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Explorations of the application of cyanine dyes for quantitative α-synuclein detection

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Abstract

We examined the practical aspects of using fluorescent mono (T-284) and trimethinecyanine (SH-516) dyes for detecting and quantifying fibrillar α-synuclein (ASN). We studied the interaction of cyanine dyes with fibrillar proteins using fluorescence spectroscopy and atomic force microscopy. The commercially available classic amyloid stain thioflavin T (Thio T) was used as the reference dye. T-284 and SH-516 dyes can be used for fluorometric quantification of fibrillar wild-type ASN at concentrations of ~1.5–20 μg/ml. Both dyes appeared suitable for step-wise monitoring of ASN variants (wild-type and mutants A30P and A53T) aggregation into fibrils in vitro, demonstrating good reproducibility, exceeding that for the commonly used Thio T. Our assay may be used for screening in vitro of agents capable of affecting the aggregation of ASN. In addition, T-284 and SH-516 cyanine dyes were shown to recognize amyloid proteins of various amino acid compositions selectively. T-284 demonstrated particular sensitivity to wild-type and A53T ASN, while for SH 156, the fluorescence response to fibrillar proteins was nearly the same except for lysozymes. T-284 and SH-516 cyanine dyes are sensitive and specific fluorescent probes for monitoring ASN fibril formation process in vitro, quantification of fibrillar ASN in solution, and fluorescent detection of various fibrillar protein species.

Key words: α-synuclein, cyanine dyes, fluorescence quantitation

A characteristic pathological feature of neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, is the formation of insoluble protein aggregates known as amyloid fibrils. Although, there is little or no similarity in the amino acid sequence of the various precursor proteins linked to disease, the structures of the insoluble amyloid fibrils formed are similar. The fluorescent dye, thioflavin T (Thio T), is the most frequently used dye for detection of amyloid structures (Glenner 1980, Naiki et al. 1989, LeVine 1993). Upon binding to fibrils, the emission of this dye increases and undergoes a spectral shift; these spectral changes are not observed with non-aggregated protein.

Despite the widespread use of Thio T, its application to amyloid quantification often gives inconsistent and inaccurate results (Khurana et al. 2001, Eisert et al. 2006, Groenning et al. 2007, Hawe et al. 2008). Variations in spectral properties caused by buffer conditions and protein–dye ratios result in poor reproducibility, complicating the use of Thio T for quantitative assessment of fibril formation. In the absence of other more reliable assays, the literature relies heavily on the properties of Thio T as a reporter probe for amyloid protein aggregation (Masuda et al. 2006, Ono et al. 2007).

A reliable method for quantification likely would be useful not only for detecting mature amyloid fibrils, but also for monitoring the

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kinetics of fibrillogenesis, which is essential for better understanding of the underlying biophysics and mechanism of the aggregation process. Furthermore, such an assay would be a tool for discovery and development of therapeutic compounds capable of blocking protein aggregation.

Recently, we have shown that cyanine dyes exhibit a specific increase in fluorescence intensity in the presence of fibrillar α-synuclein (ASN) (Volkova et al. 2008). The greatest enhancement of emission intensity was observed for dialkylamino-substituted monomethine cyanine (T-284) and meso-ethyl-substituted trimethine cyanine (SH-516). Because these dyes demonstrated selectivity for fibrillar ASN comparable to the widely used Thio T, the cyanine dyes, T-284 and SH-516 were proposed as novel fluorescent probes for fibrillar ASN detection (Volkova et al. 2008).

In the research reported here, practical aspects of the use of T-284 and SH-516 cyanine dyes were examined including their suitability for step-wise monitoring and quantitative characterization of ASN aggregation into fibrils as observed in Parkinson's disease. Such an assay also may be used for in vitro screening of agents capable of affecting aggregation of ASN. In the study reported here, we included wild-type and the familial disease-related mutants of ASN, viz., A30P and A53T. Atomic force microscopy was used to study the morphology of the amyloid fibrils obtained. To determine potential applicability of T-284 and SH-516 dyes for detecting fibrillar proteins of various amino acid compositions, the spectral-luminescent characteristics of these cyanines in the presence of insulin and lysozyme, which are widely used as model proteins for amyloid studies, were also evaluated.

Materials and methods

Reagents

Chicken egg white lysozyme and bovine pancreas insulin were purchased from Sigma (St. Louis, MO). Recombinant human wild-type and mutant ASN were expressed and purified as described earlier (van Raaij et al. 2006). The monomethine cyanine dye T-284 was synthesized according to Hamer (1964). The SH-516 trimethine dye was kindly provided by Dr. A. Bogolyubskyi (Enamine, Kyiv, Ukraine). Structures of compounds obtained were confirmed by ^{1}H NMR and elemental analysis. Purity of the T-284 and SH-516 dyes was approximately 95%. All other chemicals, including Thio T, were purchased from Sigma-Aldrich and were of the highest grade available. Thio T was used without further purification.

Preparation of dye solutions

Stock solutions were prepared by dissolving the dyes at 2 mM in dimethyl sulfoxide (DMSO).

α-Synuclein ASN fibril formation

Monomeric ASN (wild-type, A30P, and A53T) was incubated at an initial monomer concentration of 100 μM in 300 μl 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 at 37° C with constant shaking at 1500 rpm in Eppendorf tubes in a Thermomixer (Eppendorf). All aggregations were performed in triplicate. Aliquots of the reaction mixtures were withdrawn from each tube at approximately 24 h intervals to accommodate spectral measurements using Thio T, T-284, and SH-516 as reporter dyes for aggregation (see below). For this, a 5 μl aliquot of the aggregation mixture was added to 500 μl of a 5 μM dye solution in 10 mM Tris-HCl, pH 7.4. All spectroscopic measurements were made immediately after mixing the protein and dye solutions. After the aggregation (typically by 180 h) 15 μl aliquots were taken for atomic force microscopy imaging. Samples for atomic force microscopy imaging were stored at 4° C until measurement. While studying selectivity of the dyes for the different fibrillar proteins, dye and protein concentrations in the working solutions were 1 μM and 2 μM, respectively.

Lysozyme and insulin fibril formation

Chicken egg white lysozyme was dissolved at 1 mM in 10 mM HCl (pH 2) and bovine pancreas insulin was dissolved at 170 μM in 100 mM HCl (pH 1). Fibrils were formed by shaking the protein solutions at 750 rpm in a Thermomixer (Eppendorf) at 65° C. Aggregation was monitored by Thio T binding until saturation of the fluorescence signal (for lysozyme after 90 h and for insulin after 150 min). The samples were stored at 4° C. The presence of fibrils was confirmed by atomic force microscopy. While studying dye selectivity for the various fibrillar proteins, dye and protein concentrations in the working solutions were 1 μM and 2 μM, respectively.

Spectroscopic measurements

Fluorescence excitation and emission spectra were measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA). Fluorescence spectra were measured with excitation and emission slit widths set at 5 nm and at a constant photodetector voltage. Spectra were corrected automatically for the wavelength dependence of the excitation source intensity, but were not corrected for the sensitivity of the Cary Eclipse detection system. Spectra of free dyes and dye–protein complexes were corrected for buffer contributions. Emission measurements were performed in quartz cells (0.5 cm) at room temperature at the respective excitation maxima of each dye, i.e., 441 nm, T-284; 558 nm, SH-516; and 442 nm, Thio T.

Atomic force microscopy

For measurements, aggregation aliquots were diluted in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, adsorbed onto mica, washed twice with 50 μl Milli-Q water and gently dried under nitrogen gas. Tapping mode atomic force microscopy height images were made on a custom built instrument as described earlier (van Raaij et al. 2006).

Results and discussion

Spectral properties of dyes in buffer and in the presence of monomeric and fibrillar insulin, lysozyme, and ASN

Earlier, we reported that T-284 and SH-516 cyanine dyes (Fig. 1) can be efficient fluorescent probes for detecting of fibrillar wild-type ASN (Volkova et al. 2008). The fluorescence characteristics of monomethine cyanine T-284 and trimethinecyanine SH-516 dyes in buffer and in the presence of fibrillar proteins are shown in Table 1. The benzothiazole dye, Thio T, commonly used for selective fluorescent detection of amyloid fibrils, was used as a reference dye (Glenner 1980, Naiki et al. 1989, LeVine 1993). Excitation maxima for the free dyes in aqueous buffer are 441 and 558 nm for T-284 and SH-516, respectively, and 442 nm for Thio T. The intrinsic fluorescence maxima are for T-284, 563 nm; for SH-516, 569 nm; and for Thio T, 486 nm (Table 1).

Upon addition of fibrillar and monomeric species of the ASN variants, insulin, and lysozyme, the excitation maxima of T-284 were red-shifted by up to 29 nm and located between 452 and 470 nm. For SH-516, a bathochromic shift of 16–22 nm was also observed in presence of proteins ($\lambda_{ex} \sim 574$–580 nm). For Thio T, excitation maxima shifted to the long-wavelength region by up to 9 nm and were then located between 442 and 451 nm.

By contrast to the excitation maxima, the fluorescence emission maxima of T-284 and Thio T dyes in the presence of all protein species remained nearly unaltered or were blue-shifted by up to 14 nm (Table 1). For SH-516, the emission maxima were red-shifted by up to 21 nm compared to the free dye. Emission maxima were located between 549 and 565 nm, T-284; 582 and 590 nm, SH-516; and 479–487 nm, Thio T.

At the 1 μM concentration used, all dyes demonstrated low intrinsic fluorescence (Fig. 2), while a noticeable increase in fluorescence was observed in the presence of fibrillar proteins. By contrast, addition of monomeric proteins scarcely affected the emission intensities of the dyes (data not shown). For monomethine cyanine T-284, similar fluorescence intensities of 24–29 arbitrary units (a.u.) were obtained with fibrillar insulin, lysozyme, and A30P ASN. The fluorescence emission was more than twice as great with wild-type ASN fibrils (84 a.u.) and fibrillar A53T ASN (80 a.u.). The trimethinecyanine dye, SH-516, demonstrated comparable fluorescence intensities (49–61 a.u.) with all fibrillar proteins, with values more than 25 times higher compared to SH-516 in buffer or with monomeric proteins. This dye, however, appeared to be nearly insensitive to fibrillar lysozyme. Thio T fluorescence intensities ranged from 20 to 36 a.u. for fibrils of all ASN variants, an increase of 14–24 fold. The largest Thio T fluorescence response was

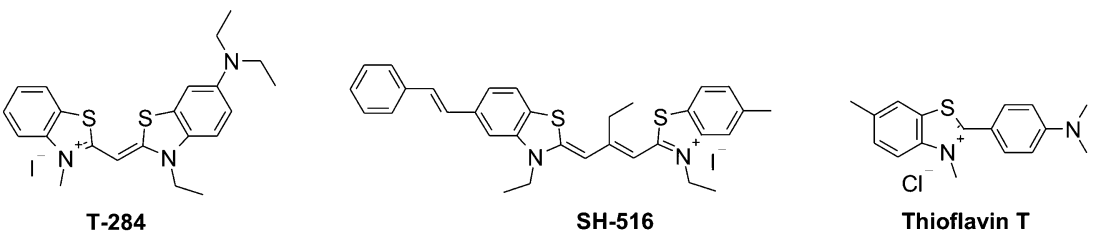

Fig. 1. Structures of T-284, SH-516, and Thio T.

Table 1. Spectral properties of the dyes, free in buffer and in the presence of fibrillar proteins.

	T-284		SH-516		Thio T	
	λ_{ex}*, nm	λ_{em}*, nm	λ_{ex}, nm	λ_{em}, nm	λ_{ex}, nm	λ_{em}, nm
Free dye	441	563	558	569	442	486
wtASN						
F†	470	560	574	584	449	480
M†	458	551	577	589	442	486
A30P						
F	459	563	574	584	451	480
M	458	552	578	590	442	487
A53T						
F	460	562	575	585	451	480
M	459	553	579	590	442	487
Insulin						
F	452	565	574	582	449	479
M	455	560	579	590	442	486
Lysozyme						
F	468	549	575	586	443	479
M	459	550	580	590	443	485

*λ_{ex} (λ_{em}), maximum wavelength of fluorescence excitation (emission) spectrum.
†F (M), fibrillar (monomeric) form of the corresponding protein.

observed upon interaction with fibrillar insulin (63 a.u.) and the lowest response with lysozyme fibrils (9.1 a.u.).

The monomethine cyanine T-284 appeared to be somewhat more specific for fibrillar lysozyme and A53T ASN mutant than the other dyes studied, while the trimethinecyanine, SH-516, showed only little variability among the different protein species, except for fibrillar lysozyme. In the presence of fibrillar proteins the emission intensities for both T-284 and SH-516 either exceeded or were at least comparable to intensities obtained with the classical amyloid-sensitive dye, Thio T. Taken together, both cyanine dyes are useful and specific fluorescent probes for detecting various fibrillar protein species.

Linear range for cyanine dyes compared to Thio T

Because one of the goals of the study reported here was to examine further, the practical applicability of the T-284 and SH-516 dyes as ASN-specific fluorescent probes, we tested the effective range

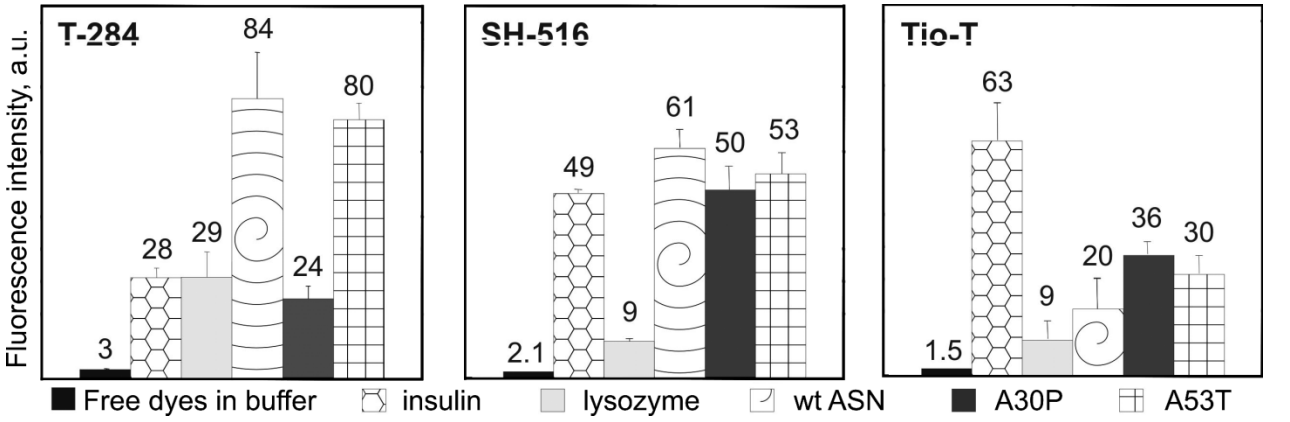

Fig. 2. Specificity of cyanine dyes compared to Thio T. T-284, SH 516, and Thio T fluorescence intensities free in solution and in the presence of different fibrillar proteins. Emission intensity values are given in arbitrary units (a.u.). The concentrations of dyes and proteins were 1 μM and 2 μM, respectively. Experiments were done in duplicate.

of these cyanine dyes for quantitation of fibrillar wild-type ASN by titrating a 10 μM solution of T-284 and SH-516 with increasing amounts of aggregated protein. We used the commonly used commercially available benzothiazole dye Thio T as a reference dye.

Fluorescence emissions of dyes in their free state and in the presence of fibrillar ASN were excited at the maximum of the fluorescence excitation spectrum. In case of cyanine dyes, the free dye fluorescence was excited at 441 nm (T-284) and 558 nm (SH-516) and recorded at 563 and 569 nm, respectively (Table 1). For Thio T, excitation and fluorescence maxima were at 442 and 486 nm, respectively. Upon addition of the wild-type ASN fibrils, the excitation maxima of both cyanine dyes were red-shifted to 470 (T-284) and 574 nm (SH-516), respectively. For T-284 and SH-516, emission maxima in presence of aggregated ASN were registered at 560 and 584 nm, respectively. In the presence of fibrillar protein, Thio T demonstrated excitation and fluorescence maxima at 449 and 480 nm, respectively. The linear ranges and detection limits for each dye were calculated from the measured fluorescence intensities. The lower limit of detection was considered to be the protein concentration that resulted in twice the fluorescence intensity of the free dye.

Fig. 3 shows the enhanced fluorescence intensities proportional to the amount of fibrillar ASN added for all dyes. The insets of Fig. 3 show enlargements of the results obtained for 0–1.5 μg protein per ml (0–3 μg/ml for Thio T). From the insets it can be seen that T-284 and Thio T display a linear fluorescence intensity increase starting at a concentration of 0.14 μg/ml of ASN, while the emission of SH-516 begins to rise at protein concentrations greater than 0.3 μg/ml (Fig. 2, inset). Both cyanine dyes allow determination of as little as 1.5 μg/ml of fibrillar ASN. For Thio T, this detection limit was not so low (2 μg/ml). The linear range required for quantitative determination of fibrillar protein extended to about 35 μg/ml for Thio T, and up to 28 μg/ml and 19 μg/ml for SH-516 and T-284, respectively.

The detection limits obtained are comparable to and even exceed those for amyloid-specific compounds described in the literature. The fluorescence of the Congo red analog (trans, trans)-1-bromo-2,5-bis-(4-hydroxy) styrylbenzene (K114) increases linearly up to 70 μg/ml in the presence of ASN fibrils, while the dye concentration was 10 times higher than for the cyanine dyes studied

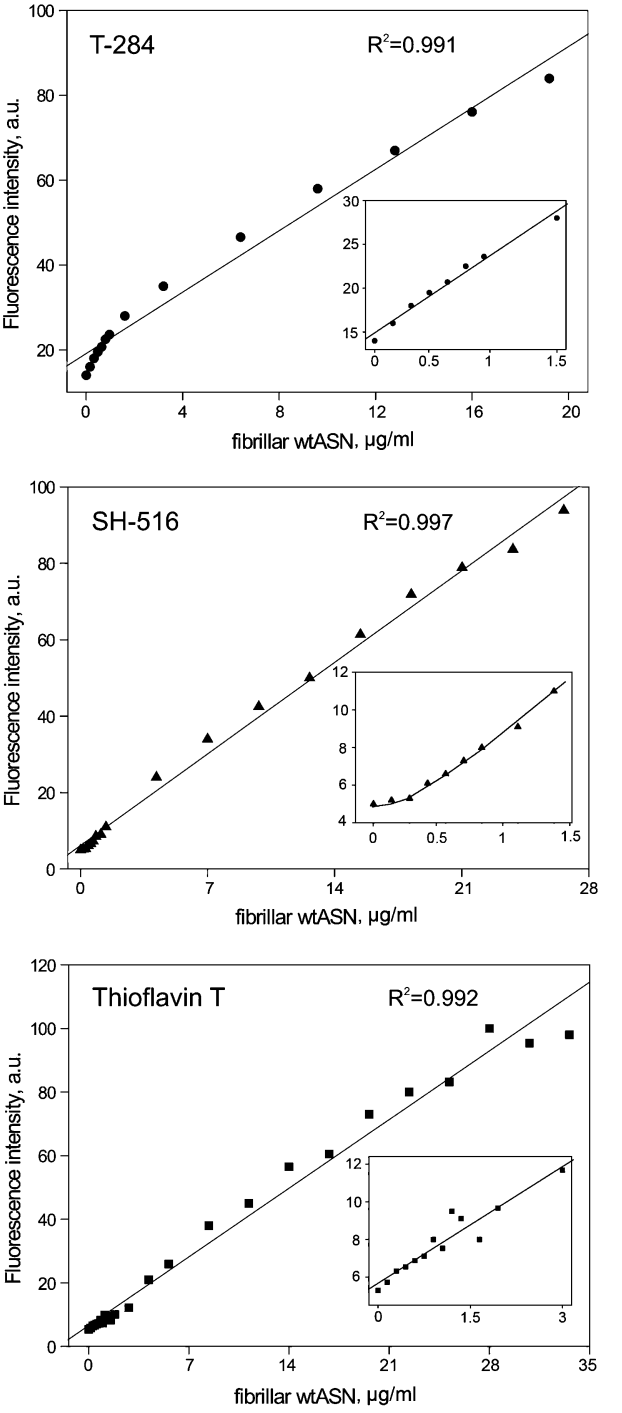

Fig. 3. Effective linear range of cyanine dyes compared to Thio T. SH-516, T-284, and Thio T fluorescence intensities relative to the concentration of fibrillar wild-type ASN. The concentration of the dyes was 10 μM. Insets show enlargements of the results obtained (0–1.5 μg of protein/ml for cyanines, 0–3 μg/ml for Thio T) and illustrate the detection limit of ~1.5–2 μg/ml. A best-fit line and the correlation coefficient (R^2) are shown for each graph.

Table 2. Morphological characteristics of the wild-type, A30P, and A53T α-synuclein fibrils.

	N fibrils	Fibril height, nm
WtASN	34	6.3±1.0
A30P ASN	49	7.4±1.0
A53T ASN	24	10.8±1.3

N is the number of fibrils analyzed. Height values are averaged over all *N* fibrils, the standard deviation is used as measurement error.

here (Crystal et al. 2003). Another amyloid-specific agent, trans-resveratrol (a major phenolic constituent of red wine) at the same concentration as the cyanine dyes studied could be used for ASN detection starting from 20 μg/ml of fibrillar protein (Ahn et al. 2007). We conclude from these data that both cyanine dyes studied can be used for fluorometric quantification of fibrillar wild-type ASN, particularly at concentrations below 20 μg/ml.

Aggregation kinetics of α-synuclein (ASN) assessed with cyanine dyes and Thio T

To determine whether cyanine dyes could be used to follow step-by-step transition of the monomeric ASN into fibrils in vitro, the fluorescence of T-284 and SH-516 was monitored during ASN (wild-type, A30P, and A53T) aggregation and compared to Thio T. Figure 4 shows an emission intensity increase in time for all dyes, reflecting in vitro wild-type ASN fibrillogenesis. The enhancement of T-284, SH-516, and Thio T fluorescence intensity in the aggregation assay correlated with the formation of amyloid fibrils, which was confirmed by atomic force microscopy (Fig. 4, inset). The aggregation curves obtained for A30P and A53T ASN were similar to those obtained for wild-type ASN (data not shown).

Quantitative analysis of atomic force microscopy images (made in tapping mode) of fibrils made from wild-type ASN and mutants A30P and A53T ASN after 120 h incubation revealed the average heights of fibrils to be 6.3±1.0, 7.4±1.0, and 10.8±1.3 nm, respectively (Table 2). This agrees with the heights of mature ASN fibrils described in the literature (Segers-Nolten et al. 2007).

Amyloid fibrils are considered cross-beta filaments, where the protein molecules making up the β-sheet are arranged perpendicular to the long axis of the fibril. Here, the side chains on each side of the β-sheet form neat rows, ''binding channels,'' running in the direction of the β-sheet. The width of the binding channels is the distance between every second residue, which in a β-sheet is 6.5–6.95 Å (Pauling and Corey 1951, Salemme 1983). A general model for amyloid-specific dye binding to amyloid fibrils was proposed by Krebs et al. (2005) based on Thio T. Confocal microscopy studies using polarized light and Thio T molecular dimension calculations showed that the dye binds to the fibrils with its long axis parallel to the fibril axis via insertion into this ''binding channel'' running along the fibril.

Previously, based on the literature (Krebs et al. 2005) and by comparing data of fluorescence studies and molecular dimension calculations, we suggested that T-284 and SH-516 cyanine dyes have favorable molecular dimensions to be inserted into ''binding channels'' with their long axes parallel to the fibril axis, closely surrounded by the side chains (Volkova et al. 2008).

While almost no fluorescent response was observed for dyes in the presence of monomeric ASN or at the beginning of the fibrillization reaction, we

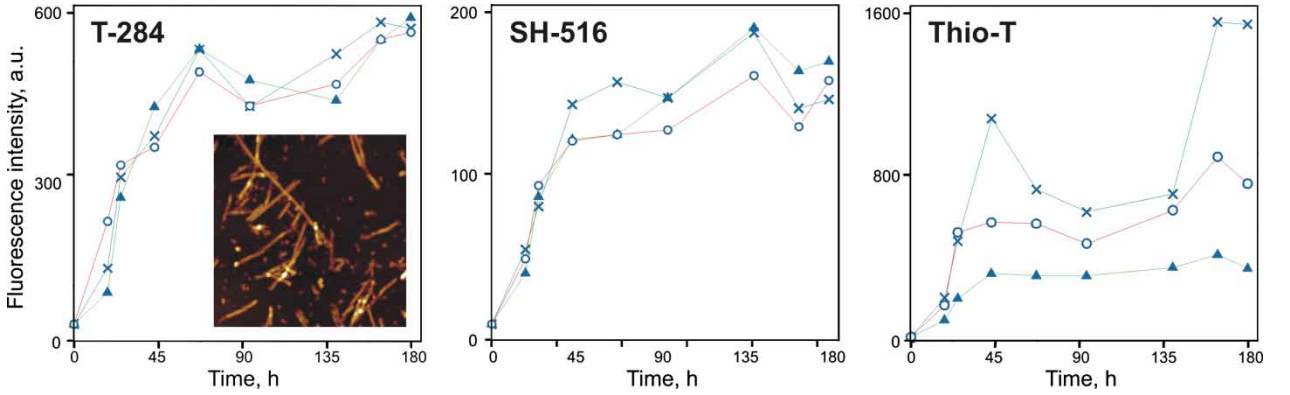

Fig. 4. Kinetics of wild-type human ASN aggregation assessed with SH-516, T-284, and Thio T fluorescence emission using a 5 μM dye concentration. Experiments were performed in triplicate (crosses, circles and triangles in each of the graphs). Inset shows an atomic force micrograph of fibrillar aggregates of wild-type ASN at 120 h.

suggest that enhancement of the dyes' fluorescence was observed after formation of beta-pleated ASN aggregates in the incubated mixture.

Fig. 4 shows that reproducibility within triplicate measurements is better for the cyanine dyes T-284 and SH-516 than for Thio T. Despite the widespread use in amyloid detection, unsatisfactory Thio T reproducibility has been described in the literature (Eisert et al. 2006). The superior reproducibility of the T-284 and SH-516 cyanine dyes suggests that they may be more useful for quantitative monitoring of aggregation of wild-type and disease-related mutant ASN into amyloid fibrils than Thio T. This property is crucial for developing cyanine dye-based fluorescent assays for in vitro screening of agents capable of inhibiting aggregation of ASN.

The T-284 and SH-516 cyanine dyes appear suitable for quantitative detection of as little as ~1.5 μg/ml of fibrillar ASN, which is comparable to the detection limit for commercially available dyes for protein quantification in solution (Crystal et al. 2003, Ahn et al. 2007). The upper detection limit for SH-516 and T-284 in fluorometric quantification was 28 μg/ml and 19 μg/ml of fibrillar protein, respectively.

To sum up, the reproducibility offered by the cyanine dyes, T-284 and SH-516, permits development of reliable fluorometric assays for in vitro monitoring wild-type, A30P, and A53T ASN amyloid fibril formation. Such an assay also may be used for screening potential inhibitors of ASN aggregation in vitro. We demonstrated that both cyanine dyes are applicable for detection of various fibrillar protein species. T-284 demonstrated particular sensitivity to wild-type and A53T ASN, while for SH-156, the fluorescence response to fibrillar proteins was almost the same, except for lysozyme.

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