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Abstract Fluorescence spectroscopy is one of the most powerful tools for characterization of a multitude of biological processes. Of these, the phenomenon of protein oligomerization attracts especial interest due to its crucial role in the formation of fibrillar protein aggregates (amyloid fibrils) involved in etiology of so-called protein misfolding diseases. It is becoming increasingly substantiated that protein fibrillization *in vivo* can be initiated and modulated at membrane-water interface. All steps of membrane-assisted fibrillogenesis, viz., protein adsorption onto lipid bilayer, structural transition of polypeptide chain into a highly aggregation-prone partially folded conformation, assembly of oligomeric nucleus from membrane-bound monomeric species and fiber elongation can be monitored with a mighty family of fluorescence-based techniques. Furthermore, the mechanisms behind cytotoxicity of prefibrillar protein oligomers are highly amenable to fluorescence analysis. The applications of fluorescence spectroscopy to monitoring protein oligomerization in a membrane environment are exemplified and some problems encountered in such kinds of studies are highlighted.

Keywords Protein prefibrillar oligomers · Protein-lipid interactions · Fluorescence techniques

Introduction

Among a huge variety of biological processes probed by fluorescence spectroscopy one of the most extensively studied over the past decade is protein aggregation [1, 2]. This phenomenon is ubiquitous in biomedical research, pharmaceutical, food industries and biotechnology. The propensity for self-association is an inherent property of polypeptide chain. The tendency of hydrophobic amino acid residues to minimize their contacts with polar solvent (hydrophobic effect), along with hydrogen-bonding and ion pairing are responsible for protein folding to compact structured native state. However, this state is marginally stable because opposing factors, such as the loss of configurational entropy and repulsive electrostatic interactions disfavor protein compactization [3]. For this reason, any change in external conditions (temperature, pH, ionic strength, the presence of surfaces or interfaces, etc.) may induce protein transition into highly aggregation-prone conformation. Protein aggregation is frequently considered as alternative folding [4], since intrachain and interchain interactions are driven by the common forces. There exist numerous types of protein aggregates which can be categorized into classes using different criteria, such as functional relevance (naturally occurring/pathological), morphology (amorphous/ordered), solubility (soluble/insoluble), stability (covalent/noncovalent), reversibility (reversible/irreversible) [5]. One type of the ordered protein aggregates, amyloid fibrils currently attracts the greatest attention due to its implication in molecular etiology of a number of so-called conformational diseases, including Alzheimer's, Parkinson's, Huntingtons diseases, type II diabetes, spongiform encephalopathies (prion diseases) [6–8]. These aggregates are featured by a core cross- β -sheet structure in which polypeptide chains are

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oriented in such a way that β -strands run perpendicularly to the long axis of the fibril, while β -sheets propagate in its direction. Amyloid fibrils usually contain from 2 to 6 laterally associated or twisted protofilaments, each 2–5 nm in diameter. In electron and atomic force microscopy images mature fibrils are commonly seen as unbranched filaments of 4–13 nanometers in diameter and up to a micrometer or more in length [9, 10].

A distinctive kinetic feature of the protein fibrillization is the existence of a slow nucleation (lag phase) and fast elongation (growth phase). During the lag phase protein monomers assemble into oligomeric nuclei, being the highest energy species along the polymerization pathway. Once a critical nucleus has formed, its further elongation becomes energetically favorable, thereby resulting in the exponential fibril growth [11].

Fibrillization-favoring conditions implicate structural transformation of polypeptide chain into a highly aggregation-prone partially folded conformation [12]. In vitro, these conditions are created by lowering pH, elevating temperature, adding organic solvents or denaturants, while in vivo abnormal partial unfolding may arise from mutations, oxidative or heat stress or protein adsorption at interfaces [13]. It is the presence of large amount of interface, formed by cellular membranes, that determines the principal difference between in vitro and in vivo amyloid growth. Both the formation of amyloid fibers and their cytotoxic action are thought to be membrane-related processes [14–16]. On the one hand, lipid bilayer, a basic structural element of biological membranes, may act as an effective catalyst of fibrillogenesis, providing an environment where protein molecules adopt conformation and orientation favoring their self-assembly. On the other hand, cell membrane represents a direct target for the toxic oligomeric species which may cause membrane destabilization and subsequent cell death via i) formation of non-specific ionic channels [17, 18], ii) modification of the intracellular redox status and free calcium level [19, 20] and iii) uptake of lipids into the growing fiber [21].

Membrane-related prerequisites for protein oligomerization include: i) protein accumulation at lipid-water interface, commonly electrostatically-driven; ii) structural transition of polypeptide chain into aggregation-competent conformation enriched in the exposed areas complementary in terms of hydrophobicity, charge topography, hydrogen-bonding capability; iii) electrostatic attraction between the dipoles of antiparallel α -helices; iv) protein self-association promoted by hydrophobic mismatch; v) aggregation-favoring arrangement of the solvent exposed and bilayer-buried portions of polypeptide chain.

Both oligomer assembly and the mechanisms of their toxicity are highly amenable to fluorescence analysis. Fluorescence techniques, both traditional (steady-state and

time-resolved fluorescence, quenching, anisotropy measurements, resonance energy transfer) and advanced (single molecule fluorescence spectroscopy, total internal reflection fluorescence microscopy, fluorescence correlation spectroscopy, etc.) being employed with intrinsic and extrinsic protein fluorophores; amyloid-specific fluorescent dyes or membrane fluorescent probes can provide unique structural and kinetic information on the protein-membrane binding, protein conformational changes, disposition of polypeptide chain relative to lipid-water interface, the type of oligomeric species and the extent of protein fibrillization [22, 23].

Fluorescence probing of protein-membrane binding

Membrane interactions of numerous amyloidogenic proteins and peptides, including A-beta peptide ($A\beta$), α -synuclein, prion protein (PP), transthyretin (TTR), islet amyloid polypeptide (IAPP), immunoglobulin light chain (IG), lysozyme (Lz) have been examined by a multitude of fluorescence methods (Table 1), based on the measurements of fluorescence intensity, anisotropy, lifetime, resonance energy transfer. Quantitative characteristics of the protein-membrane binding are commonly derived from the analysis of adsorption isotherms which in the simplest way can be obtained by detecting the changes in fluorescence intensity of intrinsic or extrinsic fluorophores upon varying protein or lipid concentration. In such kind of studies it is naturally to expect that the binding curves per se would contain information on the protein oligomerization state.

To exemplify, I will dwell on our data concerning membrane interactions of lysozyme, a multifunctional protein, displaying in its native state antimicrobial, anti-tumor, and immunomodulatory activities. Lysozyme is a hydrolytic enzyme which catalyzes cleavage of the peptidoglycan layer in bacterial cell wall, thereby promoting cell aggregation and loss of their viability. Point mutations in human lysozyme render it inclined to incorrect folding and forming amyloid fibrils, associated with a familial systemic amyloidosis, a disease in which aggregated protein tends to form deposits in liver, kidneys and spleen [48]. Importantly, all biological activities of lysozyme can be modulated by its lipid-associating abilities. Membrane binding of this protein is driven by ionic and hydrophobic interactions [49, 50]. Due to its positive charge over a broad pH range (pI ca. 11.0), lysozyme has a highest affinity for anionic phospholipids. We studied lysozyme adsorption onto liposomes composed of zwitterionic lipid phosphatidylcholine (PC) and varying amount of anionic lipids—phosphatidylglycerol (PG), phosphatidylserine (PS) or cardiolipin (CL). To detect protein-membrane binding, lysozyme was labeled with fluorescein (Fl), a widely used environmentally sensitive fluorophore. The complexation of tagged protein with

Table 1 Fluorescence-based analysis of membrane interactions of amyloidogenic proteins and peptides

Protein or peptide	Membrane constituents	Fluorescence technique	Ref.
α -synuclein	PC, PS, PA, PE, SM, Chol	Fluorescence correlation spectroscopy	[24]
α -synuclein	SM, PC, PG	DPH anisotropy	[25]
α -synuclein	PC, PS, PG	Laurdan, NBD, Tyr fluorescence	[26]
A β peptide	GM1, Chol, SM	DAC labeling RET between DAC and BODIPY	[27]
A β peptide	Total brain lipids	DPH anisotropy	[28]
A β peptide	Erythrocyte membrane	DPPE-ANS anisotropy	[29]
A β peptide		DPH, TMA-DPH, Laurdan	[30]
A β peptide	PC, gangliosides	Trp fluorescence	[31]
A β peptide	PA, PS, PI, PIP, PIP ₂ , CL, PC, PE, SM, Chol, DG, gangliosides	NBD-PA, NBD-PE fluorescence	[32]
A β peptide	PA, PG	RET fluorescein—Texas Red	[33]
A β peptide	GM1, Chol, SM	ANS, ThT assays	[34]
A β 42 peptide	Total brain lipids	Laurdan generalized polarization DPH anisotropy	[35]
Prion protein	PS	Trp fluorescence	[36]
Immunoglobulin light chains	PA	Trp fluorescence ThT assay	[37]
Transthyretin	PC, PE, PS, SM, Chol	DPH anisotropy	[38]
Serum amyloid A	PC	Trp fluorescence	[39]
Endostatin	PC, PS, PG	Trp fluorescence, RET Trp—dansyl	[40]
Islet amyloid polypeptide	PC, PG	RET ThT—BODIPY-PC ThT assay Alexa Fluor 568 labeling	[41]
Islet amyloid polypeptide	PG/PC vesicles, liposomes from pancreas lipids	ThT assay, Rhodamine-IAPP anisotropy	[42]
Surfactant protein C	PC, PG, PE	RET Tyr—bis-ANS	[43]
Insulin	PC, PE, PS	ThT assay	[44]
α -lactalbumin	PC, PG, Chol	di-8-ANEPPS	[45]
Lysozyme	PC, PG, PS, CL	DPH anisotropy, Trp fluorescence, FITC labeling	[46]
Peptide from apoC-II	PC	ThT assay	[47]

CL cardiolipin; *DG* diacylglycerol; *Chol* cholesterol; *PA* phosphatidic acid; *PG* phosphatidylglycerol; *PS* phosphatidylserine; *PC* phosphatidylcholine; *PE* phosphatidylethanolamine; *PI* phosphatidylinositol; *PIP* PI 4-phosphate; *PIP2* PI 4,5-P₂; *SM* sphingomyelin, *IAPP* islet amyloid polypeptide; *BODIPY-PC* 1-hexadecanoyl-2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-sn-glycero-3-phosphocholine; *ThT* thioflavin T; *DPH* 1,6-diphenyl-1,3,5-hexatriene; *NBD* 2-N-(4-nitrobenzo-2-oxa-1,3-diazole); *ANS* 1-anilinonaphthalene-8-sulfonic acid; *DAC* 7-diethylaminocoumarin-3-carbonyl; *DPPE-ANS* N-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; *di-8-ANEPPS* 1-(3-sulfonatopropyl)-4-[beta[2-(di-n-octylamino)-6-naphthyl]vinyl] pyridinium betaine; *TMA-DPH* trimethylammonium-diphenylhexatriene; *RET* resonance energy transfer

liposomes manifested itself in the decrease of fluorescein emission. This effect may be a consequence of fluorophore transfer into interfacial membrane region where pH is lower compared to the bulk phase because of proton accumulation near negatively charged surfaces. Fluorescein emission is known to display a complex pH dependence, reflecting equilibrium between several ionic forms, of which only monoanion and dianion forms are fluorescent [51]. The shift of equilibrium between the fluorescent and non-fluorescent fluorescein species upon the association of labeled lysozyme with liposomal membranes is likely to account for the observed fluorescence decrease. Monitoring the changes in fluorescence intensity as a function of lipid concentration allowed us to obtain adsorption isotherms for liposomes differing in chemical nature and proportion of anionic phospholipids. It appeared that at the molar fraction of

anionic phospholipid not exceeding 10 mol % the binding curves have typical hyperbolic shape. However, upon further increasing the surface charge of liposomal membranes the shape of adsorption isotherms was found to change from hyperbolic to sigmoidal. To explain this pattern, we have analyzed fluorescence binding data in terms of the adsorption models purposefully developed for protein-lipid systems on a basis of scaled particle and double layer theories [46]. These models offer the advantages of allowing for excluded area interactions between the adsorbing protein molecules and electrostatic effects (*i.e.* dependence of association constant on surface coverage). Comparison of several data treatment strategies showed that only the model assuming self-association of the adsorbed protein is capable of describing the behaviour of binding curves upon increasing the mole fractions of anionic phospholipid. A minimum number of the

protein molecules in aggregate required for reproducing sigmoidal shape of the adsorption isotherms was estimated to be four, implying that tetramers represent a preferential form of lysozyme oligomers. This example illustrates that even the simplest kind of fluorescence experiment based on intensity measurements can capture subtle details of protein-membrane binding and provide the proofs for protein oligomerization hardly achievable by other spectroscopic techniques or direct methods such as ultracentrifugation or gel filtration. The recovered strengthening of lysozyme aggregation propensity upon increasing membrane charge seems to be of interest in the context of its amyloidogenic properties suggesting that fibril nucleation may occur on a membrane template.

Fluorescence monitoring of protein structural changes

Another aspect of the problem, in which fluorescence spectroscopy may prove helpful, concerns structural transition of interfacially adsorbed proteins into aggregation-competent state. It is becoming increasingly substantiated that the highest propensity to form oligomers is inherent to a partially folded protein conformation resembling premolten globule state [12]. Lipid bilayer can lower the activation energy barrier for protein unfolding providing an environment with reduced pH and decreased dielectric constant whose concerted action enhances side chain charge repulsion, thereby giving rise to a more open structure with exposed aggregation-prone areas. Fluorescence parameters which can be correlated with protein unfolding and self-assembly in an aqueous phase include emission maximum wavelength and intensity, lifetime, anisotropy, quenching and resonance energy transfer efficiencies [52]. However, for proteins confined to a lipid phase situation becomes more complicated because the observed spectral effects may arise from several interfering processes, viz.: i) specific orienting of the adsorbed protein; ii) insertion of certain polypeptide segments into bilayer core; iii) modification of the protein structure; iv) formation of the protein dimers and higher order oligomers. Furthermore, since all these processes occur on a quasi two-dimensional surface, their patterns depend not only on the protein concentration, but also on lipid concentration. Accordingly, relative contributions of spectral responses from monomeric and oligomeric protein species would vary with lipid-to-protein molar ratio (L:P). For instance, while examining the quenching of lysozyme intrinsic fluorescence by water-soluble collisional quenchers iodide and acrylamide, incapable of penetrating into the hydrophobic membrane core, we found solvent accessibility of tryptophan residues to increase at low surface coverage ($L:P > 100$), suggesting partial unfolding of the protein molecules in a membrane environment. However, at high surface coverage ($L:P \sim 5-50$), where

lysozyme oligomers are supposed to prevail, tryptophan residues exposed upon unfolding seem to be shielded from the aqueous phase due to formation of intermolecular contacts between monomeric species, thereby resulting in the opposite effect—decrease of the quenching efficiency.

Detection of protein aggregates with extrinsic fluorophores

Complementary to intrinsic fluorophores in characterization of various types of the protein aggregates are extrinsic fluorescent dyes, of which most extensively used are classical polarity-sensitive probes, such as 8-anilino-1-naphthalenesulfonate (ANS), 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonate (bis-ANS), Nile Red (NR), molecular rotor 4-(dicyanovinyl)-julolidine (DCVJ), traditional amyloid-specific dyes Congo Red (CR) and Thioflavin T (ThT) [23, 53–55]. New classes of dyes are continuously testing for amyloid specificity [56–58]. The probes like ANS or Nile Red are highly suitable for assessment of surface hydrophobicity which is directly related to the protein aggregation tendency [23]. Importantly, fluorescent probes can display selectivity to protein aggregates differing in their type and size, like molten globule intermediates, early oligomers, spherical aggregates, protofibrils and mature fibrils, as was demonstrated, particularly, for transthyretin [59].

In this context it is important to note that correct fluorescence analysis of membrane-mediated protein oligomerization and fibrillization requires discriminating between spectral signals from the fluorophores buried in the hydrophobic membrane core, located in the nonpolar interior of a protein or residing in the contact area between two associating protein molecules [60]. The fact that in protein-lipid systems most fluorescent probes exhibit preferential binding to a lipid bilayer, may substantially complicate detection of conformational transitions and self-association of interfacially-adsorbed proteins. Ideal fluorophore for sensing conformationally altered aggregation-prone protein states should not respond for any perturbations in a membrane structure. It seems that some fluorophores meet this requirement. For instance, according to our observations, xanthene dye rhodamine 101 (R101) proved to be indifferent to the presence of liposomes. However, in hemoglobin (Hb)—lipid systems devoid of free protein by gel-filtration emission maximum of R101 exhibited short-wavelength shift coupled with fluorescence intensity changes. To explain this finding, we assumed that R101 senses lipid-induced unfolding of Hb tertiary structure and splitting of heme-globin bonds.

Another example involves classical amyloid-specific dye Congo Red. We found that this dye can effectively interact with neutral and positively charged bilayers, with maximum in differential absorption spectra being observed at 524 nm,

the value different from that characteristic of amyloid-bound dye (545 nm). However, no Congo Red binding to negatively charged bilayers has been detected. These findings suggest that this dye can be used for detection of amyloid growth in protein-lipid systems, especially for identification of amyloid fibrils induced by anionic lipids.

On the contrary, applicability of the most widespread amyloid-specific probe, Thioflavin T for detection of fibrillar aggregates at lipid-water interface seems to be limited by the capacity of this probe for non-specific association with both lipid bilayer and proteins containing β -sheet structures in its native state, as was demonstrated in our studies for lysozyme. These properties of Thioflavin T must be taken into account while monitoring the process of fibrillogenesis in protein-lipid systems where proportion of amyloid aggregates can be relatively low and incorrect interpretation of the dye spectral responses may lead to false positive result.

Optimal strategy for fluorescence analysis of protein aggregation

In general, the most sensible strategy for fluorescence-based analysis of the protein oligomerization in a membrane environment lies in correlating the data from a multitude of fluorescence methods. Furthermore, it is advisable to use each method with both intrinsic and extrinsic fluorophores differing in their physicochemical properties, responsiveness to the perturbations in protein or membrane structure, sensitivity to the changes in protein aggregation state, etc. For example, while employing resonance energy transfer technique to determine the influence of anionic lipids on oligomerization state of lysozyme, we collected information from several donor-acceptor pairs produced by fluorescent labeling of the protein: tryptophan—pyrene, pyrene—fluorescein and fluorescein—rhodamine. All these pairs reported enhancement of energy transfer with increasing total protein concentration, thereby providing arguments in favor of lysozyme self-association at the negatively charged lipid-water interface. Quantitative analysis of the experimental data in terms of the model of energy transfer in two dimensions revealed average distance between fluorescein and rhodamine tags to be two times as large as the dimensions of lysozyme molecule suggesting that oligomeric species are represented mainly by the protein dimers and tetramers. It is also worth mentioning that the most pronounced increase of energy transfer efficiency was observed at lipid-to-protein molar ratio about 40, pointing to the existence of a certain surface coverage critical for the formation of lysozyme self-associates.

At last, it should be noted that fluorescence techniques offer a versatile platform for analyzing protein oligomerization from the viewpoint of its coupling with other membrane processes, for instance, lipid segregation into lateral domains.

Specifically, comparing the efficiencies of energy transfer between intrinsic protein fluorophores and anthrylvinyl-labeled lipids led us to propose that lysozyme and another basic protein, cytochrome *c* give rise to demixing of neutral and anionic lipids in the binary model membranes [61, 62]. Notably, lipid segregation and lysozyme self-association were observed under similar experimental conditions (lipid-to-protein molar ratios falling in the range 10–70), lending support to the idea that gathering of conformationally altered and specifically oriented proteins within membrane domains may trigger the assembly of polypeptide chains into oligomeric nuclei, critical for protein fibrillization.

Concluding remarks

To sum up, the considered examples highlight the following benefits of fluorescence-based approaches in gaining further insights into membrane-mediated protein oligomerization: i) the possibility of experimentation within the protein and lipid concentration range where most other techniques appear powerless; ii) the use of high-quantum-yield environmentally-sensitive tags enables revealing the subtle peculiarities of the protein-membrane interactions, like sigmoidality of the binding curves, being a hallmark of protein oligomerization; iii) the opportunity for obtaining information about structural state of the protein and membrane under the same experimental conditions; iv) detection of small amounts of various types of aggregates, unamenable to chromatographic analysis; v) monitoring the events behind the lag phase of protein fibrillization.

The most challenging future applications of fluorescence spectroscopy in this research area include: i) development of methodological approaches to structural characterization of oligomeric intermediates; ii) searching for the dyes indifferent to modifications of lipid phase, but sensitive to protein unfolding and conversion from the disordered state to β -strands or β -sheets; iii) fluorophore screening for their selectivity to unfolded states, early oligomers, protofibrils and mature fibrils; iv) correlating the lipid demixing propensity of interfacially adsorbing protein with its aggregation tendency, to name only a few.

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