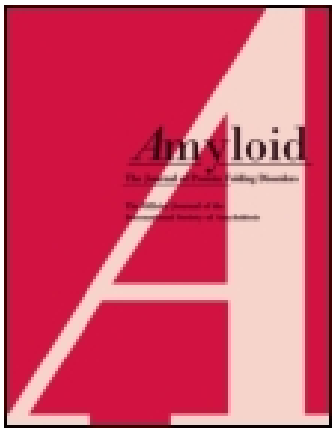

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Neurotoxic protein oligomers—what you see is not always what you get

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Neurotoxic protein oligomers — what you see is not always what you get

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Keywords: *Amyloid, oligomer, SDS-PAGE, protofibril*

Abbreviations: *AD = Alzheimer's disease; TTR = transthyretin; SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis; LMW = low molecular weight; PF = protofibrils; DLS = dynamic light scattering; AFM = atomic force microscopy; EM = electron microscopy; SEC = size exclusion chromatography*

Abstract
An increasing body of evidence suggests that soluble assemblies of amyloid proteins are the predominant neurotoxic species in many amyloid-related diseases. Consequently, the focus of research on pathologic mechanisms underlying amyloidoses has shifted from amyloid fibrils to oligomers. Biophysical characterization of oligomers is difficult due to their metastable nature. The most popular experimental method for detection of oligomers has been SDS-PAGE. However, we provide experimental evidence that SDS-PAGE is not a reliable method for characterization of amyloid protein oligomers and discuss alternative approaches. In addition, we discuss how inconsistent nomenclature has obfuscated our understanding of the process and products of protein assembly. The goals of this paper are to identify pitfalls associated with the methods and language used to study protein oligomers and to provide alternatives, thereby facilitating successful elucidation of the mechanisms controlling amyloid protein oligomer assembly and toxicity.

Introduction

Amyloidoses are a group of human diseases characterized by harmful accumulation of protein aggregates termed ''amyloids.'' In amyloidoses, proteins of various native structures and functions transform into amyloid. In their amyloid form, these proteins share a structure characterized by fibrillar morphology and cross β-sheet conformation [1] and deposit both intra- and extracellularly causing cytotoxicity and tissue damage.

Amyloidoses are classified by anatomic distribution and protein type. Systemic amyloidoses include light-chain amyloidosis, dialysis-related amyloidosis, and senile systemic amyloidosis. These diseases are caused by aggregation and deposition of immunoglobulin light chains, β_2-microglobulin, and transthyretin (TTR), respectively [2]. Other amyloidoses affect particular organs. Brain-specific diseases include Alzheimer's Disease (associated with the amyloid β-protein (Aβ) and tau), Parkinson's disease (α-synuclein), Huntington's disease (huntingtin), transmissible spongiform encephalopathies (prion proteins), and amyotrophic lateral sclerosis (superoxide dismutase) [3].

Amyloidogenic proteins normally exist in a soluble form. For some, the physiologic function is known and the three-dimensional structure of the native state has been determined [4—11]. However, in each amyloidosis, the pathologic lesions characterizing the disease comprise mainly aggregates of the respective protein. When isolated from lesions or prepared from synthetic or recombinant sources, the amyloid protein fibrils are cytotoxic, regardless of the specific protein from which they are derived [12–19]. For this reason, fibrils historicially were considered to be the causes of the respective disease. In the Alzheimer's disease (AD) field, this was a key element of the ''amyloid cascade hypothesis [20]''. However, an increasing body of evidence from studies in humans, normal rodents, transgenic mice, cultured cells, and *in vitro* systems suggests that soluble assembly intermediates of amyloidogenic proteins are the primary pathogenetic effectors in AD and other amyloidoses (Figure 1). A paradigm shift in the field [21], and an updated version of the hypothesis [22], thus have emerged. This pardigm shift has had two significant consequences: (1) biophysical studies have moved from the investigation of fibril elongation kinetics and thermodynamics to identification

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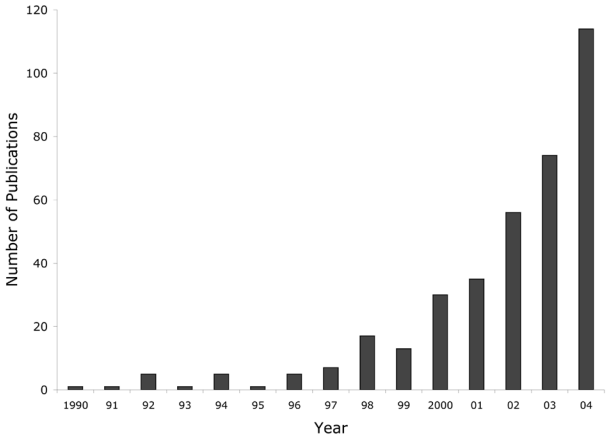

Figure 1. Number of publications on amyloidogenic protein oligomers per year between 1990—2004. The numbers were obtained by a PubMed Boolean search for the terms ''amyloid*'' and ''oligomer*,'' where ''*'' is a wild card designator. Data for the first 6 months of 2004 were extrapolated linearly to 12 months.

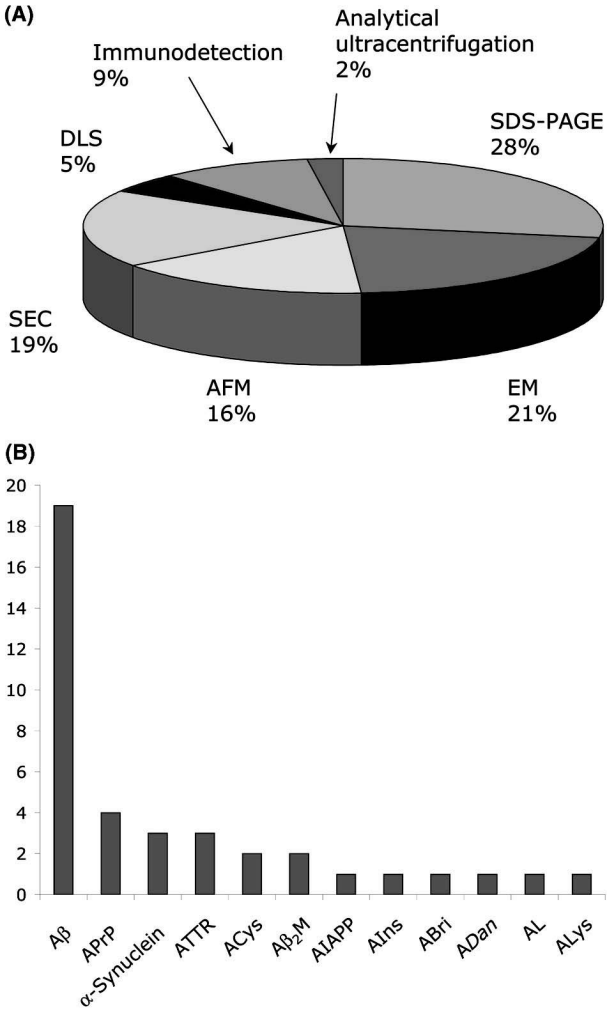

Figure 2. Biophysical methods used for studies of amyloidogenic protein oligomers. Within the first six months of 2004, 49 publications contained both the words ''amyloid*'' (1239 publications) and ''oligomer*'' (804 publications). Ten of these publications were not included in the analysis because they either were review articles or were related to non-amyloidogenic proteins. The charts represent the remaining 39 publications. (A) Methods used for oligomer detection and characterization. (B) Proteins studied. Amyloid protein names [79] are: Aβ, amyloid β-protein; APrP, prion; ATTR, transthyretin; ACys, cystatin C; Aβ_2M, β_2-microglobulin; AIAPP, islet amyloid polypeptide; AIns, insulin; ABri, the amyloid protein derived from the precursor ABriPP; ADan, the amyloid protein derived from the precursor ADanPP; AL, immunoglobulin light chain; ALys, lysozyme. ADan and ADanPP are tentative designations [79].

and characterization of oligomeric assemblies of amyloidogenic proteins; and (2) biological studies addressing protein toxicity now emphasize the assembly state of the protein, focusing on small, soluble assemblies. These shifts pose a challenge, particularly for laboratories specializing in functional biological studies. Amyloidogenic protein oligomers are often metastable and oligomers of various sizes exist in heterogeneous mixtures with monomers. Therefore, oligomers are difficult to analyze by classical methods used for studies of stable protein assemblies or of unassembled proteins. This complicates structure–activity studies, which are central for correct targeting of therapeutic agents. In this paper, we discuss: (1) caveats associated with identifying small oligomers using the ubiquitous protein fractionation technique SDS-PAGE; and (2) inconsistent nomenclature used to describe particular assemblies. The lessons learned with Aβ are equally relevant to studies elucidating the assembly pathways and function of other amyloidogenic proteins.

Results and discussion

Potential pitfalls in characterization of oligomers of amyloidogenic proteins

A survey of the recent literature reveals that the most common method for characterization of toxic protein oligomers is SDS-PAGE (Figure 2A). In studies of several different amyloidogenic proteins (Figure 2B), the sole evidence for the existence of oligomers is the presence of gel bands of apparent molecular mass higher than that of the monomeric protein. How reliable are these data and the conclusions based upon them? Biochemical and chemical data suggest that in the absence of covalent stabilization of protein oligomers, SDS-PAGE is unsuitable for revealing distributions of native oligomers.

SDS-PAGE has been an attractive analytical method for many reasons. It is a well-established, common, and inexpensive method for protein fractionation that offers high resolution and the ability to observe and quantify oligomers of similar molecular weight. In comparison, the other methods

presented in Figure 2A provide lower resolution and require specialized equipment or reagents. SDS binds to proteins through its hydrophobic dodecyl tail, leaving its sulfate head group solvent exposed and creating a negatively charged ''envelope'' surrounding the protein molecule [23]. An absolute requirement for molecular weight determination by SDS-PAGE is that this binding denatures the protein and prevents protein–protein interactions [24]. In doing so, a constant SDS:protein weight ratio is established, resulting in electrophoretic migration rates determined solely by polypeptide chain length (mass). Proper complexation underlies the exquisite resolving power and general applicability of SDS-PAGE.

A caveat of SDS-PAGE that requires greater recognition, especially in the study of protein oligomerization, is that not all proteins bind stoichiometric amounts of SDS. In some cases, rather than denaturing a protein, SDS can induce or stabilize secondary and quaternary structures. For example, numerous studies have established that proteins may form α-helices in the presence of SDS [25]. The effect of SDS on protein quaternary structure is less well understood. Most protein complexes and oligomers dissociate when treated with SDS. Observation of monomers or of low molecular weight oligomers by SDS-PAGE, therefore, does not reveal whether larger assemblies existed under native conditions *pre facto,* i.e., in the absence of SDS. SDS may also have the opposite effect, inducing artificial oligomerization, particularly of amphipathic peptides and proteins. The basis for this phenomenon is partitioning of the amphipathic polypeptide into SDS micelles followed by kinetically-controlled formation of oligomers due to the high local monomer concentration within the micelle. In the absence of micelles, if the peptide concentration is kept below a certain threshold, monomer assembly is too slow to be observed. But in the presence of SDS micelles, oligomerization occurs rapidly. Oligomers formed in the presence of SDS micelles may be stable during SDS-PAGE, thus misrepresenting the natural assembly state of the peptide or protein prior to treatment with SDS. Artifactual aggregation induced by SDS has been observed for Hepatitis B surface antigen polypeptides [26], bradykinin [27], β(2)-glycoprotein I [28], and collagen [29]. In addition to aggregation, conformational changes in the presence of SDS may cause aberrant electrophoretic mobility, a phenomenon that has been reported for various proteins [30,31], including Aβ [32].

Amyloidogenic proteins may be more prone to SDS-induced artefactual assembly than non-amyloidogenic proteins due to stabilization of partially (un)folded structures with high propensities for self-association. For example, transthyretin and prions have stable, native three-dimensional structures, but under pathologic conditions can undergo partial unfolding leading to oligomerization and aggregation [33,34]. ''Natively unstructured'' proteins, such as α-synuclein and Aβ, partially fold during amyloid assembly [35]. Pathologic oligomerization and fibril formation of both types of proteins thus involve formation of partially folded structures [36]. Such partially folded structures may be stabilized or induced by SDS [37], leading to misrepresentation of the *bonafide* assembly state of the protein.

SDS-PAGE induces formation of high molecular weight Aβ assemblies

In our studies of early Aβ oligomerization using SDS-PAGE, a question arose as to whether a particular gel band represented a genuine oligomer or an SDS-induced artefact. Using 10–20% gradient, Tris-tricine gels, the 40-amino acid alloform of Aβ (Aβ40) migrated as a monomer [38], whereas the 42-amino acid alloform (Aβ42) yielded monomer and a diffuse band consistent with a mixture of trimer and tetramer (Figure 3A) [39]. Aβ is an amphipathic protein that has been reported by many groups, including our own [40], to form SDS-stable oligomers. In fact, SDS-induced aggregation of Aβ has been used to purify the peptide from brain homogenates [41]. Similarly, sodium dodecyl sarcosinate, a derivative of SDS, was found to induce prion protein aggregation [42].

To examine whether the observed Aβ42 oligomers (Figure 3A) were formed artefactually, we used size-exclusion chromatography (SEC) to separate proteins by size. The separation mechanism of SEC, Stokes' radius-dependent differential gel permeation [43,44], does not depend on SDS binding and can be performed both in the absence and the presence of SDS. In SDS-PAGE, the sample loading buffer usually contains 2% (w/v: 69 mM) SDS, a concentration above the critical micellar concentration (cmc) of SDS ($\sim$8.5 mM). The running (tank) buffer usually contains 0.1% (w/v) SDS. To study the SDS concentration-dependence of Aβ assembly, SEC was performed in the presence of either 0.1% (w/v: sub-cmc) or 2% (w/v: supra-cmc) SDS. In the absence of SDS, Aβ42 yielded a small peak containing protofibrils (PF) and a large peak containing low molecular weight (LMW) Aβ (Figure 3B) [39]. LMW Aβ has been defined as a single peak in SEC that has an elution position consistent with monomer or low-order oligomers [40]. Addition of 0.1% (w/v) SDS to the mobile phase produced aggregation leading to the elution of $>$ 95% of the peptide in the void volume or the PF peak. The remaining peptide migrated as LMW Aβ (Figure 3B). In the

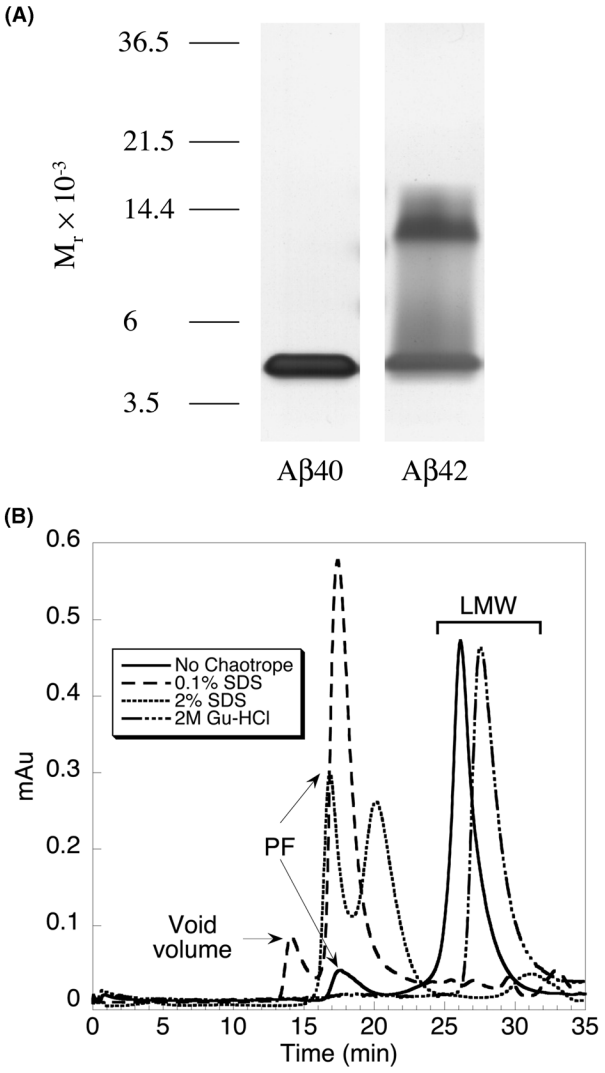

Figure 3. Analysis of Aβ by SDS-PAGE and SEC. (A) SDS-PAGE of low molecular weight (LMW [40]) Aβ40 and Aβ42. Positions of molecular weight markers are shown on the left. (B) SEC analysis of Aβ42 using a 10/30 Superdex *75* HR column (Amersham Biosciences) eluted at 0.5 ml/min with 10 mM sodium phosphate, pH 7.4, in the presence of 0.1% (w/v) SDS, 2% (w/v) SDS, 2M Gu·HCl, or no additives. The void volume, protofibril (PF), and low molecular weight (LMW) peaks are indicated.

presence of 2% SDS, a large ($\sim$30%) PF peak was observed and $\sim$60% of the peptide migrated as a broad peak between PF and LMW Aβ. A small ($\sim$10%) LMW peak was also observed. Similar results were obtained for Aβ40 (data not shown). This aggregation effect was not observed using a non-surfactant-type chaotrope, guanidine hydrochloride (Gu·HCl). At all concentrations of Gu·HCl examined (0.5–8.0 M), Aβ42 migrated as a single peak in the LMW region, consistent with monomer behavior (Figure 3B). These results demonstrate that Aβ assembles rapidly into high molecular weight aggregates when treated with sub-micellar concentrations of SDS, whereas in the presence of SDS micelles, PF and intermediate-sized aggregates form. In SDS-PAGE, during electrophoresis, these aggregates may partially dissociate to give rise to the diffuse Aβ42 trimer/tetramer band observed.

SDS- and non-SDS-based methods for monitoring oligomerization

The data presented above and other published work, indicate that SDS-PAGE is not a useful method for identification and quantitation of non-covalently associated protein oligomers. SDS-PAGE can be used to detect oligomers when the oligomers are stabilized by covalent cross-linking [38,45,46]. Once cross-linked, oligomers do not dissociate in the presence of SDS. However, in principle, small, cross-linked protein oligomers may form higher order assemblies induced by the presence of SDS in a similar fashion to non-cross-linked proteins. In fact, when cross-linked Aβ40 oligomers are purified using SEC and then analyzed by SDS-PAGE, each oligomer shows, in addition to a band corresponding to the expected molecular mass, a small band corresponding to double that mass (i.e. the dimer produces small amounts of tetramer, the trimer produces small amounts of hexamer, etc. (data not shown)). Native (non-SDS) PAGE is an alternative to SDS-PAGE for characterization of amyloid protein oligomers. However, because different oligomers and monomer may have identical mass-to-charge ratio, the resolution of native gels is inferior to that of SDS-PAGE [47,48]. Improved molecular weight determination in the absence of SDS may be achieved by systematically changing the polyacrylamide concentration (Ferguson gels [49]) or by using urea-containing gels.

As discussed above, SEC is a very useful chromatographic method for oligomer characterization, and may in fact, be the best non-SDS-based method for doing so. However, SEC is a relatively low resolution method, especially compared to SDS-PAGE.

Ultracentrifugation is a highly sensitive method for study of amyloid proteins [50—52]. However, the resolution provided by the method may not be sufficient for distinguishing among oligomers of small proteins that are close in molecular weight. Based on experimental analytical ultracentrifugation data fitted to theoretical multistate association models, Huang *et al.* suggested that Aβ40 exists as an equilibrium mixture of monomer, dimer, and tetramer [53]. However, other equilibrium systems. including monomer–dimer, monomer–trimer, or monomer–tetramer [53] produced equivalent residuals (errors), making determination of the oligomerization state of the peptide difficult.

A spectroscopic method that measures assembly size directly is dynamic light scattering (DLS) [54]. DLS offers the advantage of being non-invasive and therefore is most suitable for kinetic measurements. Analysis of polydisperse scatterer populations is challenging with DLS and the method has an intrinsic bias in favor of large assemblies (which can be advantageous when observation of scarce large assemblies on a background of abundant small assemblies is desired). Other spectroscopic methods commonly used for characterization of fibrillar protein assemblies, such as circular dichroism and thioflavin T fluorescence, measure protein conformation rather than size and therefore are not useful for oligomer size determination.

Electron microscopy (EM) and atomic force microscopy (AFM) provide powerful tools for distinguishing fibrillar, protofibrillar, and small oligomeric species and therefore are becoming increasingly popular for defining protein preparations as ''oligomeric.'' It is important to note that EM and AFM detect only that portion of the protein sample that adheres to the appropriate support (e.g., carbon-coated grids or mica, respectively) and that different assemblies have distinct adherence properties that can skew the observed results. EM and AFM have relatively low size resolution and cannot distinguish well among similarly-sized oligomers of small proteins (e.g., between dimers and trimers).

Recently, immunoglobulins capable of recognizing protein oligomers, but not monomers or fibrils, have been developed by several laboratories [55–57]. These antibodies are highly effective for detection of oligomers *in vivo* and also can be used for oligomer identification *in vitro*. They are not oligomer size specific and do not provide information about the presence or absence of other assemblies.

In summary, the resolution of SDS-PAGE is superior to that of the other methods listed above for separation and characterization of protein oligomers. However, because SDS-PAGE may misrepresent the native oligomerization state of particular proteins, the use of additional methods, including non-SDS-PAGE, SEC, ultracentrifugation, DLS, EM, AFM, immunochemical detection, or chemical cross-linking, as discussed above, is required for accurate determination of oligomerization state.

Assembly nomenclature

The use of proper terminology to describe particular amyloid assemblies is essential. This semantic issue is important because imprecise nomenclature leads to confusion among laboratories and to strategic and experimental planning based on false premises. The use of multiple names for the same entity and the use of a single term for multiple entities are two major problems that occur frequently. For example, apolipoprotein J commonly is designated not only by the acronym ApoJ, but also by the names complement-associated protein SP-40, complement cytolysis inhibitor (CLI), clusterin, sulphated glycoprotein 2 (SGP-2), dimeric acid glycoprotein (DAG), and glycoprotein III (GpIII) (see The Dictionary of Cell and Molecular Biology, http://www.mblab.gla.ac.uk/~julian). Although this multiplicity of terms is confusing, researchers in the ApoJ field generally are aware of the problem and know that all terms refer to the same protein. Such is not the case in studies of the structural biology of Aβ. Aβ self-assembly, a process archetypical for amyloidosis-related protein folding and aggregation, produces a myriad of neurotoxic oligomeric forms, including PF [58,59], dimers and trimers [60], Aβ-derived diffusible ligands (ADDLs) [61], and ADDL-like spherical aggregates [62,63]. A toxic conformer of monomeric Aβ also has been reported [64]. Additional oligomeric forms of Aβ were described without establishing their biological activity directly, including paranuclei [39] 'βamyballs'' [65], annular, pore-like structures [66], and an α-helix-rich assembly intermediate [67]. All these structures are oligomers. However, using the single term ''oligomers'' to describe these assemblies may be misleading for at least three reasons: (1) the morphologies of each of the assemblies are unique; (2) the pathways of formation of each assembly may differ (e.g., some assemblies are ''on pathway'' for fibril formation whereas others are not); and (3) the biological activities of each assembly may differ and similar activities, e.g., neurotoxicity, may be mediated through different pathways (e.g., direct membrane damage, redox activity, receptor-mediated induction of apoptosis).

Distinguishing oligomers of similar morphology prepared in different laboratories is also problematic. For example, ADDLs [61], amylospheroids [63], paranuclei [39], and spheroidal oligomers reported by the LaDu [62] and Glabe [55] laboratories are all Aβ oligomers with spheroidal morphologies. However, they may differ in the number of Aβ monomers they contain or in their toxicity. A systematic nomenclature enabling distinction among such assemblies does not exist. In such cases, the general term ''oligomer'' is appropriate, provided that an adequate description of the unique biological and structural features of the assembly is given (e.g., annulus, protofibril, α-helix-rich intermediate). Otherwise, only references to the general morphologic class of non-monomeric, non-fibrillar assemblies should be included under the rubric ''oligomer.''

The (mis)use of the term ''protofibril'' also illustrates inappropriate generalization of a specific

term. A variety of assemblies have been termed ''protofibrils,'' though they exhibit morphologies distinct from that upon which the term protofibril was based [40,68]. For example, annular, pore-like structures were designated ''annular protofibrils'' [69,70]. Spherical oligomers have also been designated protofibrils. PF were described originally as elongated, fibril-like Aβ assemblies that had curvilinear morphology, a diameter of $\sim$5 nm, and length not exceeding 100–200 nm [40,68], compared to the $\sim$10 nm diameter and indeterminate lengths (often exceeding 1 μm) of mature fibrils. PF also displayed specific secondary structure characteristics [40] and biological activity [71]. Protofibrillar structures have been described for most amyloidogenic proteins, including α-synuclein [72] huntingtin [73], islet amyloid polypeptide (IAPP) [74], prion proteins [75], and TTR [76]. In all cases the PF were cytotoxic. Annular and spherical structures differ morphologically from protofibrils, have different assembly pathways (by definition), and may cause neuronal injury by distinct mechanisms. Therefore, the use of the term ''protofibril'' to describe these assemblies is inaccurate and misleading.

The nomenclature of polymer chemistry

We as amyloidologists are indebted to polymer chemists for the terms we use to describe amyloid assemblies. These terms have been standardized by the Commission on Macromolecular Nomenclature (now the Subcommittee on Macromolecular Terminology of Division IV) of the International Union of Pure and Applied Chemistry (IUPAC) [77] and provide a solid foundation for understanding critical aspects of amyloid monomer self-assembly. The definitions of polymer (*a molecule of high relative molecular mass, the structure of which essentially comprises the multiple repetition of units derived, actually or conceptually, from molecules of low relative molecular mass*) and oligomer (*a molecule of intermediate relative molecular mass, the structure of which essentially comprises a small plurality of units derived, actually or conceptually, from molecules of lower relative molecular mass*) are particularly relevant to the prior discussion. However, what are the definitions of the relative terms ''high'' and ''intermediate''? For non-monomeric amyloid assemblies, we suggest that the appellation ''oligomer'' be applied to a specific assembly if the assembly is *not* a ''polymer''. All fibrils are polymers, both because of their high relative molecular mass, but also, and importantly, because addition or removal of monomer units does not alter their morphological or functional properties. This is not the case for low-order assemblies, including paranuclei [39], ADDLs [61], and annuli [78]. For PF, and other high-order assemblies, a distinction between oligomer and polymer is more difficult, but also unnecessary, if one defines the moiety using a clearly defined combination of biophysical characteristics (see above).

Conclusions

The recognition that protein oligomers may be the primary cytotoxic agents in amyloid-related diseases makes them key targets for biological research and drug development. For these efforts to be successful, the structural details of the target oligomers must be elucidated. It is thus imperative that methodologies be used which can reveal native assembly states and resolve oligomer order. Equally important is a systematic and consistent linkage between the defining structural and functional characteristics of each assembly type and its appellation. The use of proper terminology is critical for describing and distinguishing structural relationships among different amyloidogenic proteins and for directing future studies of the adverse consequences of these assembly events. No better advice can be proffered than ''be specific!''.

Acknowledgments

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