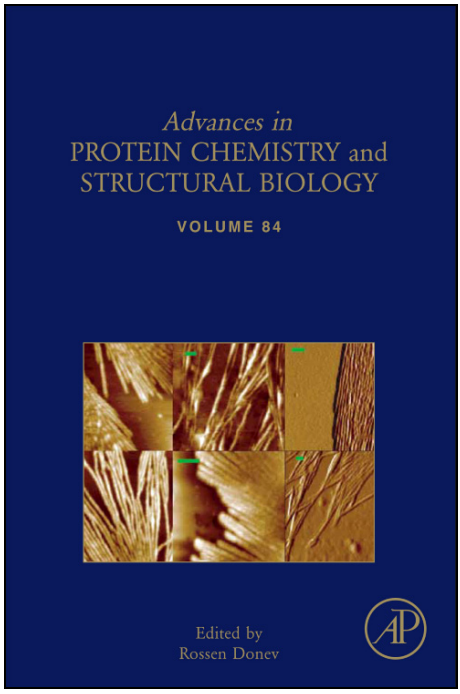

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PROTEIN AGGREGATION IN A MEMBRANE ENVIRONMENT

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Abstract

Biological membranes are featured by a remarkable ability to modulate a wide range of physiological and pathological processes. Of these, protein aggregation is currently receiving the greatest attention, as one type of the ordered protein aggregates, amyloid fibrils, proved to be involved in molecular etiology of a number of fatal diseases. It has been hypothesized that nucleation of amyloid fibrils and toxic action of their precursors is mediated by lipid–protein interactions. Lipid bilayer provides a variety of environments in which aggregated state of polypeptide chain appears to be more thermodynamically favorable than its monomeric form. The major factors responsible for the enhanced self-association propensity of membrane-bound proteins include (i) structural transition of polypeptide chain into aggregation-prone conformation; (ii) protein crowding in a lipid phase; (iii) particular aggregation-favoring orientation and bilayer embedment of the protein molecules. All these factors are considered in the present review with an emphasis being put on the role of electrostatic,

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hydrophobic, and hydrogen-bonding phenomena in initiating and modulating the protein aggregation on a membrane template. Likewise, we survey the advanced experimental techniques employed for detection and structural characterization of the aggregated species in membrane systems.

I. Introduction

Among a plethora of unique structural properties of polypeptide chain, its inherent propensity for self-assembly is currently in a focus of especially intense research efforts, representing a new frontier in unraveling the enigmas of protein molecules. This propensity may manifest itself either in intramolecular interactions, accounting for protein folding into functionally active native state, or in intermolecular interactions, giving rise to protein aggregation. The latter phenomenon can be regarded as alternative folding (Zbilut et al., 2003), because the formation of intrachain and interchain contacts is driven by the common forces, implicating hydrophobic effect, hydrogen-bonding, charge attraction, and van der Waals interactions (Seelig, 2004). It is believed that in the majority of cases, protein aggregation is triggered by the transition of polypeptide chain into unstable aggregation-competent conformation (Gsponer and Vendruscolo, 2006). This is readily achievable because the compactness of native state is compromised by the loss of configurational entropy during polypeptide folding and repulsive electrostatic interactions (Dill, 1990). Due to small difference between stabilizing and destabilizing forces, native protein structure is only marginally stable, so that any variation in physicochemical properties of polypeptide surroundings may appear critical for protein transition from monomeric to aggregated state. Accumulating evidence suggests that, in biological environment, protein self-associating tendency can be enhanced by cell membranes (Stefani, 2008; Hebda and Miranker, 2009). In a physiological context, aggregation of membrane-associated protein molecules has been recognized to play a crucial role in signal transduction, immune response, controlling cell shape, etc. (Yeow and Clayton, 2007; Morris et al., 2009). Along with naturally occurring protein self-association, there exists so-called pathological aggregation leading to the formation of amyloid fibrils (Fink, 1998; Stefani, 2004). These are highly ordered assemblies with a core cross-β-sheet structure in which β-strands run perpendicularly to the long axis of the fibril, while β-sheets propagate in its direction (Serpell, 2000; Kelly, 2002). During the past decade, this type of protein aggregates

attracts enormous interest due to its implication in pathogenesis of numerous disorders, including neurological diseases, type II diabetes, spongiform encephalophathies, etc. (Dobson, 2003; Stefani, 2008). A wealth of recent data corroborates the hypothesis that protein oligomers formed on a membrane template serve as nuclei for amyloid fibril growth on the one hand (Stefani and Dobson, 2003; Aisenbrey et al., 2008a; Stefani, 2008), while on the other hand, they represent toxic species producing membrane destabilization and subsequent cell death (Sparr et al., 2004; Relini et al., 2009).

The principal membrane-related factors promoting protein aggregation have been tentatively outlined as follows (Gorbenko and Kinnunen, 2006; Aisenbrey et al., 2008b): (i) structural transformation of polypeptide chain into aggregation-prone conformation featured by the exposed patches complementary to each other in terms of hydrophobicity, charge distribution, and hydrogen-bonding propensity; (ii) increase of protein local concentration in a lipid phase; (iii) specific aggregation-favoring orientation of a protein molecule in a lipid environment; (iv) aggregation-competent arrangement of the solvent-exposed and bilayer-buried portions of polypeptide chain; (v) electrostatic attraction between the dipoles of antiparallel α-helixes; (vi) mismatch between hydrophobic thicknesses of the lipid bilayer and the embedded protein fragment (Fig. 1). The present review is intended to cover the principal aspects of the problem of membrane-mediated protein aggregation and to provide an overview of the most essential recent findings, emerging ideas, and advanced experimental approaches in this research area. More specifically, we strove to scrutinize the above membrane processes associated with protein aggregation through analyzing the role of underlying driving forces tentatively categorized here as electrostatic, hydrophobic, and hydrogen-bonding, depending on their predominant nature.

II. The Role of Electrostatics in Membrane-Mediated Protein Aggregation

A. *Protein Confinement to Lipid–Water Interface*

An important prerequisite for self-association of membrane-bound polypeptides is provided by an increase in local concentration of interfacially adsorbed protein molecules. For water-soluble proteins, this process is

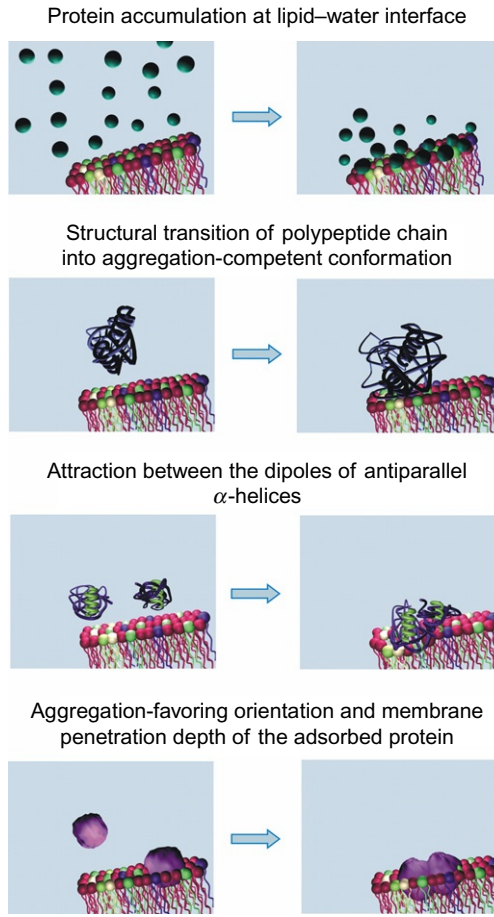

FIG. 1. Schematic representation of membrane-related factors promoting protein aggregation.

predominantly electrostatic in nature, displaying both sequence-specific and nonspecific features. The extent of nonspecific accumulation of mono- and polyvalent ions near oppositely charged surface can be roughly estimated in terms of the Gouy–Chapman diffuse double layer theory (Cevc, 1990). For instance, under physiological conditions (pH 7.4,

ionic strength 150 mM), the concentration of monovalent ions in the vicinity of a membrane with surface potential −100 mV is ca. 50 times higher than that in a bulk. Nonspecific protein accumulation near membrane surface due to long-range Coulomb attraction is followed by sequence-specific ion pairing between positively charged amino acid residues and anionic lipid headgroups (Chiti et al., 2003; Zhao et al., 2004). The formation of four or five such pairs ensures strong protein-membrane binding, with free energy contribution being ca. 4–6 kcal/mol per pair (Zhdanov and Kasemo, 2010). The recruitment of protein molecules by a membrane surface brings about significant increase in their proximity, which is inversely proportional to the square root of the number of molecules in a two dimensional (2D) space (instead of cube root in a 3D space; Aisenbrey et al., 2008a). Such a crowding effect may essentially account for the enhanced protein tendency to undergo aggregation, because (i) the compactly associated states correspond to free energy minimum of the system; (ii) lowered water activity engenders decreased protein solubility; (iii) increased medium viscosity leads to decreased diffusion rates of protein molecules (Munishkina et al., 2004; Aisenbrey et al., 2008c). It is also noteworthy that cell membranes may contain the areas of preferential accumulation of certain proteins. In electrostatic context, anionic lipid domains may be the sites for gathering of basic proteins, especially those containing the clusters of positively charged residues (Mulgrew-Nesbitt et al., 2006).

To illustrate the role of the membrane surface charge in promoting the aggregation of membrane-associated proteins, it seems reasonable to refer to our recent study into aggregation behavior of the basic protein lysozyme bound to negatively charged lipid bilayers (Gorbenko et al., 2007). By monitoring the fluorescence changes of fluorescein-labeled lysozyme, we explored its adsorption to the model membranes composed of phosphatidylcholine in mixtures with varying proportions of anionic lipids phosphatidylglycerol (PG), phosphatidylserine, or cardiolipin. As evidenced by the changes in the shape of adsorption isotherms from Langmuir-like to sigmoidal, lysozyme tends to convert from monomeric to aggregated state upon increasing membrane surface charge density from $-0.025\ C/m^2$ (10 mol% PG) to $-0.05\ C/m^2$ (20 mol% PG). Notably, this conversion occurs in the physiological range of PG membrane content suggesting that subtle variations in the proportion of anionic lipids *in vivo* may give rise to drastic changes in the aggregation state of membrane-bound proteins.

B. Structural Transformation of Polypeptide Chain

Along with acting as a matrix for recruiting and crowding protein monomers, cell membranes provide an environment favoring transition of polypeptide chain into aggregation-competent conformation (Uversky and Fink, 2004). Significant role in this structural transition is ascribed to electrostatic effects. Interfacially adsorbed proteins experience a complex environment which is highly anisotropic in terms of chemical, structural, and electrostatic properties. The network of protein intramolecular bonds may undergo substantial rearrangement in response to (i) electric field produced by the charged lipid groups; phosphocholine and ester carbonyl dipoles, and molecular dipoles of interfacial water; (ii) the changes in dielectric permittivity, ion content, ionic strength, and pH compared to the bulk; and (iii) formation of ionic contacts with specific lipid groups. Notably, the pattern of such a rearrangement may be different depending on structural peculiarities and physicochemical properties of interacting species. Due to considerable variability in the structures of naturally occurring proteins and peptides, which may be folded or unfolded, rich in α-helix or β-sheet, contain both α-helical and β-sheet regions, etc., there exists no unique way in which lipids promote transformation of polypeptide chain from native to aggregation-prone state. For natively folded proteins, the main kind of conformational transition at lipid–water interface is partial unfolding to molten globule and premolten globule states which are currently considered as most significant intermediates on the oligomerization and fibrillization pathways (Uversky and Fink, 2004). The importance of electrostatic interactions for the occurrence of such states seems to be most clearly demonstrated for basic protein cytochrome *c*, which displayed rise in the extent of unfolding with increasing proportion of anionic lipids in the model membranes (Muga et al., 1991; Salamon and Tollin, 1996; Pinheiro et al., 1997). Other examples are represented by acetylcholinesterase (Shin et al., 1997), bacterial toxins (Muga et al., 1993; Butko et al., 1997), insulin (Sharp et al., 2002; Relini et al., 2009), phospholipase A_2 (Tatulian et al., 1997), pheromone-binding protein (Wojtasek and Leal, 1999), and recombinant human prion protein (PrP; Morillas et al., 1999). Among the factors which may contribute to protein unfolding at negatively charged membrane surfaces are neutralization of the protein surface charge by anionic headgroups and enhanced side chain charge repulsion followed by the exposure of aggregation-favoring

nonpolar patches at low interfacial pH that may be up to 3 units less than bulk pH (van der Goot et al., 1991). Likewise, strong electrostatic field produced in anionic lipid domains can favor the unfolding of natively folded proteins.

On the contrary, natively unfolded proteins and peptides tend to adopt more structured conformation prior to self-association in a membrane environment, as exemplified by α-synuclein (Uversky et al., 2001; Beyer, 2007), islet amyloid polypeptide (IAPP; Kayed et al., 1999), protomyosin α (Pavlov et al., 2002), medin (Olofsson et al., 2007), and Aβ peptide (Koppaka and Axelsen, 2000). Particularly, anionic phospholipids were reported to induce α-synuclein transition from random coil to amphiphilic α-helix with contiguous hydrophobic side chains which are inclined to aggregate (Zhu et al., 2003). α-helical oligomers have been supposed to represent intermediate species on fibrillization pathway (Knight et al., 2006). Remarkably, the manner in which lipids modify protein spatial organization proved to depend on lipid-to-protein molar ratio: for instance, at low surface coverage, Aβ peptide adopts α-helical structure, but at high surface coverage, β-sheet structure becomes predominant (Terzi et al., 1995).

C. *Specific Orientation of Adsorbed Protein*

Another electrostatically controlled way in which membrane may enhance protein oligomerization potential involves specific disposition of monomeric species with respect to lipid–water interface. Due to nonuniform surface distribution of charged amino acid residues, protein molecule tends to adopt certain most energetically favorable orientation in the membrane electric field (van Klompenburg et al., 1997). One relevant example is represented by cytochrome *c*, a mitochondrial basic protein, *in situ* interacting with anionic phospholipid cardiolipin. Using resonance energy transfer technique, we obtained evidence for specific orientation of cytochrome *c* bound to cardiolipin-containing model membranes (Gorbenko, 1999). Allowing for recently reported ability of cytochrome *c* to form highly ordered amyloid-like fibrillar aggregates in the presence of negatively charged liposomes (Alakoskela et al., 2006), one can assume that this process starts with oligomerization of specifically oriented protein monomers. Numerous studies of antimicrobial and amyloidogenic peptides provide a basis for the concept of crowding-dependent

switch between in-planar and transmembrane configurations differing in aggregation propensity (Aisenbrey et al., 2008a,b,c). For instance, clustering of IAPP associated with negatively charged lipid bilayer was observed at high surface occupancy when peptide molecules became oriented normal to the membrane surface (Knight and Miranker, 2004). Protein–lipid charge interactions are considered as an important topological determinant (Bogdanov et al., 2008).

III. Hydrophobic Effect as Driving Force for Protein Aggregation

A key role in promoting a process of protein self-association and stabilization of aggregated states is attributed to hydrophobic effect. To minimize entropic cost of water ordering around nonpolar fragments of polypeptide chain exposed to solvent under certain environmental conditions, protein molecules tend to form dimers and higher order oligomers stabilized by short-range hydrophobic interactions with free energy ca. 0.1 kJ/mol/A^2. Hydrophobicity, net charge, and β-sheet propensity are considered as the major determinants of polypeptide self-association tendency (Chiti et al., 2003). All of the above aggregation prerequisites, increase in local concentration, conformational changes, and particular alignment of protein molecules may be created by hydrophobic protein–lipid interactions. Hydrophobicity *per se* may account for protein crowding in a membrane, as exemplified in multiple studies on protein and peptide partitioning into lipid phase (Seelig, 2004). Several lines of evidence indicate that phase-separated uncharged lipid domains may act as templates able to increase protein concentration and to trigger its oligomerization (Kamp and Beyer, 2006; Choucair et al., 2007).

Further, being partially or fully inserted into membrane interior, polypeptide chain tends to fold in a way, distinct from that in aqueous solution, with nonpolar lipid moieties competing with those of protein molecules for hydrophobic interactions (Haynes and Norde, 1995). The transfer of nonpolar amino acid side chains from aqueous phase to apolar membrane core produces a free energy gain which is regarded as the major driving force for folding of membrane-bound proteins (Wimley and White, 1996). Protein aggregation propensity is the interplay between the forces stabilizing the monomeric state and self-association of hydrophobic sequence fragments from different polypeptide chains into oligomeric nuclei. The balance

between intrachain and interchain interactions is controlled by the relative heights of the folding and aggregation barriers (Khare and Dokholyan, 2007). Although it seems impossible to establish unequivocal relation between the character of protein refolding in a membrane environment and the changes in its aggregation propensity, some peculiarities of these processes have been brought out. It has been demonstrated that hydrophobic and electrostatic protein–lipid interactions differently affect protein structure and aggregation extent. For example, association of PrP with negatively charged membranes resulted in the increase of β-sheet content, while its binding to zwitterionic membranes from phosphatidylcholine mixture with cholesterol and sphingomyelin in a raft-mimicking ratio induced α-helix formation (Sanghera and Pinheiro, 2002; Bogdanov et al., 2008), suggesting that electrostatic protein–lipid interactions are favorable for β-sheet, while hydrophobic interactions promote α-helical structure. Another example is represented by Aβ-peptide, whose structural changes proved to be different when membrane anchoring was mediated either by hydrophobic C-terminal or hydrophilic N-terminal part, thereby resulting in distinct flexible segments and differences in the folding pathways (Giacomelli and Norde, 2005). It was hypothesized that protein molecules attached to membrane via electrostatic interaction are more mobile and better able to reorient themselves into a position favorable for β-sheet formation than those held more tightly by hydrophobic interactions (Adams et al., 2002). Moreover, initial orientation of protein molecules at charged and neutral membrane surfaces may be different, resulting in a different tendency for intermolecular association.

Another mechanism by which membrane can modulate protein aggregation propensity involves variations in the depth of polypeptide chain penetration into bilayer interior. Electrostatic, hydrophobic, and hydrogen-bonding properties of bilayer-buried and solvent-exposed regions of the protein molecule determine the extent of its self-association (Gorbenko and Kinnunen, 2006).

An important point to bear in mind is that protein aggregation in membranes is followed by extensive dynamic reorganization of all components of the system, including protein, lipid, and hydrated water molecules. As follows from theoretical predictions, aggregation may be a consequence of mismatch in size between the hydrophobic part of the embedded protein fragment and the thickness of nonpolar membrane core, which produces a nonmonotonic force between proteins due to

membrane elastic deformation. Likewise, protein-induced local changes in membrane stiffness coupled with thermal fluctuations may give rise to a van der Waals-like long-range attraction between protein molecules (Bruinsma and Pincus, 1996; Meyer et al., 2008).

Importantly, theoretical analyses of the energetics of lipid–lipid interactions compared to lipid–helix and helix–helix interactions showed that even small increase in hydrocarbon chain order could favor oligomerization of transmembrane α-helices (Lee, 2004). Hydrophobic mismatch is assumed to promote lipid-mediated protein–protein attraction (Gil et al., 1998). Attractive interactions between interfacially adsorbed (partially inserted) α-helical amphipathic peptides were theoretically predicted on a basis of mean-field chain packing theory (Zemel et al., 2004).

IV. Hydrogen-Bonding as Determinant of Protein Aggregation Propensity

In addition to electrostatic and hydrophobic interactions, the process of membrane-assisted protein aggregation may involve hydrogen-bonding. In stabilizing the structure of membrane-bound proteins, hydrogen bonds acquire a special significance because in hydrophobic lipid bilayer environment there are no competitive relationships between water and H-bond donor/acceptor groups of polypeptide chain (Popot and Engelman, 2000; Ding et al., 2002). However, hydrogen-bonding is thought to be one of the driving forces for protein aggregation. It has been proposed that protein aggregates can be stabilized by nonspecific intermolecular H-bonds between the unprotected edge-strands (Dima and Thirumalai, 2002; Fernandez and Berry, 2002). Another mechanism by which H-bonds may participate in protein self-association is provided by the so-called dehydron hypothesis (Fernandez and Scott, 2003). Water-accessible amide–carbonyl H-bonds have been defined by Fernandez et al. (2003) as dehydrons. The basic idea is that structural motifs, such as insufficiently desolvated (underwrapped) hydrophobic patches, salt bridges, or H-bonds, represent highly reactive moieties. The removal of water out of these moieties upon a variety of protein interactions, including self-association, is energetically favorable. Although hydrophobic patches are regarded as most probable regions of intermolecular contacts in protein aggregates, such patches are sparse, as for most proteins, the ratio of hydrophilic to hydrophobic residues falls in the range 7:1–10:1 (Fernandez and Scott, 2003). Further, matching

condition between hydrophobic sites of associating protein molecules is rather difficult to satisfy, because this requires specific orientation and particular membrane penetration depth of polypeptide chain. Therefore, it seemed logical to suppose that other moieties, especially insufficiently dehydrated H-bonds, may significantly contribute to the formation of protein aggregates. Recent studies revealed a relationship between the protein aggregation tendency and the extent of underwrapping of the backbone amide–carbonyl hydrogen bonds. It was demonstrated that lower extent of H-bond protection from water correlates with higher affinity of a series of soluble proteins for phosphatidylcholine bilayer and their higher amyloidogenic propensity (Fernandez and Berry, 2003). Protein–lipid complexes, stabilized by H-bond protections, were assumed to play a significant role in nucleation of amyloid fibrils. Thus, partial exposure of hydrogen bonds to water is an important determinant of protein reactivity which must be taken into account while analyzing the molecular basis of protein aggregation.

V. Experimental Techniques Used to Study Protein Aggregation

Paramount importance of protein oligomerization in controlling the normal cell functioning and nucleation of disease-related fibrillar aggregates highlights the necessity of precise detection and correct characterization of aggregated species. Experimental strategies appropriate for the identification of protein clusters in membrane preparations can be classified according to the structural details of protein aggregates they can provide (Table I). In this section, we present the quintessence of analytical methods currently used for detection and characterization of protein self-association in a lipid bilayer. The applicability of each technique will be illustrated by some examples which represent only a compendium of practical use of these methods but not the exhaustive list of their utilization.

A. Atomic Structure

Nuclear magnetic resonance (NMR) spectroscopy is applied extensively to assess the structural details of protein self-assemblies at an atomic resolution. This tool analyzes the dependence of magnetic properties, typically isotropic chemical shielding, of a certain nucleus on its chemical

TABLE I
Analytical Methods Used to Study Protein Aggregation in Membranes

Information	Technique	Pros and cons
Atomic structure	NMR	*Advantages*: (i) measurements are performed in aqueous solutions under conditions close to physiological; (ii) does not require attachment of specific reporter groups to the protein molecule; (iii) large array of parameters extracted from resonance lines allows determining 3D molecular structure of protein aggregate. *Disadvantages*: (i) extremely expensive equipment; (ii) measurements are time consuming; (iii) peak assignment may be challenging due to peak overlap.
Secondary structure	CD	*Advantages*: (i) resolves the fine details of protein secondary structure changes; (ii) does not require large amounts of protein or extensive data processing; (iii) insensitivity to a wide range of pH and temperatures. *Disadvantages*: (i) lack of high sensitivity; (ii) working buffer absorption may contribute to CD spectra; (iii) light scattering effects may hamper the measurements.
	FTIR	*Advantages*: (i) resolves the fine details of protein secondary structure changes; (ii) quick measurements; (iii) enables detection of kinetic reactions on microsecond timescale. *Disadvantages*: (i) lack of high sensitivity; (ii) works almost with covalent bonds; (iii) water absorption may affect the results.
Tertiary and quaternary structure	EPR	*Advantages*: (i) high sensitivity, (ii) high signal-to-noise ratio, (iii) allows studying protein structural and dynamical changes with nanosecond resolution. *Disadvantages*: works only with unpaired electrons and, thus, requires the spin labeling of the biological samples.
	Fluorescence	*Advantages*: (i) high sensitivity, specificity, and informativity; (ii) wide arsenal of different modifications; (iii) noninvasive nature of measurements; (iv) immense range of measured parameters. *Disadvantages*: (i) labeling with fluorescent labels may affect the oligomerization propensity of a protein; (ii) some fluorophores are sensitive to milieu conditions (pH, ionic strength, temperature); (iii) dye degradation and photobleaching may distort the fluorescence signal.

Morphology	TEM	*Advantages*: (i) high resolution; (ii) high magnification.
		Disadvantages: (i) harsh conditions of sample preparation; (ii) unwanted sample sorption on carbon grid; (iii) high concentrations of contrasting agent may distort TEM images.
	AFM	*Advantages*: (i) high resolution; (ii) produces 3D topographical images; (iii) imaging from liquid environment.
		Disadvantages: (i) small image size; (ii) low scan speed; (iii) tip convolution effect.
	FM	*Advantages*: (i) high informativity; (ii) high selectivity; (iii) imaging from liquid environment; (iii) possibility of the imaging of different regions of macromolecule.
		Disadvantages: (i) photobleaching and degradation of fluorophore; (ii) lower resolution compared to TEM and AFM.
Size distribution	DLS	*Advantages*: allows measurement of protein aggregate size and molecular weight.
		Disadvantages: appropriate for analysis only of the small amounts of aggregated proteins.

environment (Robustelli et al., 2008). NMR occurs when the nuclei with nonzero spin quantum numbers are exposed to the magnetic field and subjected to the radiofrequency irradiation. The most informative nuclei involve ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P (Evans, 1995). Basic configuration of NMR (solution-state NMR) is successfully utilized for investigation of small molecules whose molecular weight is less than ~30 kDa (e.g., protein monomers). However, while exploring the structural and topological aspects of protein clusters, this technique encounters some difficulties connected with reduced tumbling rates and longer rotational correlation times of the polypeptide assemblies (Auger, 2000). This pitfall led to the development of the advanced modification of NMR, called solid-state NMR. Contrary to solution-state NMR spectra which are averaged over all anisotropic interactions, solid-state NMR reflects the full range of orientation-dependent interactions. To detect the monomer-to-oligomer transition, the changes in spectroscopic features, such as chemical shift, linewidth, cross-peaks, nuclear Overhauser effect, are recorded. A number of NMR studies have been performed to study protein aggregation in a membrane environment (Lindstöm et al., 2002; Grage et al., 2004; Naito and Kawamura, 2007). An example of such studies is given by the work of Ravault et al. (2005) where the results of ^{2}H and ^{31}P NMR provided an evidence for the oligomer formation while binding of Aβ peptide monomers to lipid membranes.

Despite its clear privileges, solid-state NMR suffers from several drawbacks. First, the instrumentation is extremely expensive, though some solution-state NMR spectrometers can be adapted to perform solid-state measurements. Second, the measurements are time consuming ranging from several minutes to a couple of days depending on the sample being analyzed. Finally, peak assignment can be challenging, because multiple peaks could be observed for a single nuclear site or they may overlap.

B. Secondary Structure

Details of the changes in protein secondary structure accompanying its conversion into aggregated state can be characterized using circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR), two prominent representatives of vibrational spectroscopy. Recording of the differential absorption of the left and right circularly polarized light constitutes the basis of CD spectroscopy. This allows analyzing the

secondary structural features of polypeptide chain, such as percentage of α-helices, β-sheets, or random coil regions (Greenfield, 2006). FTIR, in turn, operates via measuring the wavelengths of infrared radiation absorbed by a sample. Analysis of the characteristic absorption bands found in the protein infrared spectra, Amide I and Amide II, provides valuable information on conformational changes of polypeptide chain (Smith et al., 2002). The absorption associated with Amide I reflects the stretching vibrations of amide C═O bond, while Amide II corresponds primarily to the bending vibrations of N—H bond. Since both C═O and N—H bonds are involved in maintenance of protein secondary structure, the locations of Amide I and II are extremely sensitive even to the slight changes in polypeptide conformation. The application of vibrational spectroscopy has contributed significantly to the understanding of structural features of lipid-induced protein aggregates. Accordingly, appearance of β-sheets, a hallmark of the ordered fibrillar aggregates, has been observed upon association of Aβ-amyloid peptide (25–35) with negatively charged liposomes (del Mar Martinez-Senac et al., 1999). Further, comparison of aggregation-favoring abilities of different types of lipids showed that membranes with higher negative charge speed up the adoption of β-sheet structure by the peptide and thus accelerate its transition into oligomeric state. In elegant work of Bokvist et al. (2004), comprehensive time-dependent analysis of conformational changes of $A\beta_{1-40}$ peptide, monitored by CD, revealed that aggregation of the peptide occurs only when it is bound to the membrane surface. In the meantime, translocation of $A\beta_{1-40}$ across the lipid bilayer was found to prevent the peptide from aggregation. Despite the informativity of data achieved by CD and FTIR spectroscopy, these techniques still lack sensitivity. In addition, light scattering may hamper CD measurements while strong water adsorption affects FTIR results.

C. Tertiary and Quaternary Structure

Investigation of tertiary and quaternary structures of protein assemblies bound to lipid membranes is performed mainly with electron paramagnetic resonance (EPR) and fluorescence spectroscopy. The basic physical concept of EPR lies in characterization of the energy levels of a system with unpaired electrons in an externally applied magnetic field (Boas et al., 2008). EPR is very similar to NMR. The only difference is that

EPR measures the spin signals of electrons, while NMR mensurates the spin signals from the atomic nuclei. The absence of unpaired electrons in most biological materials appears to be the major impediment for the applicability of EPR. On the other hand, possibility of the labeling of both the lipid and the protein enables the manifold analysis of the aggregation processes occurring in the system. EPR has been applied in the early work of Marsh et al. (1982) to explore the aggregation behavior of different proteins incorporated into lipid bilayers. Analysis of the stoichiometries of the motionally restricted lipid spin label components in various systems under different lipid-to-protein molar ratios showed that protein–lipid complexation results in random intermolecular contacts between cytochrome oxidase molecules. EPR was also utilized successfully by Esposito et al. (2006) to evaluate the effect of membrane interactions on the oligomerization potential of Aβ (25–35) peptide. The authors found that C-terminal portions of the peptide monomers tend to aggregate, while the N-terminal regions extend into the aqueous medium with occasional contacts with the lipid headgroups.

Fluorescence spectroscopy seems to offer the widest arsenal of different modifications compared to the other techniques. Little or no damage to the examined matter, requirement of material micromolar concentration, rather simple methodology, involvement of relatively inexpensive instrumentation, and high sensitivity and specificity make fluorometry a broadly used research tool for studying the protein–protein interactions (Lakowicz, 2006). Various kinds of fluorescence technique, namely steady-state and time-resolved fluorescence, fluorescence polarization and fluctuation spectroscopy, stopped-flow and laser-induced fluorescence, provide the information about the structure, microenvironment, and distribution of protein complexes. The immense range of parameters measured for both intrinsic and extrinsic protein fluorophores includes fluorescence intensity, quantum yield, anisotropy and quenching, lifetime, Förster resonance energy transfer (FRET) efficiency, diffusion coefficients, to name just a few.

Intrinsic protein emitters are represented by tryptophan, tyrosine, and phenylalanine (Trp, Tyr, and Phe); however, the dominant one is Trp, which absorbs at the longest wavelength and displays the largest extinction coefficient. The well-known sensitivity of tryptophan fluorescence to environmental factors, spectral relaxation processes, rotational motions within the protein molecule or mobility of protein as a whole, the presence of

nearby quenching groups, etc. makes Trp fluorescence a valuable tool for examination of protein oligomerization. For example, detailed analysis of Trp fluorescence and anisotropy decays in the absence and presence of negatively charged lipid vesicles provided molecular insights into the lipid-controlled lysozyme aggregation (Trusova, 2009). Specifically, it was shown that protein self-association is controlled by the extent of surface coverage, balance between electrostatic and hydrophobic protein–lipid interactions, and protein orientation relative to membrane plane. However, while utilizing the intrinsic protein fluorophores as reporter molecules, one should bear in mind that their spectral characteristics may be polluted by intramolecular energy transfer (e.g., from Tyr to Trp).

Direct output of structural parameters of protein oligomerization at the lipid–water interface is furnished by another modification of fluorescence spectroscopy—FRET, which is based on radiationless dipolar coupling between donor and acceptor molecules. The characteristic nanometer distance scale of energy transfer phenomenon renders this technique particularly sensitive in quantitative analysis of protein clustering. Moreover, FRET can be considered not only as quantitative but also as qualitative method. Very often, the fact of presence or absence of FRET itself may be an indication of protein self-association. An excellent example of this approach is described by Li et al. (1999), who studied the aggregation of phospholamban in a membrane environment. Detailed quantitative analysis of FRET between fluorescent labels covalently bound to the protein yielded precise estimates of the oligomerization degree, distance between the monomers within the protein cluster, and fraction of protein present as monomers. Successful application of FRET in monitoring the protein–protein interactions has been reported also while exploring the aggregation of membrane-bound mellitin (Hermetter and Lakowicz, 1986; John and Jähnig, 1991), calcium ATPase (Fagan and Dewey, 1986), 5-HT1A receptor (Woehler et al., 2009), glycophorin A (Adair and Engelman, 1994), and β_2-adrenoceptor (Fung et al., 2009). Yet, FRET between labeled proteins must be used with caution, as protein labeling may affect its oligomerization propensity.

Classical example of the use of extrinsic fluorescent probes for the detection of self-associated proteins, especially ordered fibrillar aggregates, is benzothiazole dye Thioflavin T (ThT). Characteristic bathochromic shift of ThT excitation and emission spectra to 444 and 482 nm, respectively, and more than 100-fold increase in its fluorescence upon

its binding to the β-sheets, abundant in filamentous aggregates, is thought to be one of the gold standards for protein fibril detection (Nilsson, 2004). One explanation for such a specificity of ThT involves its peculiar location in protein aggregates. It has been proposed that ThT binds to fibrils via insertion into channels formed between every other row of side chains, with the dye long axis being parallel to the fibril axis (Krebs et al., 2005). This results in significant restriction of the dye mobility and, as a consequence, enhancement of its fluorescence by the orders of magnitude. The evidence for the protein aggregation upon association with the lipid membranes was obtained with the use of ThT while investigating the membrane binding of immunoglobulin light chains (Meng et al., 2008), IAPP (Domanov and Kinnunen, 2008), insulin (Grudzielanek et al., 2007), peptide from apoC-II (Hung et al., 2008), etc. However, despite being widespread, ThT assay is not devoid of shortcomings associated, particularly, with (i) dependence of ThT spectral characteristics on fibrillar aggregate morphology (Murakami et al., 2003), pH (Lindgren et al., 2005), ionic strength (LeVine III, 1997); (ii) ThT ability to affect aggregation kinetics and stability of different intermediates (Mauro et al., 2007); (iii) sensitivity of ThT fluorescence to the presence of exogenous compounds, for instance, polyphenols (Hudson et al., 2009).

Detection and characterization of protein aggregates from the viewpoint of the macromolecule diffusion can be performed with fluorescence correlation spectroscopy (FCS). FCS rests on the analysis of intensity fluctuations of labeled protein conjugates at nanomolar concentration in a femtoliter volume (Benda et al., 2003). Strong dependence of these fluctuations on a variety of factors, including translational and rotational diffusion, macromolecule dynamics and molecular weight, number of fluorescing species in the excitable volume, makes FCS particularly useful for quantification of protein interactions. The obtained data are interpreted in terms of auto- or cross-correlation function. Analysis of autocorrelation curves of Alexa-labeled α-synuclein incorporated into the lipid vesicles uncovered that increasing protein-to-lipid molar ratio and preferential polypeptide association with anionic lipid bilayers resulted in α-synuclein oligomerization (Rhoades et al., 2006). The main disadvantages of FCS approach may be itemized as follows: (i) dependence of the detection volume on concentration of the sample and the diameter of the excitation laser beam; (ii) photobleaching effects (Kim and Schwille, 2003).

D. Morphology

High resolution microscopic techniques such as atomic force microscopy (AFM), transmission electron microscopy (TEM), and fluorescence microscopy (FM) are indispensable for distinguishing the various morphologies of self-assembled aggregates. Historically, TEM was the first microscopic tool which was able to visualize the pathological aggregates of proteins extracted from the tissues (Cohen and Calkin, 1959). Nowadays, due to its extremely high resolution, TEM is generally used for quantitative and qualitative description of ultrastructural organization of protein oligomers. 2D images obtained with TEM represent grayscale microphotographs, analysis of which allows extracting the length and width of protein aggregates as well as their morphology. Application of TEM appeared to be successful in deciphering the role of lipid membrane composition and milieu conditions in polypeptide self-association (Choo-Smith et al., 1997; Kazlauskaite et al., 2003). For example, no aggregation of PrP was observed upon its interactions with neutral lipid membranes, while introducing of anionic lipid PG into membrane composition induced protein clustering (Choo-Smith et al., 1997; Kazlauskaite et al., 2003). In the same study, TEM was also capable of unraveling the pronounced impact of pH on the morphology of membrane-induced oligomers of PrP. Specifically, large amorphous aggregates of PrP bound to PG-containing lipid vesicles were identified at pH 7. In contrast, decreasing the pH value to 5 resulted in the formation of protein aggregates with the appearance of protofibrillar structures.

The major drawback associated with TEM is harsh conditions of sample preparations—the samples must be dried and stained before study. This means that the images are produced by dehydrated samples in their nonnative state. By comparison, sample preparation for AFM or FM experiments is relatively easy and enables imaging from liquid environment, thereby maintaining the samples close to their native hydrated state. The main advantage of AFM is its ability to produce 3D topographical photos. This advantageous property makes AFM one of the foremost tools for imaging, measurements, and manipulation at the nanometer scale. The information is gathered by probing the surface of the studied system with a flexible cantilever (Binnig et al., 1986). As cantilever scans the surface in three directions, a topographical map of the sample, depicting the distributions of length, width, and height of protein aggregates on the

lipid bilayer surface, emerges. Analysis of such maps provided important information on the kinetics of protein aggregate growth (McLaurin et al., 2002), role of cholesterol (Yip et al., 2002), and oxidative stress (Tabner et al., 2005) in lipid-assisted polypeptide oligomerization, etc. The limitations connected with the use AFM may be summarized as follows: (1) the image size is much smaller than that of TEM; (2) the scan speed is relatively low (about several minutes for a typical scan); (3) the tip of the cantilever is not ideally sharp. As a consequence, when the radius of the tip curvature is greater or smaller than the size of the surface region under study, AFM image does not reflect the true sample topography but rather represents a smoothed contour of tip interaction with the sample (so-called tip convolution effect).

The distinctive feature of FM is the attractive possibility of visualizing only the objects of interest against the black background (Lakowicz, 2006). Selective excitation of intrinsic fluorophores of polypeptides or fluorescent labels, covalently attached to protein molecules, permits constructing the real image of the protein aggregate structure and morphology. Further, wide range of available fluorescent markers with different spectral properties allows simultaneous imaging of different parts of the sample. Rapidly advancing innovations in laser scanning confocal and two-photon microscopy in couple with relative simplicity, convenience, and noninvasive nature of sample preparations nominate FM for one of the most informative approaches to seeing the microscopic details of oligomer structure in three dimensions. The power of FM is brilliantly illustrated, for instance, in the works of Zhao et al. (2004) and Pandey et al. (2009). Using dual labeling of the protein and the lipids, the authors showed that (i) clustering of α-synuclein is accompanied by formation of lipid domains enriched in anionic PG which represent the site of protein oligomer nucleation and growth (Pandey et al., 2009); (ii) there is a strong causative link between the presence of anionic lipids and protein gathering into highly ordered aggregates (Zhao et al., 2004). Moreover, the presence of lipids in the filamentous aggregates of lysozyme, insulin, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, transthyretin, cytochrome *c*, histone H1, and α-lactalbumin was revealed (Zhao et al., 2004). The factors that may reduce the quality of fluorescence images are (i) fluorophore concentration, which should be sufficient to produce clear image; (ii) photobleaching and degradation of the fluorophore; (iii) lower resolution compared to AFM and TEM (Lichtman and Conchello, 2005).

E. Size Distribution

Quasielastic light scattering, also known as dynamic light scattering (DLS), is currently used for gathering the information about size distribution of the aggregated species in a lipid bilayer. The principle of DLS lies in calculation of the diffusion coefficients of particles (protein monomers and oligomers) undergoing Brownian motion. DLS measures the fluctuations of light scattering by solute particles as a function of time. These fluctuations contain information about particle size, shape, and flexibility (Lomakin et al., 1999). DLS can resolve the monomeric and dimeric state of a protein if the shape of the protein is known, but it cannot distinguish among small oligomers when their hydrodynamic radii differ by less than a factor of 2 or between, for example, a compact dimer and an extended monomer. Further, due to the direct proportionality between the intensity of scattered light and the square of the particle mass, DLS is appropriate for analyzing of the small amounts of aggregated proteins. Application of DLS by Janson et al. (1999) allowed the authors not only to calculate the size of IAPP aggregates in the presence of membranes but also to determine the dimension of IAPP clusters, which becomes crucial for membrane stability (Janson et al., 1999). Another example of DLS contribution in clarifying the mechanisms of protein aggregation in membrane systems is provided by Martins and coworkers who showed that lipids cause the disassembly of mature fibrillar aggregates of Aβ peptide into protofibrillar structures (Martins et al., 2008).

VI. Concluding Remarks

To summarize, current view on the problem of membrane-mediated protein aggregation relies on the following main concepts that have obtained solid experimental and theoretical substantiation.

(i) Protein aggregation propensity is ultimately dictated by the amino acid sequence, but it is highly susceptible to the changes in environmental conditions, which *in vivo* can be produced by polypeptide transfer from aqueous to membrane phase. It is believed that different sequence fragments do not contribute equally to the overall aggregation tendency—there exist amino acid stretches (hot spots) serving as facilitators of self-association process (Ventura et al., 2004; Chiti and Dobson, 2006; Conchillo-Solé et al., 2007).

(ii) Lipid bilayer represents 2D anisotropic liquid providing a variety of environments which can profoundly alter protein structure and dynamics via hydrophobic, electrostatic, and hydrogen-bonding interactions. These interactions may exert influence not only on the accessibility of hot spots on the protein surface but also on the relative strengths of intermolecular forces stabilizing the protein aggregates, thereby significantly enhancing polypeptide aggregation potential.
(iii) Membrane-related factors, such as increased local concentration, decreased diffusion rate, and specific orientation of the adsorbed protein, create strong prerequisites for self-association of polypeptide chain.
(iv) Modulating effect of membranes on the protein aggregation propensity seems to be controlled not only by the general physicochemical characteristics of a lipid bilayer, such as surface charge, hydrophobicity, dielectric permittivity and viscosity profiles, elasticity, curvature, etc. but also by the exact chemical nature of membrane constituents and their conformational characteristics.

Despite considerable advances achieved in elucidating the driving forces and molecular mechanisms of membrane-mediated protein aggregation, there are still a number of outstanding issues to be resolved. Currently, the accents are crucially shifted to clarifying the role of membranes in pathological protein aggregation resulting in the formation of amyloid fibrils. Since amyloid-forming propensity is thought to be a generic property of polypeptide chain (Dobson, 2004), gaining further insights into molecular level details of amyloid self-assembly on a membrane template is of utmost importance for ascertaining the fundamental principles of the protein aggregation behavior.

Notably, the present review does not pretend to provide complete coverage of all relevant ideas and experimental findings shedding light on multifaceted problem of membrane-mediated protein self-association. In particular, concerning the kinetic mechanisms of protein aggregation, we can refer the reader to the excellent survey of Morris et al. (2009). Aggregation behavior of amyloidogenic proteins at lipid–water interface and its putative physiological implications are comprehensively reviewed by Stefani (2008), Aisenbrey et al. (2008a,b,c), Byström et al. (2008), and Relini et al. (2009).

The most challenging questions in this field of research that have not yet been adequately addressed concern (i) the nature of lipid-induced conformational transitions of polypeptide chain; (ii) the role of acidic phospholipids, sphingolipids, and cholesterol in enhancing the protein tendency to aggregate; (iii) kinetic peculiarities of membrane-promoted protein aggregation; (iv) structural characterization of aggregated species; (v) destabilizing effect of protein oligomers on the lipid bilayer structure; (vi) chemical and physical factors capable of preventing the growth of amyloid fibrils in a membrane environment, to name only a few.

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