Phasor-FLIM analysis of Thioflavin T self-quenching in Concanavalin amyloid fibrils

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Abstract

The formation of amyloid structures has traditionally been related to human neurodegenerative pathologies and, in recent years, the interest in these highly stable nanostructures was extended to biomaterial sciences. A common method to monitor amyloid growth is the analysis of Thioflavin T fluorescence. The use of this highly selective dye, diffused worldwide, allows mechanistic studies of supramolecular assemblies also giving back important insight on the structure of these aggregates. Here we present experimental evidence of self-quenching effect of Thioflavin T in presence of amyloid fibrils. A significant reduction of fluorescence lifetime of this dye which is not related to the properties of analyzed amyloid structures is found. This result is achieved by coupling Fluorescence Lifetime Imaging Microscopy with phasor approach as suitable model-free methods and constitute a serious warning that have to be taken in account if is dye is used for quantitative studies.

The deposition of inclusion bodies that contain abnormal aggregated proteins, in form of amyloid aggregates, is one of the fingerprints of a large population of neurodegenerative disorders, such as Alzheimer's and Parkinson's (Knowles, Vendruscolo, & Dobson, 2014). Many years of studies have elucidated general laws regulating their growth in vitro and the mechanism by which they generate cells dysfunction but a large debate is still open. It is well assessed that almost all proteins, in specific conditions, may assemble in amyloid structures that are ordered supramolecular elongated aggregates (width of 2–10 nm and length up to hundreds μm) stabilized by H-bonds (Serpell, 2000; Sunde & Blake, 1997). Nonhomologous proteins can form amyloid fibrils with analogous structural features that is cross-β structures in which β-strands run perpendicularly to the long axis of the fibrils. Interestingly, amyloid state represents the most stable state that proteins can adopt (Baldwin et al., 2011; Thirumalai & Reddy, 2011). Even if historically related to pathological conditions, these structures were found to perform a diverse range of biological functions in living organisms (Chiti & Dobson, 2006; Fowler, Koulov, Balch, & Kelly, 2007; Lamour et al., 2017). Due to their peculiar properties as highly ordered structure at the nanoscale, high thermal stability, extraordinary mechanical strength and resistance to harsh conditions, amyloids have revealed their high potential for design and realization of materials for drug delivery (Ping et al., 2017) and tissue engineering (Ping et al., 2017; Shimanovich et al., 2015) as well as electronics (Scheibel et al., 2003) and water purification (Bolisetty & Mezzenga, 2016).

Although sharing common features amyloid fibrils do not present a unique morphology and different fibril polymorphs may present dissimilarities at the nano-micro- or mesoscopic level that can be tuned via changing the growth conditions during in vitro protein aggregation (Fändrich et al., 2018; Tycko, 2015; Vetri & Foderà, 2015). Such differences in morphology often reflect different molecular arrangements (Andersen et al., 2010; Carrotta et al., 2011; Gelenter et al., 2019; Heise et al., 2005) and can determine the physical–chemical features (Madsen, Christiansen, Giehm, & Otzen, 2019; Vetri et al., 2018).

One of the most common tools to analyze formation of amyloid structures is the benzothiazole dye Thioflavin T (ThT) that has revealed an enormous potential (with some caveat [D'Amico et al., 2012; Di

Carlo et al., 2015; Foderà et al., 2008]) for the analysis of supramolecular assembly kinetics of these aggregates in vivo and in vitro (Ban, Hamada, Hasegawall, Naiki, & Goto, 2003). ThT shows high selectivity for amyloid fibrils and, upon binding to amyloid aggregates, it shows a bright fluorescence emission in the visible region. The increase of magnitude in ThT fluorescence intensity makes it an unusually sensitive and efficient reporter as roughly related to the amount of fibrillary structure in solution (Biancalana & Koide, 2010; LeVine, 1999).

Although its widespread usage for studies of amyloid fibrils, the details of ThT photophysical properties and its interactions with fibrils are not yet fully understood. Nowadays, it is commonly accepted that ThT binds to the side chain channels along the long axis of amyloid fibrils and the quantum yield enhancement has been attributed to the rotational immobilization of the central C─C bond connecting the benzothiazole and aniline rings (Biancalana & Koide, 2010). Since the first application, ThT kinetic assay has become widespread for the mechanistic study of amyloidal supramolecular assembly and was largely used also for drug screening applications (Michaels et al., 2018). However, it is important to note that the phenomenon of fibrils polymorphism has to be taken in account when trying to extrapolate quantitative information from ThT kinetics since the simultaneous presence of multiple aggregate morphologies in solution implying different structure directly translates into potentially different ThT binding sites whose nature may affect the quantum yield of this molecule (Andersen, Otzen, Christiansen, & Rischel, 2007; Lindberg, Wranne, Gilbert Gatty, Westerlund, & Esbjörner, 2015). The interest in this dye, in its photo-physics and in the accessible information available is growing and a number of studies are also dedicated to setup of experimental conditions in order to avoid artifacts (Hudson, Ecroyd, Kee, & Carver, 2009; Xue, Lin, Chang, & Guo, 2017; Ziaunys, Mikalauskaite, & Smirnovas, 2019).

When trying to quantify the mass of aggregates formed during aggregation kinetics it is important not to forget technical issues that have to be taken in account ranging from solution conditions that is, pH and temperature effects which may alter plateau value and the shape of the kinetics. Moreover, in steady state measurements scattering issues may occur if the massive aggregation leads to high turbidity of the sample, intrinsic sample inhomogeneity due to aggregation mechanisms, and in some instance light reabsorption issues if the dye concentration is not wisely selected may hinder results interpretation. Importantly, the role of dye concentration and dye/protein molar ratio were largely investigated in the past, revealing that fluorescence signal depends mostly on the total ThT concentration, rather than amyloid to ThT ratio (Xue et al., 2017).

For this reason, in the last years, time resolved fluorescence measurements were also proposed as tool for analyze amyloid formation kinetic, also giving information on the microenvironment of the dye which in turn gives suitable information on amyloid molecular structures and on dye/protein interactions. Indeed, the significant increase of ThT quantum yield upon interaction with amyloid structures is attributed to a dramatic reduction of the nonradiative decay rate (Amdursky, Erez, & Huppert, 2012) that is also reflected in the growth of fluorescence lifetime from ps to ns timescale (Rovnyagina et al., 2019). Fluorescence lifetime of a molecule indeed may give separated information, polarity, rigidity, or other specificities of the molecular environment as the presence of fluorescence quenchers.

Moreover, time resolved fluorescence measurements bring about a number of advantages as they do does not strictly depends on concentration of fluorescent molecules or on the experimental set up as intensity measurements, and minimize artifacts due to scattering and inhomogeneity effect. Importantly, they can be coupled with spatial mapping of the sample using Fluorescence Lifetime Imaging Microscopy (FLIM), giving back the map of fluorescence decays at pixel resolution, this being of utmost importance for the analysis of largely spatially heterogeneous samples as amyloid aggregates in the most cases.

In particular, in this article, we show by means of FLIM on Concanavalin A (Con A) amyloid fibrils using phasor approach for data analysis that self-quenching mechanisms of Thioflavin T fluorescence may occur that may alter the quantitative interpretation of data.

In previous studies, we have shown that Concanavalin A fibrils are formed in aqueous solution at basic pH by a simple noncooperative elongation mechanism, which do not imply nucleation. Amyloid growth is characterized by the formation of intermolecular β-sheet structure, which constitutes a rate-limiting step of the process. Kinetics are highly repeatable and the morphology and the structure of the final aggregates are largely homogenous. This represent an ideal model sample to elicit the effect of ThT photo-physical properties due to the homogeneity of binding sites (Vetri et al., 2007; Vetri, Carrotta, Picone, Di Carlo, & Militello, 2010).

Phasor analysis details are reported elsewhere (Digman, Caiolfa, Zamai, & Gratton, 2008; Ranjit, Malacrida, Jameson, & Gratton, 2018). Briefly, phasor approach is a Fourier domain technique that allows the transformation of the signal in every pixel in the image to a point in the phasor plot. Every possible lifetime is mapped in a phasor plot and all possible single exponential lifetimes lie on the "universal circle," defined as the semicircle going from point (0, 0) to point (1, 0), with radius 1/2. Point (1, 0) corresponds to $\tau = 0$, and point (0, 0) to $\tau = \infty$. Longer lifetimes are mapped near the origin and shorter lifetimes are mapped on the circumference toward the bottom right intersection with the x axis. Complex decays are represented by phasors within the universal circle. Usually results are visualized as clouds of points representing the fluorescence lifetime distributions. Pixels in the phasor plot can be selected using a colored cursor and the corresponding pixels are mapped back with selected color to the image pixels.

Although already assessed in literature we report in Figure 1 the experimental evidences of Con A aggregation state and structure used for the present study (Materials and Methods in Data S1). In Figure 1a representative confocal image of Con A fibrils stained with ThT is reported, as can be seen large micron-scale fibrillar structures positive to ThT are present. It is evident that within measurements spatial resolution sample is characterized by homogeneous fluorescence intensity independent on aggregate size and morphology. To characterize Con A amyloid structure FTIR measurements in the amide I′ region is also reported in Figure 1b in comparison with Con A native structure. As it is well known in the amide I′ (1,570 and 1,730 cm^{-1}) consists of several spectral components, which give quantitative information on samples secondary structures (Barth, 2007). Reported FTIR spectra presents expected features with respect to native proteins which shows the typical shape of an all beta protein. Con A fibrils present an intense absorption peak between 1,610 and 1,620 cm^{-1}, typical for aggregate β-sheets with very strong intermolecular hydrogen bonds that it is considered a marker for amyloid structures (Piccirilli et al., 2015; Zandomeneghi, Krebs, McCammon, & Fändrich, 2009). Several aliquots were measured and no heterogeneity was found within the experimental error.

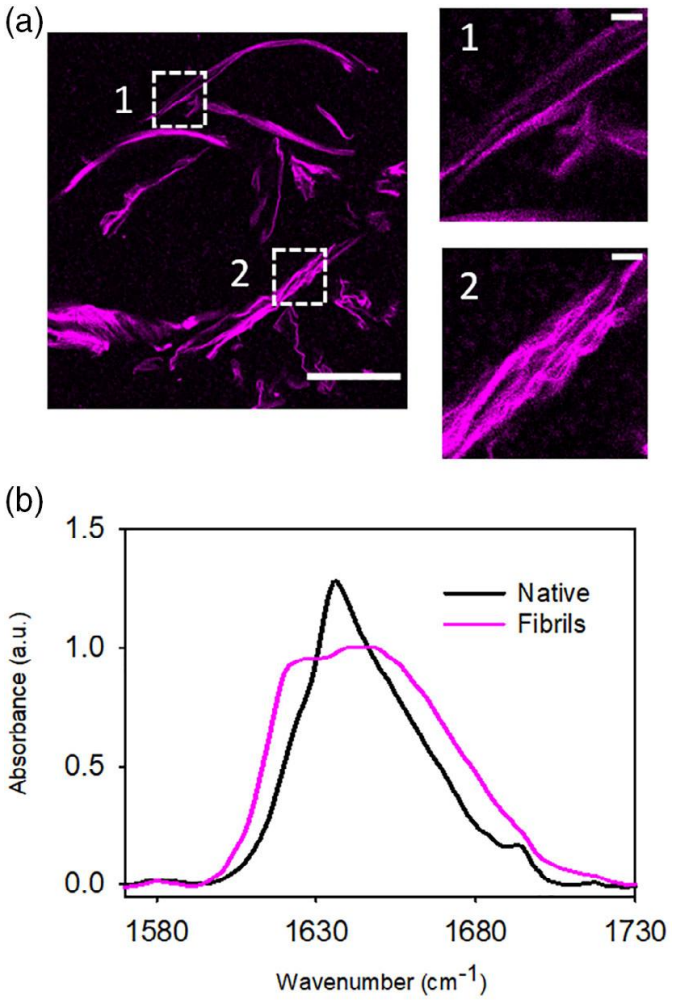

FIGURE 1 Con A aggregation state and structure. (a) Con A fibrils stained with ThT (scale bar 50 μm, scale bar inset 5 μm). Large micronscale fibrillar structures positive to ThT are present. (b) FTIR measurements in the amide I0 region of fibrils (purple) and native protein (black) in D2O. Con A fibrils present an intense absorption peak between 1,610 and 1,620 cm-1, typical for aggregate β-sheets [Color figure can be viewed at wileyonlinelibrary.com] Con A amyloid structure FTIR measurements in the amide I0 region is also reported in Figure 1b in comparison with Con A native structure. As it is well known in the amide I0 (1,570 and 1,730 cm-1) consists of several spectral components, which give quantitative information on samples secondary structures (Barth, 2007). Reported FTIR spectra presents expected features with respect to native proteins which shows the typical shape of an all beta protein. Con A fibrils present an intense absorption peak between 1,610 and 1,620 cm-1, typical for aggregate β-sheets with very strong intermolecular hydrogen bonds that it is considered a marker for amyloid structures (Piccirilli et al., 2015; Zandomeneghi, Krebs, McCammon, & Fändrich, 2009). Several aliquots were measured and no heterogeneity was found within the experimental error.

In Figure 2 the phasor analysis of FLIM measurements on Con A fibrils stained with ThT at increasing concentration of the dye. For each sample four representative 256 × 256 pixels images are reported for seven different ThT molar concentrations (5, 10, 20, 42.5, 85, 170, and 340 μM).

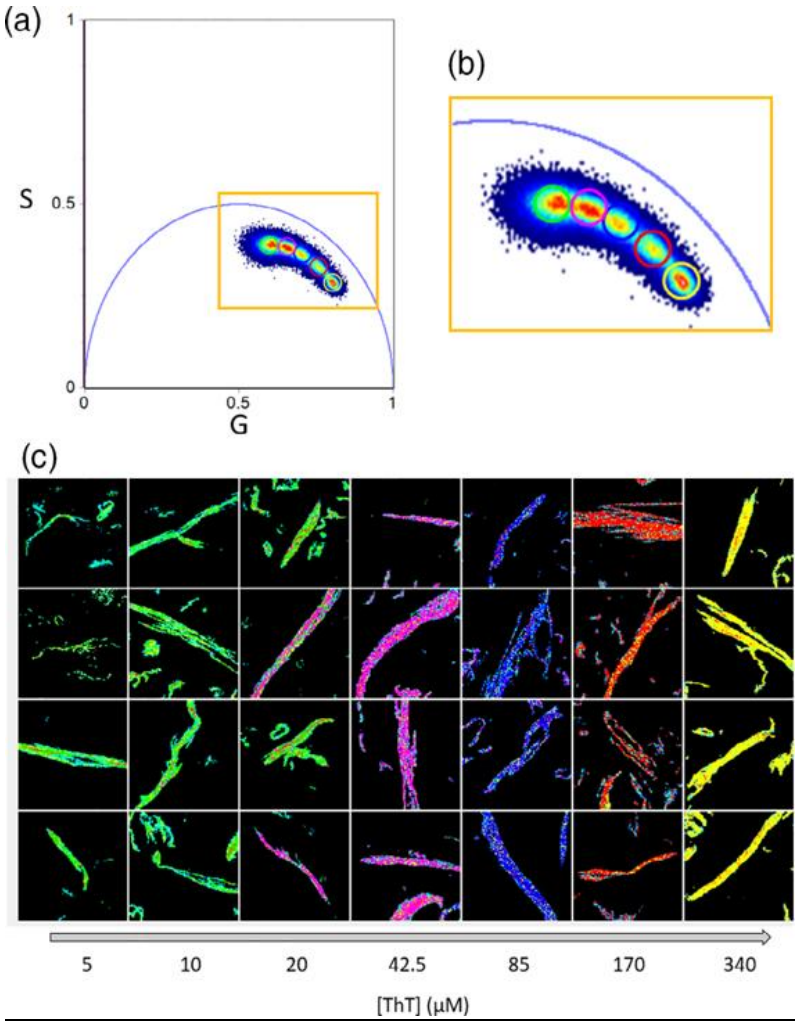

FIGURE 2 Phasor analysis of 256 × 256 pixel FLIM measurements on ThT signal in Con A fibrils (samples in water stained with 5, 10, 20, 42.5, 85, 170, 340 μM ThT). (a) Phasor plot where fluorescence lifetime distributions are highlighted using colored cursors ranging from green (higher lifetime lower ThT concentration to yellow lower lifetime higher ThT concentration. (b) expansion of the region in the phasor plot highlighting that fluorescence lifetime distributions maximum regularly decrease along a curve line typical of a quenching trajectory. (c) Phasor color map in which each pixel is colored according to the color of the corresponding cursor in the phasor plot. The choice of the size and the position of the cursor is arbitrary and it is used to highlight average properties of the lifetime distributions. ThT concentration ranging from 5 to 20 μM shows an almost overlap (green), indicating that ThT fluorescence decay is characterized by the same lifetime distribution, at increasing concentration, the measured lifetime distribution changes toward shorter lifetimes and samples at each concentration are uniformly colored and clearly distinguishable.

Figure 2a shows the phasor plot analysis of Con A amyloids fibrils as a function of ThT molar concentration. In Figure 2b the expansion of the phasor region where all phasors lie is shown. For each measurement, pixels with specific lifetime were selected in the image using a colored circle and corresponding pixels appear colored with the same color code on the phasor map reported in Figure 2c. Phasor plot method allows distinguishing, without fitting procedures, distributions of multi-lifetime species which result in the appearance of clouds inside the universal circle. In this specific case, measurements at

each concentration are characterized by a single phasor, each one lies inside the universal circle indicating that ThT fluorescence lifetime, for all the measured ThT concentrations, is characterized by non-single exponential decay. This observation is in line with the literature where multi (2 or 3) components analysis was applied to ThT fluorescence lifetime in different systems ranging from aqueous solution in the presence of crowding agents, high viscosity solvent and of course different amyloid fibrils samples (Lindberg et al., 2015; Rodina et al., 2017; Stsiapura, Maskevich, Uversky, Kuznetsova, & Turoverov, 2008; Sulatskaya et al., 2018). Multiple exponential have been interpreted, together with other evidences, as an indication that the fluorescence signal growth is determined by the steric hindrance of the internal rotation of the ThT aromatic rings relative to each other, which block transition to nonfluorescent state so that ThT quantum yield depends also on the solvent viscosity (Sulatskaya, Maskevich, Kuznetsova, & Uversky, 2010) and on the rigidity of its microenvironment. The complexity of this decay may also arise from different structural properties of the β-aggregate structure, including chain length or H-bond strength, which may modify the binding mode of ThT to amyloid structures (Ivancic, Ekanayake, & Lazo, 2016).

Each Phasor is selected with a colored cursor which is mapped back in the corresponding images in Figure 2c. According to the selected color code, phasor plot corresponding to the lower concentrations (5–10 μM) almost overlaps, the same lifetime distribution is observed also at 20 μM concentration with some broadening. This indicates that ThT lifetime distribution in these samples are almost indistinguishable and that in this range of concentration fluorescence lifetime does not change. Importantly, for each measured concentration, we observe a single homogeneously distributed phasor cloud (Figure S1). As the ThT concentration increases, phasors obtained from each sample result in well distinguishable lifetime distribution with progressively decreasing average lifetimes. We here describe the observed behavior in the phasor plot selecting five main components which are clearly distinguishable by eye, this underlying the simplicity of the method. Data in panels (a) and (b) clearly show that as ThT concentration increases above 20 μM, the phasor follows a specific curved trajectory from low concentration (left-green cursor) to high concentration (right-yellow cursor). This is also evident in panel (2b) where the expansion of the phasor region is shown. Data in Figure 2 clearly show that as ThT concentration increases, the phasor follows a specific curved trajectory from low concertation to high concentration. Curved trajectory in phasor plot have been attributed to energy transfer and FRET mechanisms (Chen et al., 2017) leading to the reduction of lifetime and to self-quenching processes of fluorescent species due to high concentration. Due to the homogeneity of the selected model fibrils we are able to rule out that the different lifetimes are not due to structural differences. Results indicate that as local concentration of the dye bound to fibrils increases fluorescence lifetime decreases following quenching trajectory this occurring above 20 μM. In line with our results, Thioflavin T self-quenching mechanisms at high ThT concentration were already proposed (Lindberg et al., 2017) in more insulin amyloid structures by means of time resolved measurements in solution coupled with linear dichroism and Atomic forced microscopy measurements.

Reported results reveal FLIM advantages in amyloid characterization as it allows separating fluorescence signal in aggregate species from fluorescence background (Sulatskaya, Lavysh, Maskevich, Kuznetsova, & Turoverov, 2017; Xue et al., 2017), highlighting when needed, spatially heterogeneous structures within the diffraction limited resolution. In addition, considering ThT complex fluorescence decay, the use of phasor analysis is highly advantageous as it avoids the choice of a model which may drive data interpretation. In this specific instance, the reported measurements in a simplified amyloid system allowed highlighting specific photophysical properties of the ThT dye, which have to be taken in account both in spectroscopy and in fluorescence microscopy measurements.

To the best of our knowledge, this is the first analysis of Thioflavin T self-quenching by Phasor-FLIM analysis. The present work fosters the attention on the experimental condition when Thioflavin T is involved, avoiding artifacts and misleading results. Thanks to the selected approach, reported results univocally reveal that ThT is subject to substantial self-quenching that result in a significant change of fluorescence signal that is not related to aggregate structural details. Measured lifetime decreases as the

concentration of ThT increases this constituting a serious warning when attempting quantification and comparison between samples in different conditions. Depending on selected conditions, steady state fluorescence measurements may not reflect the amount of dye bound to the fibrils and/or the plateau value in kinetics may be the results of the superimposition of multiple effects. Moreover, this should be also taken in consideration when using ThT lifetime as reported of molecular structure in complex polymorph aggregates.

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