Chapter 13

Structure-Specific Intrinsic Fluorescence of Protein Amyloids Used to Study their Kinetics of Aggregation

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Chapter Outline

INTRODUCTION

Fluorescence techniques are among the most powerful in the study of proteins because of their versatility, ease of application, sensitivity and non-invasive nature [1]. In particular, the study of the intrinsic fluorescence from proteins permits sensitive information about the native protein to be obtained with little or no structural modification, and is therefore particularly valuable in biochemical research. Proteins containing aromatic amino acids – phenylalanine (F), tyrosine (Y), and tryptophan (W) – absorb and fluoresce in the ultraviolet (UV) range (250–400 nm), and it is this phenomenon that is commonly referred to as 'protein fluorescence' [1]. Fluorescence from aromatic residues reveals a wealth of information about the structure, folding and binding interactions of proteins [1–4]. The method is, however, not universally applicable, and it is limited to proteins that contain the aromatic amino acids natively or via non-native residue substitutions.

Recently, there have been reports of a different type of intrinsic protein fluorescence that is specific to structures containing a high proportion of β-sheets. Fluorescence emission of this kind occurs in the visible range, and appears to be independent of the presence of aromatic residues within the polypeptide structure. It has been observed from a range of protein aggregates and crystals [5–7], and most notably also from protein amyloids that have been associated with protein misfolding diseases, including Alzheimer's disease, Parkinson's disease and various types of amyloidosis [8,9]. The discovery of intrinsic amyloid fluorescence has enabled the process of amyloid formation from disease-relevant polypeptides to be monitored in a label-free manner and with high specificity [9].

These findings raise important fundamental questions about the electronic charge distributions in amyloid structures, and signal a need for a deeper understanding of the effect of amyloidogenesis on electronic transitions. All chromophores that are known to absorb and fluoresce in the visible range contain extensively delocalized electron clouds, usually through the presence of aromatic rings and conjugated double bonds. For example, Figure 13.1 (A and B) shows the skeletal structures of the common fluorescent dyes rhodamine 6G and fluorescein isothiocyanate (FITC). Extensive electron delocalization via multiple bond conjugation lowers excitation energies in such dyes and this shifts the fluorescence emission into the visible range. In contrast, smaller aromatic structures such as indole, benzene and phenol (the side chains of tryptophan, phenylalanine and tyrosine, respectively; Fig. 13.1, C, D and E) contain fewer

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conjugated bonds, and their fluorescence occurs in the UV range. For protein aggregates, it has been hypothesized that the formation of extensive arrays of hydrogen bonds may also give rise to electron delocalization such that optically excitable energy states arise [5]. Experimental studies that have emerged so far are consistent with this hypothesis, but to our knowledge no attempts have been made to study the phenomenon via theoretical calculation. A thorough understanding of the underlying physical mechanisms that give rise to the phenomenon promises to yield new information on the fundamental properties of amyloid systems. Not only would this benefit practical applications, such as the development of diagnostic assays for amyloidogenesis, but a better understanding of the electronic configuration of self-assembling amyloid systems will also help the rational design of novel functional biomaterials such as bionanowires [10–12].

In this chapter, we review current evidence for this recently discovered amyloid-specific intrinsic fluorescence, and explore possible directions for future research. We outline studies of protein aggregation kinetics using intrinsic protein fluorescence, which open up exciting new opportunities for the study of protein misfolding diseases with optical methods. The discussion is focussed solely on the intrinsic fluorescence from proteins; readers interested in the use of extrinsic fluorescent probes, or modification of proteins to make them amenable to optical studies, should refer to other reviews [13,14].

CHARACTERIZATION OF OLIGOMERS USING TRYPTOPHAN AND TYROSINE FLUORESCENCE

The emission from aromatic amino acids that are naturally present in proteins offers useful information to detect protein conformation and changes in protein structure, which occurs during the earliest stages of protein aggregation [4,15]. The process of aggregation is widely accepted to proceed through various intermediate stages between the native fold and the stable amyloid form. The protein in the native fold destabilizes into partially misfolded states due to various denaturing conditions, forms oligomers of up to tens of monomeric units, and finally transforms into stable amyloid fibrils [16–18]. Tryptophan and tyrosine have easily detectable quantum yields when unquenched (~0.10 in neutral aqueous solution), but their fluorescence properties are highly sensitive to the local environment, thus offering a method to identify different protein conformations. The emission maximum of tryptophan is particularly dependent on solvent polarity, displaying a red-shift in increasing polar solvents [19]. Thus, the emission spectrum of tryptophan is often sensitive to the folding and unfolding of proteins. Upon protein folding, tryptophan is often buried in hydrophobic cores, thus reducing exposure to the solvent molecules. In aqueous solution its spectrum therefore red-shifts as the protein goes from a folded to an unfolded state.

Tryptophan and tyrosine fluorescence has proved useful also for the characterization of β-amyloid (Aβ) and α-synuclein oligomers prior to fibril formation [15,20-22]. The fluorescence decay of tyrosine in Aβ has, for example, been used to distinguish oligomers from monomers and to determine the kinetics of early aggregation, a phenomenon that is not detectable by thioflavin T fluorescence [15,22]. Quenching of tryptophan by solvent exposure was used to detect oligomerization of α-synuclein and to identify the region involved in the core of the aggregate [20,21]. A mutant of α-synuclein designed to contain one tryptophan and one tyrosine, Y125W/Y133F/Y136F α-synuclein, was used to detect the kinetics of oligomerization via Förster resonance energy transfer (FRET) from tyrosine to tryptophan [23]. There are, however, limitations to these methods. The intrinsic fluorescence of aromatic residues, and their changes in fluorescence upon conformational change, are not specific to amyloid structures. Often, non-native substitutions are required, when no multiple fluorophores are present in the region of interest in the protein, or if multiple

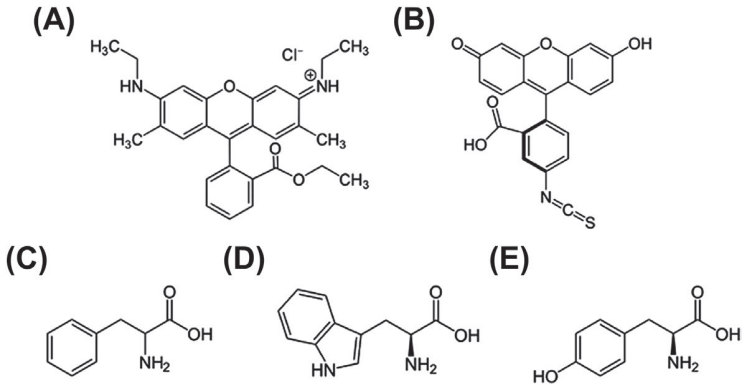

FIGURE 13.1 Skeletal structures of fluorescent dyes. (A) rhodamine 6G (absorption/emission maximum at 530 nm/566nm). (B) Fluorescein isothiocyanate (FITC) (495 nm/521nm). (C) Tryptophan (280 nm/348 nm). (D) Phenylalanine (257 nm/282 nm). (E) Tyrosine (274 nm/303 nm).

fluorophores are present in variably exposed domains of the protein [20,23]. In this case, prior knowledge is necessary to determine where the aromatic residue should be placed, and special care is needed to verify that the substitutions do not significantly alter the folding properties of the protein.

CERTAIN PROTEIN CRYSTALS AND AGGREGATES DEVELOP INTRINSIC FLUORESCENCE IN THE VISIBLE RANGE

In addition to the intrinsic fluorescence from aromatic residues in proteins described so far, certain protein aggregates have been reported to fluoresce in the visible region upon excitation with UV light. This phenomenon was first observed by Shukla et al from a range of protein crystals and aggregates, including thermally precipitated gamma-II crystallin from ovine lenses and crystals of hen egg white lysozyme (Fig. 13.2) [5]. They also showed that such fluorescence could not be observed from salt crystals, and suggested that the fluorescence was specific to proteins. Later reports from del Mercato et al and Sharpe et al showed that fibrillar amyloid-like structures formed from the elastin-related peptides, poly(VGGLG) and GVGVAGVG respectively, also exhibited intrinsic fluorescence in the visible blue region (Fig. 13.2C) [6,7]. Notably, both peptides do not contain any aromatic residues, suggesting that the phenomenon is independent of the intrinsic fluorescence of aromatic residues. These observations thus draw attention to a previously unknown form of intrinsic fluorescence from proteins, and when understood thoroughly, will reveal new molecular-level information about the structures of protein aggregates. Shukla et al raised the hypothesis that fluorescence results from low-energy transitions caused by the delocalization of electrons in the peptide bonds through backbone-to-backbone hydrogen bonds in β-sheet structures, which is also supported by evidence in later reports by other authors [6,7,9]. In particular, Sharpe et al verified – using Fourier transform infrared spectroscopy (FTIR) – that aggregates of the GVGVAGVG peptide contained a predominantly β-sheet conformation [7].

INTRINSIC FLUORESCENCE ARISING FROM DISEASE-RELATED PROTEIN AMYLOIDS DURING AGGREGATION

If the hypothesis of Shukla et al is true, it is reasonable to expect that protein amyloids, which are associated with numerous human diseases, including Alzheimer's disease, Parkinson's disease and type II diabetes, also exhibit intrinsic fluorescence in the visible spectrum, because their structures are also rich in β-sheet content. Indeed, two reports of photoactivity from amyloid structures of disease-related peptides and proteins have recently emerged [8,9]. The peptides and proteins shown to exhibit intrinsic fluorescence include α-synuclein, β-amyloid 1–40 and 1–42 (referred to as Aβ40 and Aβ42 hereafter), Tau and lysozyme, covering the most dominant aggregation-prone species involved in Alzheimer's disease and Parkinson's disease.

Tcherkasskaya measured the emission spectrum of α-synuclein fibrillar aggregates and suggested that chemical modifications of aromatic residues in α-synuclein during amyloid formation may lead to the formation of an intrinsic choromophore like that of GFP [8]. This is an alternative hypothesis to that of Shukla et al, but it is specific to the amino acid sequence of α-synuclein and would thus not be consistent with findings in other peptides, particularly those lacking aromatic residues yet exhibiting similar fluorescence properties.

The first systematic report to characterize the properties of intrinsic fluorescence from disease-related protein amyloids was published by Chan et al [9]; this presented fluorescence microscopy images and fluorescence lifetime data from amyloid structures of Aβ40 and Aβ42, Tau and lysozyme (Figures 13.3 and 13.4, respectively).

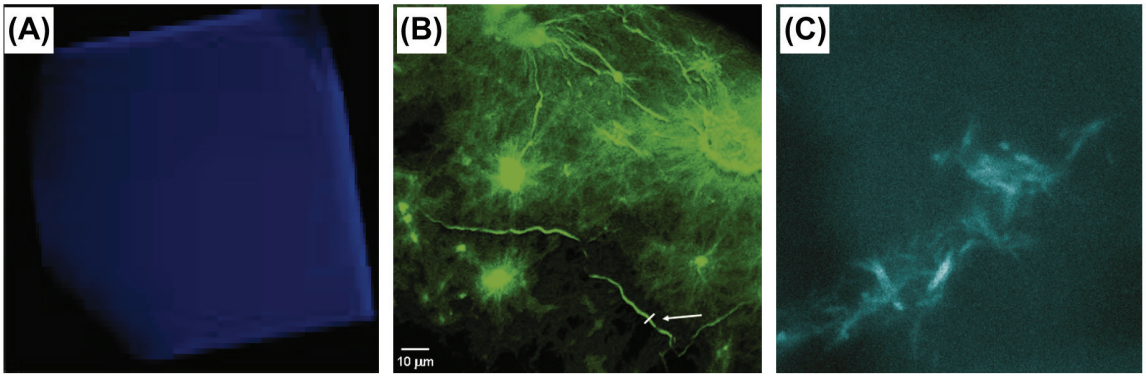

FIGURE 13.2 Intrinsic fluorescence of protein crystals and aggregates. (A) Crystal of hen egg white lysozyme (excitation at 351 and 364 nm; emission at 470 nm). Reproduced with permission from reference 5. (B) Fibrillar aggregates of poly(VGGLG) (excitation at 405 nm; emission at 465 nm) [6]. (C) Fibrillar aggregate of GVGVAGVG (excitation at 358 nm; emission at 460nm [7]. Reprinted with permission from Sharpe S, Simonetti K, Yau J, Walsh P. Biomacromolecules 2011;12(5):1546–1555. Copyright 2013 American Chemical Society.

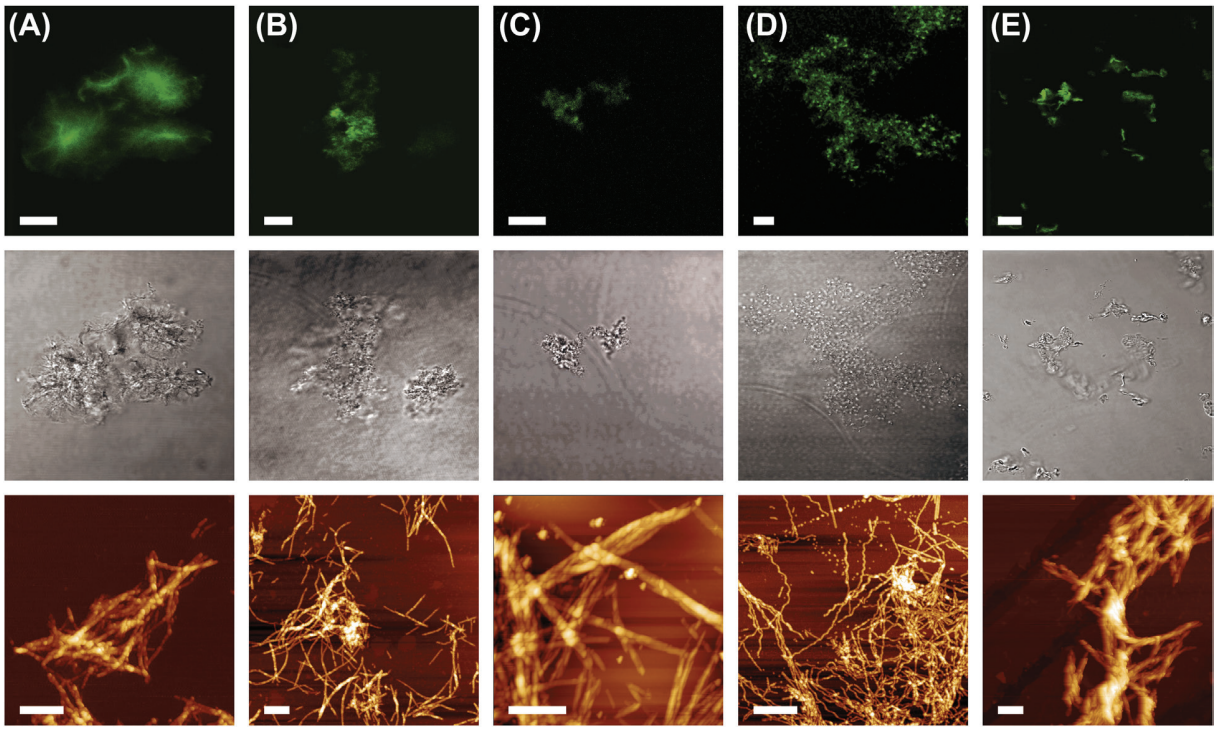

FIGURE 13.3 Intrinsic fluorescence of disease-related protein amyloids. Rows 1–3 show confocal microscopy images, corresponding phase-contrast images and atomic force microscopy (AFM) images, respectively, of Aβ40 (A), Aβ42 (B), Aβ [33–42] (C), K18 tau (D) and I59T human lysozyme (E) (excitation at 405 nm; emission at 450–500 nm). Scale bars represent 10 μm for microscopy images, and 500 nm for AFM images [9]. Reproduced by permission of The Royal Society of Chemistry.

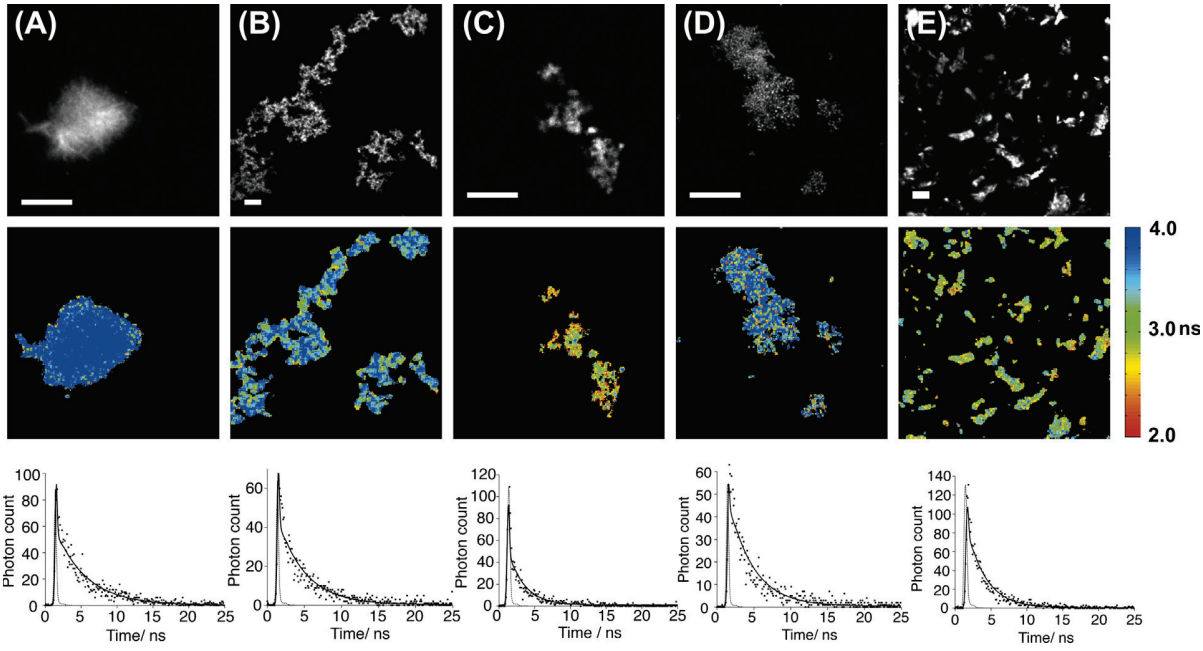

FIGURE 13.4 Rows 1–3 represent fluorescence intensity images, fluorescence lifetime images and fluorescence intensity decay curves of the amyloid form of Aβ40 (A), Aβ42 (B), Aβ [33–42] (C), K18 Tau (D), and I59T human lysozyme (E) (excitation at 450 nm; emission at >488 nm) [9]. Reproduced by permission of The Royal Society of Chemistry.

Measurements of the time-resolved fluorescence intensity were performed using time-correlated single photon counting fluorescence lifetime imaging (TCSPC-FLIM), and revealed fluorescence emission in the ns range (rows 2 and 3, Fig. 13.4). Possible artefacts due to scattering, such as Mie, Rayleigh and Raman scattering, could thus be excluded as possible origins for the phenomenon, as the former occur on a picosecond timescale. Furthermore, the emission observed from the aggregates is Stokes-shifted from the excitation wavelength and photobleachable [9], bearing all the hallmarks of a fluorescence process. Collectively, the evidence suggests that intrinsic fluorescence is a generic property of amyloid structure, featuring a characteristic emission lifetime in the [2–4] ns range for each polypeptide investigated.

Figure 13.5 shows the emergent fluorescence properties during the aggregation of hen egg white lysozyme (HEWL) as a representative example. It shows HEWL excitation and emission spectra at various time-points during the aggregation process, which demonstrate that amyloid formation is closely associated with an increase in the intensity of its intrinsic fluorescence signature (Fig. 13.5).

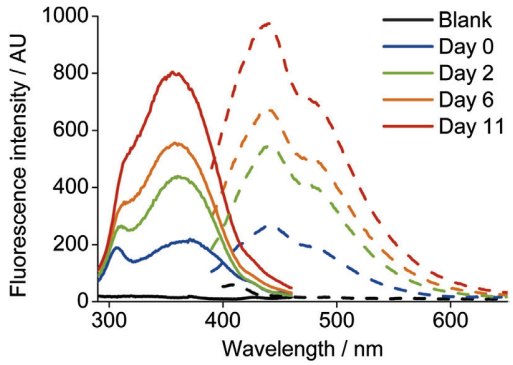

FIGURE 13.5 Excitation and emission spectra of hen egg white lysozyme (HEWL) at various time-points during the aggregation process show the increase in intrinsic fluorescence intensity with amyloid formation (at 0, 2, 6 and 11 days; solid line: excitation, dashed line: emission). Adapted from Chan et al [9], reproduced by permission of The Royal Society of Chemistry.

DOES THE INTRINSIC FLUORESCENCE OF PROTEIN AMYLOIDS PROVIDE NOVEL INSIGHTS INTO THEIR REMARKABLE STABILITY?

This intrinsic fluorescence phenomenon observable in protein amyloids raises fundamental questions about the electronic transitions that occur upon amyloidogenesis. The major common conformational change that occurs when proteins of different native structures convert to the amyloid state is the formation of extensive arrays of ordered β-sheets. It is therefore reasonable to speculate that this β-sheet structure is responsible for the phenomenon. The question of how the β-sheet structure brings about the electronic transitions that give rise to the visible blue emission observed is thus an important question yet to be answered. Shukla et al hypothesized that such electronic transitions could arise from delocalization of electrons in the peptide bonds through backbone-to-backbone hydrogen bonds in β-sheet structures (illustrated in Fig. 13.6). Although the hypothesis is yet to be backed by theoretical calculations, evidence exists in the literature to support the role of backbone-to-backbone hydrogen bonds in the enthalpic driving force for the conversion of proteins from the native monomeric form to the supramolecular amyloid form. It has been suggested that amyloid structures are mainly stabilized energetically by backbone-to-backbone hydrogen bonds [24,25]. Furthermore, quantum mechanical studies on model amyloid systems show that hydrogen bonds in the amyloid state are cooperative and contribute to the stability of the cross-β structures [26,27]. These hint at a potentially more significant role for backbone-to-backbone hydrogen bonds in the energetics of amyloid structures than would be expected from a classic view of electrostatically interacting point charges.

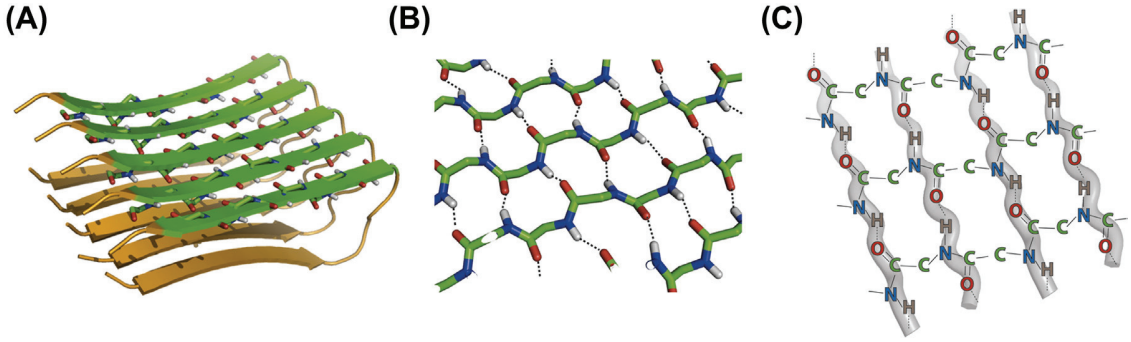

FIGURE 13.6 Formation of hydrogen bonds may give rise to delocalization of peptide orbital electrons participating in hydrogen bonding. (A) Structural model for Aβ42 amyloid fibrils (2BEG) in which the backbone atoms of one of the β-sheets have been highlighted (carbon in green, nitrogen in blue, oxygen in red and hydrogen in gray). (B,C) Close-up view of the network of hydrogen bonds established between amide hydrogens and carbonyl oxygen atoms. The possible electron delocalization network is highlighted in gray [9]. Reproduced by permission of The Royal Society of Chemistry.

INTRINSIC AMYLOID FLUORESCENCE INFORMS ON THE KINETICS OF AMYLOID FORMATION AND MECHANISMS OF PROTEIN MISFOLDING DISEASES

In addition to the fundamental interest in the molecular structure of amyloids, the ability to visualize protein amyloid structures in a label-free manner provides opportunities for the development of kinetic aggregation assays to study the behavior of these biomedically important protein assemblies *in vitro*. Such label-free assays are not susceptible to perturbations of the aggregating protein by dyes or labels and do not require non-native substitution by aromatic residues. Chan et al showed that amyloid self-assembly reactions can be tracked directly using measurements of the intrinsic fluorescence of amyloid structures [9]. In Figure 13.7, fluorescence intensity and lifetime images of I59T lysozyme at various time-points during the aggregation process show the increase in the number of fluorescent aggregates with time. Graphs of the mean number of fluorescence-containing pixels and mean emission lifetime in images of the aggregates provide a robust readout of the aggregation time course of the protein without any requirement for external fluorescent probes.

This label-free aggregation assay would also be suitable for evaluating the effects of inhibitors of aggregation that could be potential therapeutics for protein misfolding diseases, where an absence of, or subdued increase in, intrinsic fluorescence indicate that amyloid formation has been inhibited (Figs 13.8 and 13.9). Traditional assays have relied on extrinsic labels such as thioflavin T (ThT) [28], Congo Red (CR) [29] and 1-anilino-8-naphthalene sulphonate (ANS) dyes [30]. These systems have been of great value, but in the search for small molecules as inhibitors of aggregation these dyes can influence the kinetics and mechanisms of the aggregation process, as labels can interfere with the binding of the inhibitor molecule [31–33] or the inhibitor molecule can quench the fluorescence of the label [34,35]. It is noted, however, that any potential inhibitors tested which are themselves fluorescent, or are fluorescence quenchers, could affect the effectiveness of this label-free technique in certain cases. In Pinotsi et al the ability of a label-free assay to quantify amyloid growth was demonstrated in a situation where ThT was known to be challenging [36]. In this work the aggregation of α-synuclein was studied in the presence and absence of 100 μM lacmoid (Fig. 13.8), a small molecule which binds to amyloidogenic proteins and which had previously been reported to act as an inhibitor of aggregation. The aggregation process was monitored using either ThT (solid lines) or the intrinsic fluorescence assay (dots). The intrinsic fluorescence was seen to rise in

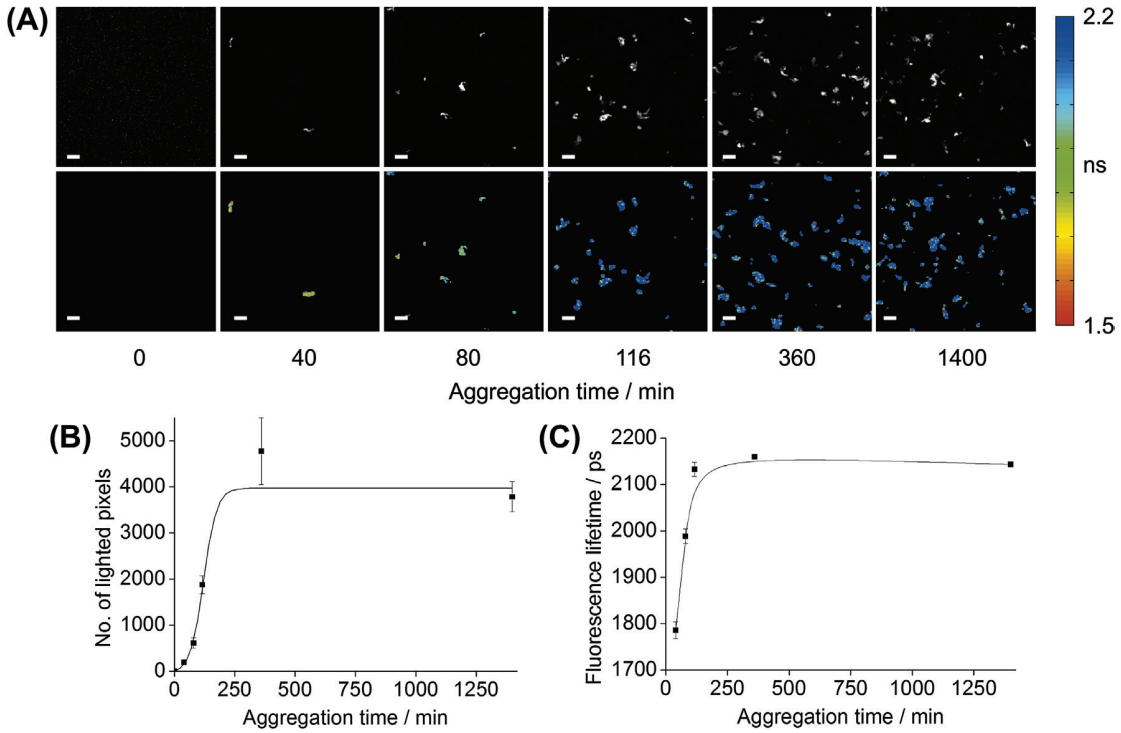

FIGURE 13.7 (A) Fluorescence intensity and lifetime images (rows 1 and 2 respectively) of I59T lysozyme aggregates at various time-points during the aggregation process permit the direct tracking of amyloid self-assembly reactions. (B,C) Graphs of the mean number of fluorescence-containing pixels and mean emission lifetime in images of the aggregates shown in A [9]. Reproduced by permission of The Royal Society of Chemistry.

an almost identical fashion whether lacmoid was present (red dots), or absent (blue dots), and in agreement with earlier reports [35] it was thus verified that at the stated concentration, and in the presence of seed fibrils, lacmoid causes no inhibition of α-synuclein aggregation. The ThT fluorescence traces, however, varied significantly between the presence (red line) and absence (blue line) of lacmoid. The observed dramatic decrease in fluorescence intensity (ca. 5-fold) could be mistaken for an inhibitory action of lacmoid but is likely to be caused either by a quenching interaction between ThT and lacmoid, or by competition between ThT and lacmoid for free binding sites on the amyloid fibrils. The capability to detect and quantify amyloidogenesis *in vivo* is essential for elucidating the role of protein aggregation in protein misfolding diseases. The direct detection of the intrinsic fluorescence of amyloid structures *in vivo* is, however, likely to be challenging due to competing autofluorescence from living cells and organisms. Still, specific and sensitive *in vivo* probes of amyloid structures could be developed using external fluorophores covalently attached to the amyloid backbone [37]. Such external fluorophores can participate in Förster resonance energy transfer (FRET) with intrinsic energy states of amyloid structures if present, providing a readout in the form of a reduced fluorescence lifetime of the external fluorophores (Fig. 13.9). Readers can refer to Chapter 13 in this volume for an account of the development of this assay for live models of protein misfolding diseases.

CONCLUSIONS

In this chapter, we have reviewed recent experimental evidence on an intrinsic fluorescence phenomenon that occurs in protein crystals and amyloids and appears in the visible range. The fluorescence is independent of the presence of naturally occurring aromatic amino acid residues, whose own intrinsic fluorescence is emitted in the UV range. It has been shown that protein amyloids associated with common human diseases, including Alzheimer's disease and Parkinson's disease, exhibit this intrinsic fluorescence, thus providing opportunities for the development of sensors that provide direct readouts to amyloidogenesis *in vitro* and *in vivo*. Such assays are immensely useful for the study of aggregation kinetics and the evaluation of potential inhibitors of aggregation *in vitro* in a label-free manner, as well as for the study of amyloidogenesis *in vivo* in live models of protein misfolding diseases [37,38]. These studies would contribute significantly to an understanding of the pathologic role of protein aggregation in such diseases. A theoretical foundation to explain this apparently generic phenomenon of amyloids remains to be established, and it is anticipated

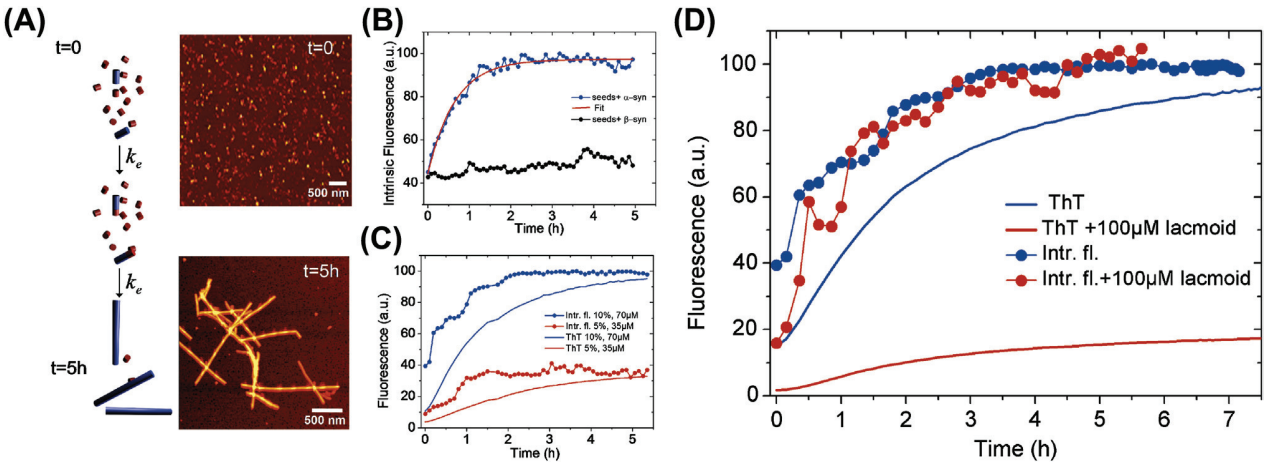

FIGURE 13.8 (A) *Left*. Simple schematic diagram illustrating the experimental design of the aggregation assay. Blue rods denote α-synuclein fibrillar seeds and red rods denote monomeric protein. The rate k_e is the elongation rate constant. *Right*. AFM images of sonicated seed fibrils and of the α-synuclein fibrils formed at the end of the reaction in the experiment shown in B. Scale bars 500 nm. (B) *Blue curve*. Kinetic trace of the change in intrinsic fluorescence intensity over 5 hours during seeded aggregation of α-synuclein. Seeds at 7 μM concentration are incubated at 37°C with 70 μM of α-synuclein monomeric protein. *Black curve*. Intrinsic fluorescence intensity over 5 hours for α-synuclein seeds at 7 μM concentration incubated with 70 μM of β-synuclein monomeric protein, where no increase in the intrinsic fluorescence and hence no seeded growth is observed. (C) Comparison between ThT and intrinsic fluorescence assays to follow the kinetics of fibril elongation of α-synuclein. The assays were performed for two different concentrations of fibril seeds and monomers to examine their scaling behavior. *Continuous lines*. Intrinsic fluorescence. *Dashed lines*. ThT fluorescence. *Blue curves*. 10% of seed concentration and 70 μM of monomeric α-synuclein protein. *Red curves*. 5% of seed concentration and 35 μM of monomeric α-synuclein protein. Kinetic constants scale with concentration in the expected fashion for both assays. (D) Comparison of intrinsic fluorescence and ThT assays for α-synuclein elongation in the presence of lacmoid. *Red and blue points*. Intrinsic fluorescence intensity (red and blue: 37°C, 7 μM seed concentration, 70 μM monomer concentration; red only: addition of 100 μM lacmoid). *Continuous red and blue lines*. Corresponding ThT fluorescence. Reproduced with permission from Pinotsi et al [36].

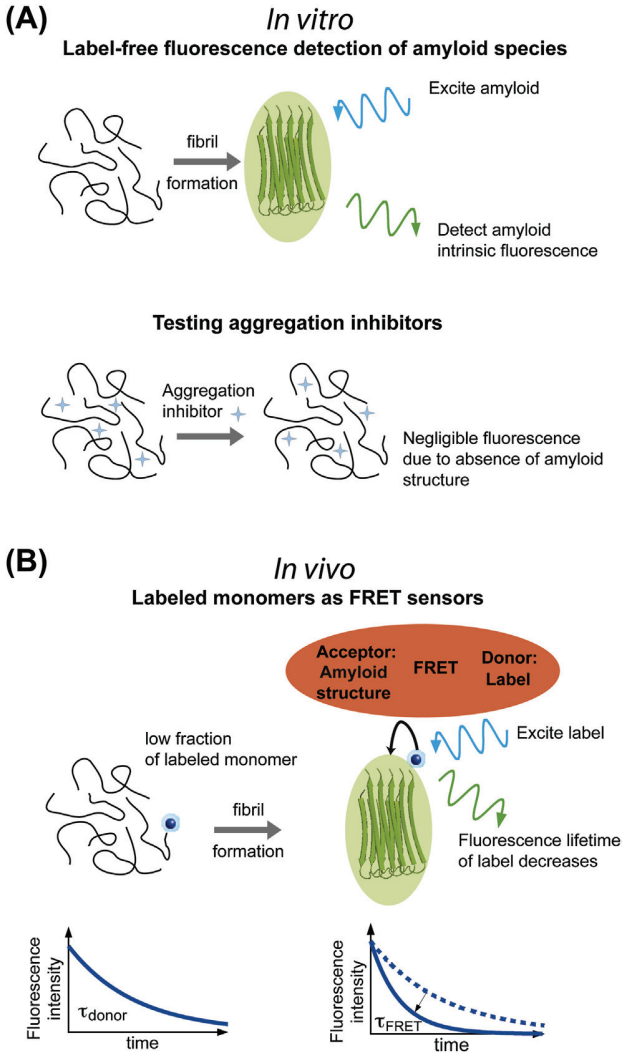

FIGURE 13.9 Strategies for sensor development using amyloid intrinsic fluorescence (A) *in vitro* and (B) *in vivo* [9]. Reproduced by permission of The Royal Society of Chemistry.

that future studies on the excited electronic states of a wider range of amyloid systems will yield new insight into their structure at the molecular level.

FUTURE WORK

Reports that have emerged so far represent just the first handful of accounts on the phenomenon, and many avenues exist for possible future research. Some suggestions are presented here and are by no means exhaustive. Further characterization of the intrinsic fluorescence would provide a deeper understanding of the molecular structure of protein amyloids. For example, attempts to correlate the properties of the intrinsic fluorescence such as quantum yield and fluorescence lifetime with the morphology and β-sheet content of particular amyloids would be informative. Also, attempts to detect intrinsic fluorescence from early oligomers are also worth pursuing, as they have been suggested to be responsible for toxicity to cells in many cases [39–41]. Fluorescence studies of other amyloid systems which play a functional role in nature could reveal how amyloid formation is regulated by nature, with possible implications for the development of therapeutics for protein misfolding diseases. Furthermore, development of the label-free fluorescence assay for the detection of amyloid structures could be applied to high-throughput screening of potential inhibitors of aggregation, providing a more direct assessment on the performance of potential inhibitors than dye-binding assays.

ACKNOWLEDGMENTS

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