system contributing to the normal activity of the brain [35]. Aβ monomer homeostasis plays a crucial role in the control of synaptic functions. In neurons, Aβ is involved in different stages of the synaptic vesicle cycling (SVC) and the release of neurotransmitters [36]. Extracellular amyloid accumulation occurs when there is an unbalance between Aβ production and degradation [37]. Enzymatic degradation is the main mechanism for the removal of excess Aβ from the brain [38]. Aβ clearance by proteases could occur through intra and extracellular hydrolysis by brain proteases or by proteolytic removal after transport from the brain to peripheral districts. Aβ is degraded by several metalloproteases (MPs) and it is not clear which of them is more important for Aβ clearance in vivo [39]. Insulin-degrading enzyme (IDE), pre-sequence peptidase (PreP), Neprilysin (NEP), endothelin converting enzymes (ECE-1 and ECE-2), matrix metalloproteases (MMPs), angiotensin-converting enzyme (ACE) and plasmin have been demonstrated to be able to cleave Aβ. The failure of Aβ removal led to a significant cerebral accumulation of Aβ, which is believed to trigger the disease process [40–42]. Aβ aggregation is considered to play a causal role in the pathogenesis of AD [43–46], as suggested by several works evidencing its neurotoxicity both in vitro and in vivo [42,47], and many reports point to development of aggregation inhibitors as therapeutic agents [48–50]. Aβ peptides change their secondary structure from random coil to β-sheet rich, highly ordered states, endowed with cytotoxic properties [51]. β-Sheet structures aggregate into oligomers, protofibrils, and finally mature fibrils (Figure 2) with distinct morphologies [52,53]. The presence of two C-terminal hydrophobic amino acids, i.e., isoleucine and alanine, in its primary sequence induces Aβ1-42 to have a higher tendency to aggregate with respect to the more abundant Aβ1-40 [54]. Several studies suggest that small-sized Aβ oligomers represent the most toxic form of the peptide [55], supporting the view that intermediate aggregates rather than mature amyloid fibrils, might be responsible for neuronal loss [56].

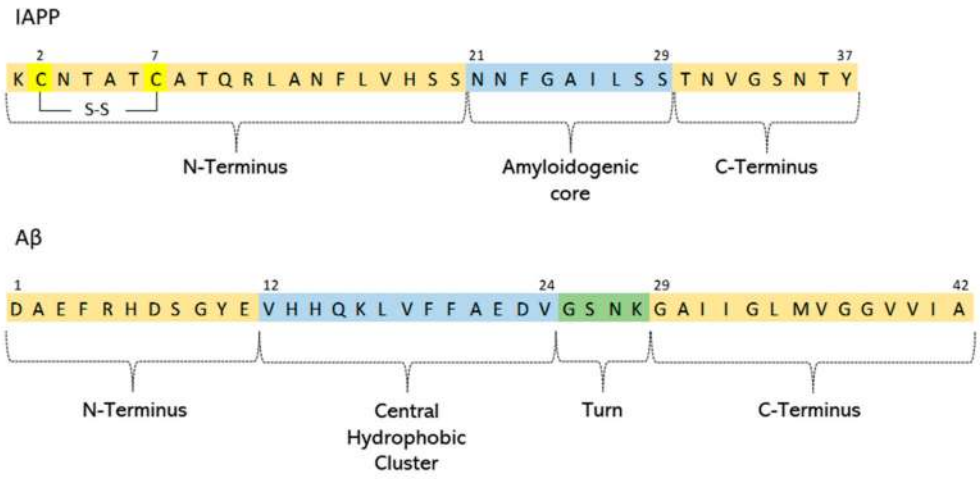

Figure 1. Representations of the human IAPP (top) and Aβ (bottom) sequences. The disulfide bridge connecting residues C2 with C7 is indicated as S-S. The amyloidogenic core of IAPP, the central hydrophobic cluster, the turn regions of Aβ, and the C- and N-terminal regions of both peptides are highlighted in different colors.

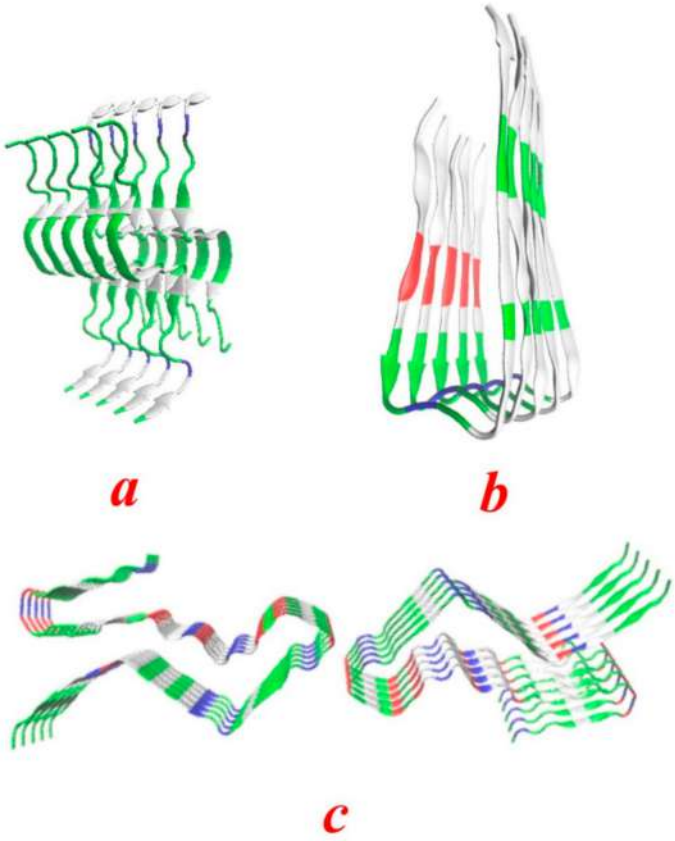

Figure 2. Cartoon representation of fibrillar structures of IAPP (**a**, pdb: 6vw2); Aβ42 (**b**, pdb: 2beg); and prion protein (**c**, pdb:6lni). Color code: blue, basic; green, polar; white, non-polar; red, acidic residues.

3. Islet Amyloid Polypeptide (IAPP)

Islet amyloid polypeptide (IAPP) or amylin is a peptide of 37 amino acids that includes a disulfide bridge linking residues C2 and C7 (Figure 1, up). IAPP is co-expressed and co-secreted in pancreatic β-cells with insulin, in response to glucose intake [57]. IAPP is initially expressed as a pre-prohormone which is cleaved by endoplasmic reticulum (ER) peptidases, thus producing the prohormone which, in turn, is processed by the pro-hormone convertase2 (PC2), pro-hormone convertase 1/3 (PC1/3) and carboxypeptidase E (CPE) [58]. This process is strictly dependent on pH variations and occurs in the late Golgi apparatus and the secretory granules [59,60].

The physiological role of IAPP is related to activate endocrine actions through β-cell signaling [61]. IAPP and insulin are both involved in glucose homeostasis and metabolism. As insulin regulates the transfer of glucose from the blood into tissues, IAPP is implicated in the regulation of glucose uptake by controlling the gastric emptying [58]. Likewise, IAPP acts as a satiety signal which controls appetite, restricts the consumption of food and, in turn, regulates body weight [62]. Increased blood glucose levels activate a compensatory mechanism enhancing both insulin and IAPP synthesis and secretion. However, it is possible that throughout these stressed circumstances there is an augmented risk for abnormal pro-hormone processing, with a concomitant secretion of nonfunctional hormones. In accordance with this scenario, TEM analysis of the intracellular amyloid deposits in mouse and human pancreatic islets using with specific antibodies confirmed the presence of abnormally processed proIAPP [63]. A malfunction of the cellular proteostasis system can ignite IAPP misfolding and, eventually, led to the toxic accumulation of amyloid deposits in pancreatic β-cell [64,65]. Hyperglycemia is also known to promote a nonenzymatic glycation of proteins forming advanced glycation end products (AGEs). Abnormal accumulation of AGEs may interfere with normal activity of their receptor (RAGE), causing immune

and endothelial cell failure. Notably, it has recently been evidenced that toxic aggregated forms of IAPP may also bind and upregulate RAGE in β-cells [65]. IAPP is known to be highly aggregative both in vitro and in vivo [66]. Moreover, when dissolved in water, IAPP readily forms amyloid fibrils that may be observed by electron or atomic force microscopy (Figure 2). IAPP fibrils formed in aqueous solutions do exhibit typical amyloid features since as definite β-sheet structures are seen by spectroscopy and classic birefringence upon staining with Congo red [67]. Likewise, an analysis of the X-ray diffraction pattern of hIAPP fibrils indicate a cross-β organization characterized by a typical 4.7 Å meridional signal in accordance with what observed in other amyloid fibrils [68]. Amyloid fibril formation by IAPP was observed not only in human patients, but also in animal models. As an example, islet samples from transgenic mice show the presence of deposits within the β-cells. Post-mortem pancreatic tissues from T2DM patients evidenced extracellular amyloid deposits [69]. From a comparison with data from mice models, it was ascertained that IAPP amyloid may be formed both intracellularly and extracellularly [70].

IAPP is an intrinsically disordered protein (IDP), as it presents a broad conformational diversity in aqueous solution: random coil, extended antiparallel β-hairpin, unstructured with transitory α-helix conformation, mixed α-helix and short antiparallel β-sheet, and a compact helix-coil structure [71]. Even though IAPP may explore many different conformations, it predominantly adopts a random coil structure in the monomeric state. Many Circular Dichroism (CD) and computational studies carried out on IAPP allowed a thorough characterization of its various of secondary structures components. As indicated in Figure 1, the segment S21–S29 is termed the amyloidogenic core and plays an essential role in amyloid aggregation. Conformational changes of this domain toward β-rich structures ignite protein aggregation, fibril growth and, ultimately, toxicity. In fact, the non-amyloidogenic rat IAPP (rIAPP) sequence differs from human IAPP (hIAPP) in 6 residues, with 5 of them located within the amyloidogenic core, which may thus be considered responsible for aggregation propensity of the human peptide [66,72]. Three prolines of rIAPP (in positions 25, 28, 29) are considered as β-sheet breakers, i.e., regions which may destabilize β-sheet secondary structure, rooting the different aggregation propensities of hIAPP and rIAPP [73]. The C-terminal segment T30–Y37 is also considered to contribute in IAPP fibril formation [74]. The N-terminal segment K1-S19 is implicated in the early peptide-membrane interactions and does not significantly contributes to IAPP aggregation in the presence of membranes [75,76]. By contrast, the segment A13-H18 plays an important role in IAPP amyloid growth in aqueous solution [77]. The physic-chemical properties of individual amino acid residue, including hydrophobicity, aromaticity, and electrostatic charge, are known to play a key role in driving IAPP aggregation propensity [78]. Hence, an understanding of the association between peptide sequence and fibrillogenicity is needed to describe the molecular factors underlying hIAPP amyloid growth. Experiments using hIAPP variants, including point mutations and truncated variants, have been employed to define the role of each residue in driving hIAPP aggregation propensity. The N-terminal region encompassing residues from 1 to 13 of hIAPP is not aggregative by itself [79] and is believed to be placed out of the amyloid fibril core [80,81]. For this reason, many investigations have addressed the central and the C-terminal region of IAPP [82,83]. The three positively charged amino acids Lys1, Arg11 and His18, at neutral pH make IAPP a positively charged peptide (pI = 8.90). These positive net charges drive IAPP interactions with negatively charged membranes, and this phenomenon is known to catalyze fibril formation and toxicity [84–86]. However, an increase of the positive net charge in a peptide may in general reduce its aggregation propensity due to electrostatic repulsion [78]. To single out the role played by positive residues on IAPP aggregation, several peptide variants have been investigated including ΔLys-1 [87], the mutated peptide K1E [88] and K1I [89]. Results of those studies suggested that modifications/replacements of Lys1 have little effects on IAPP amyloid formation in aqueous solution. However, all IAPP variant lacking Lys1 exhibited a diminished toxicity to INS-1 β-cells thus demonstrating the role of the N-terminal part of the peptide in driving pathogenic membrane interactions [87–89].

The residue His18 has been also intensively studied. In fact, the pH-dependent ionization of His18 affects IAPP aggregation [90]. The non-fibrillogenic rIAPP has an arginine residue in this position. An R18H residue replacement generates a "humanized" peptide that can form fibrils thus highlighting the important role played by His18 in modulating fibril formation [91]. Several variants in which His18 is replaced by residues varying in size or polarity formed amyloid fibrils albeit with a prolonged lag-time if compared to the parent peptide [92,93].

Aromatic side chains are also thought to significantly affect peptide aggregation. The role played by the three aromatic residues, i.e., Phe15, Phe23 and Tyr37, in modulating hIAPP aggregation has been broadly studied [94–98], but their real effects on peptide aggregation are still unclear. To shed light on this issue, previous studies have focused on short IAPP segments containing Phe-15 or Phe-23, i.e., peptides IAPP12-18 (LANFLVH) [94], IAPP20-29 (SNNFGAILSS) [66,95] and IAPP22-27 (NFGAIL) [96]. In these studies, Phe is normally replaced by an Ala residue. Alanine is chosen because it is not aromatic, less hydrophobic and with a lower β-sheet propensity than Phe [98]. Phe23 replacement by an alanine on the hexapeptide NFGAIL [96] abolished amyloid formation, showing that aromatic interactions can promote fibril growth, maybe through π-π interactions [99]. Amino acids Phe15 and Phe23 were also replaced by Ala in segments LANFLVH and SNNFGAILSS [94,95]. Those studies revealed that an aromatic sidechain at these two positions is not essential for amyloid growth of these two short peptides. These apparently contradictory results demonstrate that aromatic residues driving IAPP aggregation are sensitive to their exact position along the peptide sequence. Moreover, the length of the segment and the residues near the Phe in question may also play a role. However, when in the full-length peptide Phe15 and Phe23 are replaced by Ala residues the IAPP amyloidogenic propensity is maintained [97,98,100]. Nevertheless, substitution of aromatic residues affected aggregation kinetics, suggesting that these residues may play a role in the formation of the early steps of self-assembly occurring at the onset of the aggregation process [97], likely by activating long-range interactions between them [82,100,101]. Studies addressing the role of Phe15 further demonstrated the relationship existing between secondary structure and aggregation propensity of IAPP [97]. Phe15 replacement with residues forming α-helices resulted in peptides characterized by a propensity to form fibrils more rapidly [97]. This study reconciles with a proposed aggregation pathway for hIAPP, in which the early self-assembly events require an α-helix conformation [102].

Although the disulfide bridge is not directly involved in the amyloid core structure, still it plays a not negligible role in IAPP aggregation; as a matter of fact, its presence affects the fibrillation kinetics [103]. The disulfide bond in hIAPP connects Cys-2 and Cys-7 forming a six-residue loop which could restrain peptide structure and exclude the formation of a β-strand conformation in this domain. To address this issue, some variants have been created, such as the truncated peptide IAPP8-37, which lacks the disulfide bridge, and a peptide in which the disulfide bridge has been reduced and carboxyamidomethyl (CAM) blocked ($IAPP_{CAM}$). These hIAPP variants showed that the presence of the disulfide bridge is not essential for amyloid growth [103]. These IAPP variants aggregate more rapidly than the parent peptide, thus showing that this N-terminal domain may inhibit hIAPP aggregation kinetics [104]. The stabilizing role of the disulfide bridge was additionally noted in an NMR study carried out using reduced and oxidized hIAPP forms [105]. Although hIAPP amyloid aggregates are present in patients affected by T2DM, a mechanistic link between fibril formation and the observed toxicity has not yet firmly established. The addition of hIAPP aggregates is clearly cytotoxic to cultured β-cells as determined by cell viability assays including the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IAPP soluble oligomers, rather than mature amyloid aggregates, were found to be the species toxic to pancreatic β-cells. Most notably, these small-sized, transient IAPP oligomers were also shown to compromise the integrity of lipid cell membranes [106,107], thus suggesting that an abnormal peptide/membrane interaction could be the causal relationship between amyloid aggregation and toxicity [108].

4. Prion Protein

Prions are a class of proteins involved in development of transmissible spongiform encephalopathies (TSEs). TSEs are fatal neurodegenerative disorders affecting both animals and humans. Prions exist in two isoforms containing 209 amino acids: the physiological cellular (PrPc) and the disease-associated form (PrPSc) [109]. PrPc is a cell surface glycoprotein anchored to cell membranes by a glycosylphosphatidylinositol (GPI) moiety. PrPc contains an N-terminal unstructured part and a globular portion encompassing three α-helices [110]. The conformational transition from α-helix to β-sheet underlies conversion of PrPc into toxic PrPSc. Circular dichroism and Fourier transform infrared spectroscopy were used to characterize the secondary structure of both isoforms. It was found that PrPc consists of 42% α-helix and about 3% of β-sheets, whereas PrPSc contains 43% of β-sheet and α-helix amounted to 30% [74].

A structural alignment of PrPc sequences from different species shows a common β1–α1–β2–α2–α3 secondary structure motif, in which the β-strands establishes an antiparallel β-ribbon [109]. Steered molecular dynamics simulation have shown a different stability of the globular part of prion from different species. Two mammalian, human (HuPrP) and Syrian hamster (ShaPrP), and two non-mammalian, chicken (ChPrP) and turtle (TuPrP) prions were investigated. These studies have highlighted that the structure stability decrease in the order HuPrP<ShaPrP<ChPrP<TuPrP. It can be speculated that the scale of stability follows the scale of evolution of species. The most archaic species of reptiles, TuPrP, is more stable than the species in which they evolved (i.e., birds) then the mammalian hamster. The lowest stability is associated to the most evolved species, i.e., humans [111]. Moreover, it was evidenced that the intramolecular contacts between the three helices play a pivotal role in the stability of Prion proteins. This finding reconciles with X-ray investigations that report the crucial role played by hydrophobic regions of PrP in forming the pathogenic prion isoform [110]. This region spans from positions 112 to 135 and has a high tendency to acquire a β-sheet structure (Figure 2) [112,113]. Other studies report the involvement of hydrophobic regions in the PrP conformational transition, occurring when the PrPSc seed the pathogenic conformational transition of PrP [110]. Solution NMR investigations have been used to determine monomeric structures of mouse [114], Syrian hamster [115,116], murine [117] and human prion proteins [118]. The conversion process from PrPC into PrPSC is associated with the self-assembling of monomeric units [119]. Further in vitro measurements suggested a "nucleation and growth" aggregation model characterized by a lag phase of hours at the micro or sub-micromolar PrP concentrations [120,121] normally found in the brain [122].

5. Biophysical Studies of Amyloid–Membrane Interactions

5.1. Model Membranes

The cell membrane is a complex system. It demarcates the boundary between a highly ordered space (inner space cell) from the chaotic extracellular area. The cell membrane consists of two layers with an asymmetric lipid composition. It is a two-dimensional fluid with liquid-crystalline features (lyotropic liquid crystals) made of lipids and proteins with high specialization, such as specific ion-channels and receptors. Besides, the cell membrane is organized in microdomains, termed rafts, essential for the biological function of membrane proteins. From this picture, we conclude that understanding fundamental lipid–lipid and lipid–protein interactions is a complex biophysical problem with many interconnected variables. To address all the variables of such a complex system, a "bottom-up" approach is preferable, i.e., it is better to study and understand the properties of model systems containing only one lipid and then add complexity to the system one step at the time, e.g., by adding different lipids to the membrane composition. This simplified view was first proposed by Singer and Nicholson [123] in their "fluid mosaic" model of the membrane where proteins are embedded in the "sea" of the lipid bilayer. The most common artificial membranes prepared are the multi-lamellar vesicles (MLVs). MLVs are concentric multibilayer vesicles having an onion-like structure. They have an average diameter ranging

between 1 μm and 5 μm and a broad size distribution [124]. MLVs containing zwitterionic saturated phospholipids show polymorphism in the temperature range between 10 to 50 °C. At low temperatures, vesicles are in the "gel" phase; phospholipid hydrocarbon tails have all-trans configuration, low order parameter (S), and low diffusion time (DT). As temperature increases, vesicles undergo a phase transition toward the ripple phase, a physical state characteristic of saturated acyl-phosphocholine, in which S and DT do not vary appreciably, while the membrane surface becomes corrugated. As temperature increases, a phase transition, named "main transition", is observed and the liquid-crystalline phase formed. The liquid-crystalline, or disordered, phase is characterized by a low S, a fast DT. Lipid "rafts" are formed in the presence of unsaturated phospholipid or cholesterol, coexist with disordered lipids, and shows a high S and a fast DT (about 1 $\mu m^2 \cdot s^{-1}$) [125–131]. Extrusion of MLV suspensions through polycarbonate membranes with properly sized pores, originate large unilamellar vesicles (LUVs). Ultrasonication of MLVs or LUVs, produce small unilamellar vesicles (SUVs). SUVs have average dimensions ranging from 100 to 400 nm with a very narrow size distribution. SUVs show a very short curvature radius and thus are instable and show a remarkable tendency to fuse together [132]. Giant unilamellar vesicles (GUVs) [133] have a diameter larger than 5 μm [134], and have a curvature radius close to zero. GUVs are useful in many biophysical studies because they can be visualized by optical microscopy [135]. Supported lipid bilayer (SLB) are formed by deposition of SUVs on mica or SiO_2 silicon surface. SLB are used in quartz crystal microbalance (QCM-D) and atomic force microscopy (AFM) studies. QCM-D [136] detects the mass deposition on the crystal sensor by revealing the change of oscillation frequency of crystal [137]. As an example, QCM-D measurements have been employed to study the interaction between hIAPP and POPC within the lag phase [138]. AFM gives information about the topology of protein-membranes complexes. The main advantage of AFM relies on its high resolution on the perpendicular axis to the plane of the SLB (of the order of nm) but a poor resolution in the XY plane where the SLB lies. Another attractive aspect of AFM is the possibility to compare mechanical force measurements with a computational strategy called steered molecular dynamics (SMD) and therefore obtain atomistic information concerning the stability of the investigated system. Black lipid membranes (BLM) consist of a phospholipid bilayer film deposited across a 1 mm hole in a solid plate between two solution compartments [139]. This model is normally used to detect membrane pore formation consequent to protein interaction by measuring the conductance of solution in the two compartments. Nano disks (nDk) are disk-shaped nanoparticles containing a phospholipid bilayer surrounded by an amphipathic belt made of synthetic polymers. Polymers bind to the lipid bilayer interface, driven by the hydrophobic effect. Polymers have the significant advantage of being able to extract membrane proteins from their native environment. Due to their shape, they have significant advantages in NMR experiments [140–143]. Figure 3 reports a schematic representation of mostly used model membranes.

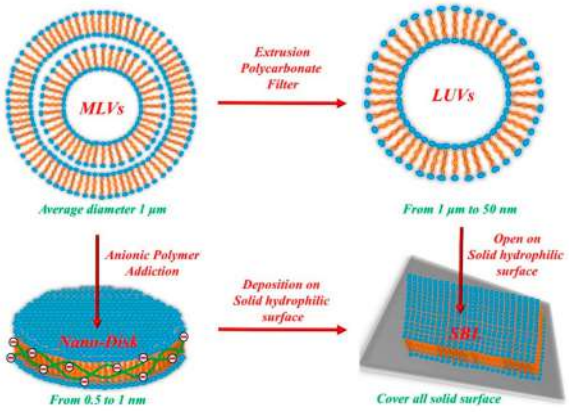

Figure 3. Schematic representation of model membranes commonly used in biophysical studies addressing amyloid–membrane interaction.

5.2. Mechanisms of Amyloid-Mediated Membrane Damage

Many in vitro studies have shown that several amyloidogenic peptides become structured upon binding to membrane surfaces, and that small-sized intermediates play a significant role in amyloid-mediated membrane damage and toxicity [144,145]. Three main membrane damage models have been proposed [146].

(i) Generation of stable transmembrane protein pores. The interaction of amyloidogenic protein, in its monomeric or oligomeric forms, with the membrane lipid bilayer led to the formation of pores which act as non-specific ion channels. It was reported that Aβ peptide, after interaction with lipid membranes, can form calcium-permeable channels that were suggested to induce cell death [147]. Consistently with this "channel hypothesis", formation of calcium channels by Aβ depends on the presence of anionic lipids and is favored by acidic solutions. Moreover, it was also demonstrated that calcium permeable channels are reversibly blocked by zinc ions and small molecules like Congo Red [148]. It has also been reported that IAPP oligomers are able to form pore-like structures in the membrane resulting in pro-apoptotic Ca^{2+} dysregulation [149,150]. Of note, similar mechanisms have been reported for α-synuclein and PrP oligomers [151,152].

(ii) Membrane destabilization via a "carpet model". According to this model, the interaction of prefibrillar species with the lipid bilayer surface results in an asymmetric pressure between both layers. Relaxation of this pressure, proximal or distal to the protein, is accompanied by leakage of small molecule, leading to membrane damage [153]. Carpeting could lead to the detergent like mechanism of membrane disruption.

(iii) Removal of lipid components from the bilayer by a detergent-like mechanism. The asymmetric pressure generated by peptides carpeting of membrane surface could lead to the removal of lipid from one or both the leaflets of the membrane. Removal of the outer leaflet may result in a transient membrane thinning, allowing the leakage of small molecules. Alternatively, removal from both leaflets results in the formation of a hole.

These three models are not mutually exclusive, and they may cooperate in triggering membrane damage (Figure 4). Indeed, recent studies have shown that membrane disruption induced by hIAPP and by Aβ may be described as a two-step process [154,155]. The first step occurs after the insertion of monomeric or oligomeric species inside the membrane hydrophobic core and leads to the formation of heterogeneous ion channels. The second step, which is independent of the first one, is related to fiber growth on the membrane surface, followed by membrane disruption through a detergent-like mechanism. Several factors have been suggested to regulate the mechanism of membrane disruption induced by amyloidogenic peptides. Lipid composition could regulate interaction between peptides and membrane surface. It has been shown, for example, that the affinity of Aβ peptides for membranes is increased in the presence of gangliosides [156] and negatively charged phosphatidylserine (PS) membranes [157–159]. IAPP–membrane interactions were investigated by using different model lipid membrane systems. It was shown that high concentrations of cholesterol, in non-raft model membranes, do not affect IAPP fibril growth kinetics but significantly reduce pore formation. On the contrary, cholesterol enhances both fiber and pore formation in raft-like model membranes [160]. Metal ions could also address membrane disruption process towards one or the other mechanism. In particular, Ca^{2+} ions promote lipid phase segregation by clustering negatively charged lipids on the membrane/water interface [161], and affect many membrane properties including the structure of membrane domains, and vesicles fusion. It was demonstrated that Ca^{2+} ions may promote the interaction of hIAPP with the hydrophobic core of PS-enriched membranes [162] and favor lipid loss via a detergent-like mechanism [163]. Moreover, it was recently demonstrated that the presence of free, non-vesicular phospholipids may promote insertion of hIAPP into membrane bilayer leading to pore formation [164]. Further biophysical and in silico studies revealed that this mechanism of membrane poration is common to other amyloidogenic

(e.g., Aβ) peptides. Based on these results, some of us proposed the "lipid chaperone" hypothesis as an additional mechanism of amyloid-mediated membrane poration [165].

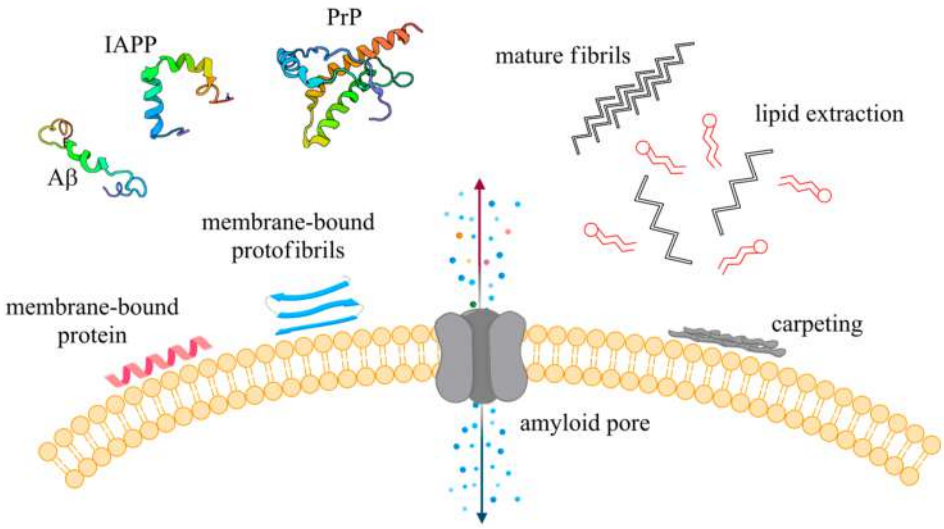

Figure 4. Representation of the different amyloid-mediated membrane disruption mechanisms. Arrows indicate the passage of ions across the amyloid pore.

6. Membrane-Bound Amyloids

6.1. Aβ/Membrane Complexes

Lipids are essential components of neuron membranes. Since APP processing by α-, β-, and γ-secretases occurs within the lipid bilayer, membrane components are believed to participate in the regulation of Aβ levels. In addition, accumulating evidence suggests abnormal lipid levels in the AD brain, indicating that aberrant interactions of Aβ amyloid with the membrane may play a significant role in AD pathogenesis [166,167]. Aβ may interact with membranes in different ways and modify their biophysical properties [168]. Aβ peptide may insert into the lipid hydrocarbon core generating a pore-like channel or may be placed (and aggregate) over the membrane surface [167]. Independently of the type of interaction, membrane-bound Aβ may damage neurons, worsen synaptic signaling, and eventually lead to apoptosis [167]. Interestingly, just as the membrane can affect Aβ fibril growth, peptide insertion may alter the physic-chemical properties of the membrane. Aβ peptide/membrane interactions are driven by both electrostatic and hydrophobic forces which cooperatively catalyze amyloid growth on the membrane surface. Zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid increases the Aβ fibril formation kinetics if compared to water [169]. Likewise, negatively charged phospholipids, such as 1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol (DMPG), speed up amyloid growth [170].

Amyloid growth on the membrane surface can affect the structure of the lipid bilayer [171]. As an example, Aβ insertion into the hydrocarbon core of 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (POPE)-rich membranes causes remarkable changes into bilayer curvature [172]. Cholesterol is known to enhance the rate of fibril formation in membranes by accelerating Aβ nucleation [173]. Gangliosides catalyze Aβ oligomerization in neuronal membranes: in fact, Aβ binds ganglioside GM1 in lipid-raft membrane [174]. Of note, Aβ fibrils formed in GM1-enriched membranes were found to be more toxic than the fibrils growth in aqueous solution [175]. Cell-free experiments carried out using neuron-mimicking total lipid brain extract (TLBE) vesicles have shown that Aβ fibril growth is not significantly faster in pure water [176,177]. An accepted hypothesis indicates that alterations in lipid bilayer owing to Aβ interactions may induce toxicity through molecular mechanisms mainly steered by electrostatic forces, in analogy to the well-known action of antimicrobial peptides [166]. Small-sized, soluble Aβ amyloid aggregates with neurotoxic activity were first described by Lambert et al. and were named

"Aβ-Derived Diffusible Ligands" (ADDLs) [178]. Aβ oligomers are highly heterogeneous in size, morphology, and toxicity. Currently, many membrane active Aβ oligomers have been reported such as dimeric [179–185], trimeric [179,180,184,186], tetrameric [180,183,184], pentameric [182,184], hexameric [183,184,186,187], decameric [183], dodecameric [186,187] and 24-meric assemblies [188–190]. Small-sized Aβ assemblies are normally short-lived and with a globular morphology. Their β-sheet content increases with their size, leading eventually to the formation of protofibrils. However, the relationship linking Aβ oligomers toxicity with their morphology is still a highly debated issue: it seems that toxicity increases with the oligomers size reaching a maximum for to dodecameric assemblies. Membrane-embedded Aβ oligomers characterized by a β-sheet annular structure have been reported to form pores in lipid bilayer and to trigger cytotoxic processes [191,192].

6.2. IAPP–Membrane Interactions

Biophysical experiments addressing IAPP amyloid growth and pore formation in model membranes with different lipid composition including DOPC, POPC, sphingomyelin, negatively charged phospholipids (POPS) and cholesterol, have demonstrated that zwitterionic phospholipids have a poor impact on amyloid growth but promote pore formation [193–197]. Cholesterol, when present in lipid membranes reduces IAPP pores and fibrils formation [198]. Concerning the role played by lipid vesicles in managing IAPP intra- and extra-cellular trafficking, some authors have investigated the interaction between IAPP, and exosomes obtained from T2DM patients and healthy people as a control. Exosomes from healthy subjects inhibited the formation of IAPP fibril growth. By contrast, exosomes extracted from diabetic individuals had no effect on fibril formation. Lipid composition of exosomes is believed to steer interactions with IAPP. In fact, differently from neuronal exosomes, no anionic lipids were found in exosomes from pancreatic tissues [199]. It is widely accepted that anionic phospholipids catalyze fibrillogenesis. However, additional experimental data are needed to propose more accurate models depicting the role of exosomes in IAPP amyloidogenesis and diabetes development. Molecular simulations of membrane-bound hIAPP from different species have been carried out [200–202] including the non-toxic, non-amyloidogenic rIAPP [203]. Molecular dynamics (MD) simulations revealed short-lived α-helical and β-sheet structures throughout IAPP adsorption onto an anionic POPG (palmitoyl oleoyl phosphatidylglycerol) surface of a lipid bilayer [201]. Membrane adsorbed IAPP monomers produced bending of the bilayer. HIAPP interaction with zwitterionic POPC (phosphatidylcholine) bilayer was investigated by MD simulations, and kinetics measurements of dye release from LUVs [164]. Both simulations and experiments demonstrated that IAPP insertion into zwitterionic membranes is assisted by non-vesicular lipids that are present in solution at their critical micellar concentration (cmc). Other authors have adopted coupled coarse-grained/umbrella sampling molecular dynamics simulations to investigate the interactions of hIAPP with ganglioside-rich membranes [200]. These simulations indicate that hIAPP locate in ganglioside-rich membrane regions due to electrostatic interactions promoting adhesion of cationic hIAPP peptides with anionic gangliosides. The three positively charged amino acids K1, R11 and H18 located in the N-terminal domain of IAPP are known to play a major role in driving interactions with negatively charged membranes [65].

6.3. Prion–Membrane Interactions

Many reports suggest that small soluble and transient oligomeric aggregates, due to their high propensity to associate with membranes, are the most active agent in driving amyloid toxicity. From these observations on IAPP, Aβ and other amyloids stemmed the "toxic oligomers hypothesis". This hypothesis was also extended to prion proteins [204–206]. Electrophysiology experiments have shown an increased conductance in membranes containing zwitterionic 1,2-diphytanoyl-sn-glycero-3-phosphocholine interacting with PrP(90–231) by pore formation [207]. Measurements on negatively charged phospholipid interaction with human prion amyloidogenic fragment PrP(185–206) described the formation of channel

but not of fibrils [208]. Studies on the PrP(180–193) fragment suggest hydrophobicity as the major driving force in protein interaction with membranes [209,210]. Additionally, PrP(106–126) and huPrP60-91 showed a noticeable tendency to penetrate the lipid bilayer with an associated conformational transition toward a β-sheet structure affected by the presence of metal ions. Copper ions favor fragment insertion, whereas zinc ions inhibit fragment transfer from the aqueous phase to the bilayer [211,212]. This finding suggests that, in the transfer PrP from the aqueous phase to the bilayer core, the electrostatic force cannot be overlooked. Additionally, some reports show that binding of PrP to artificial membrane depends on the type of lipid [213] and follows the decreasing order of affinity POPG > DPPC > rafts [214].

7. Conclusions

Current knowledge concerning the harmful role played by fibril-forming proteins on the structural integrity of plasma membranes suggests that it may be considered a key mechanism at the root of amyloid toxicity. Two distinct mechanisms have emerged over the past 20 years as the most reliable models to describe toxic amyloid–membrane interactions. According to the "channel hypothesis" amyloid peptides may self-assemble into toroidal structures that porate membranes through a mechanism resembling the activity of antimicrobial peptides. On the other hand, in the "detergent-like" mechanism, amyloid fibrils growing on the membrane surface are believed to extract lipid molecules from the bilayer. Each of these two mechanisms has been alternatively invoked to describe the effects observed by different self-assembling peptides in the presence of either artificial or natural membranes. However, despite the multitude of biophysical data gathered so far in this area, the very nature of toxic lipid/peptide complexes remains elusive. More recently, some of us have proposed a "lipid chaperone" hypothesis: non-vesicular lipids dispersed in the aqueous phase may facilitate peptide insertion within the lipid bilayer. This model, albeit extensively investigated by biophysical experiments, still needs to be verified in living cells. Because of the difficulties in obtaining stable crystalline amyloid/membrane samples, computational methods offer an unvaluable tool to describe amyloid/membrane structures at an atomistic level of detail. Indeed, details concerning the real nature of amyloid/membrane assemblies is of utmost importance due to the clinical implications in the development of new drugs aimed at interfering with membrane disruption by amyloids. In fact, when membrane damage is mediated by amyloid pores, such pathogenic effect may be alleviated by specific channel blockers designed to seal the amyloid pores. On the contrary, when nonspecific mechanisms occur, preventing membrane disruption would require different approaches focusing on the inhibition of amyloid growth and binding onto the membrane surface. In conclusion, a better knowledge of the molecular steps involved in the cascade of events at the roots of amyloid toxicity is expected to boost the research aimed at the design of molecules that can inhibit either protein aggregation or membrane disruption. Parallel computational and experimental investigations performed on even more sophisticated models will hopefully fill this gap and allow a to counteract amyloid toxicity.

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