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Selecting FRET pairs for visualizing amyloid aggregation

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ABSTRACT

In a recent work, we reported a methodology for imaging the different stages of amyloid aggregation in quantitative multiparametric dual-color fluorescence lifetime imaging (FLIM) and superresolution microscopy by using a pair of dyes capable of binding aggregates and undergoing subsequent intra-aggregate energy transfer (FRET) (RuizArias et al. *Sensors Actuat. B*, 2022, **350**:130882). In this microarticle we present the optimization process for choosing the best pair of dyes through a screening of different naphthalimides and quinolimides and other known amyloid-binding dyes.

1. Introduction

Several neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, are characterized by the abnormal aggregation of specific proteins in fibrillar plaques called amyloid fibrils [1]. Hence, novel, advanced methodologies of analysis, detection and visualization are direly needed for a better understanding of the mechanisms of aggregation and fibrillization [2,3]. Among these methods, fluorescence microscopy and, especially, high-performance variants, such as multidimensional fluorescence lifetime imaging microscopy (FLIM) [4], single-molecule microscopy and superresolution techniques [5,6], are especially relevant due to their noninvasiveness and high sensitivity. In this regard, the development of new fluorophores with enhanced capabilities for the detection of amyloid aggregates is a very active field of research [7,8].

In a recent work, we reported the simultaneous use of two fluorophores capable of binding amyloid aggregates yet in the early stages of aggregation with different affinities, and therein undergoing Förster resonance energy transfer (FRET) from the donor to the acceptor fluorophore. The FRET phenomenon within amyloid aggregates provided an extra layer of contrast in quantitative multiparametric dual-color FLIM and superresolution studies of protein aggregation [9]. In particular, we employed the solvatochromic dyes 9-azetidinyl-quinolimide (**AQui**) [10] and Nile Blue A (**NBA**) to visualize amyloid aggregates of apoferritin [11,12]. Nonetheless, during the optimization process of the previous work, we tested other potential fluorophores that, according to sufficient spectral overlap, may have constituted a suitable FRET pair for such studies. Herein, we describe additional attempts to find suitable FRET pairs for the visualization of amyloid aggregation using multiparametric FLIM.

2. Experimental section

Horse spleen apoferritin (Sigma–Aldrich) was incubated at pH 2 and heated to 90 °C in hermetically sealed glass tubes to allow amyloid fibrillization as previously described [9,11,12]. Aliquots of the incubating sample were collected after 1, 3, 9, and 24 h of incubation.

The different fluorophores were added to aliquots of apoferritin samples at a concentration of 5 μM.

Fluorescence emission spectra were obtained on a Jasco FP-8300 spectrofluorometer (Jasco, Tokyo, Japan).

Abbreviations: FLIM, Fluorescencelifetime imaging microscopy; STED, Stimulated emission depletion; FRET, Förster resonance energy transfer.

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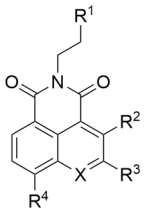

AQui: X = N; R^1 = CH_3; R^2 = H; R^3 = H; R^4 = N

1: X = N; R^1 = CH_3; R^2 = H; R^3 = H; R^4 = OMe

2: X = N; R^1 = OH; R^2 = $PO(OEt)_2$; R^3 = Cl; R^4 = OMe

3: X = N; R^1 = CH_3; R^2 = H; R^3 = H; R^4 = NH_2

4: X = N; R^1 = CH_3; R^2 = H; R^3 = H; R^4 = NHnBu

5: X = CH; R^1 = $N(CH_3)_2$; R^2 = H; R^3 = H; R^4 = $N(CH_2)_2OH$

6: X = CH; R^1 = $N(CH_3)_2$; R^2 = H; R^3 = H; R^4 = HN

Chart 1. Chemical structures of **AQui** and dyes **1**–**6**.

3. Results

3.1. Screening of quinolimide dyes as reporters of protein aggregation

In a previous work, the quinolimide dye **AQui** was found to be an excellent reporter for the aggregation of the amyloid-β peptide [10]. Before applying the concept of using a FRET pair of dyes to maximize contrast in multiparametric microscopy imaging of the aggregation of apoferritin, we screened different quinolimide and naphthalimide derivatives (Chart 1) that might be able to work as sensitive probes for protein aggregation. For this, we obtained the fluorescence emission spectrum of the different dyes in aqueous solution (pH 2, as the incubating buffer for apoferritin aggregation) and upon interaction with apoferritin (30 μM) incubated for 9 or 24 h under amyloidogenic conditions. The best features of the emission for the dyes are i) negligible emission in water and ii) an extensive enhancement of the emission upon interaction with the incubated protein. Fig. 1 shows the results of the screening of 9-methoxy-quinolimides **1** and **2** [13], 9-amino-quinolimides **3** and **4** [10] and **AQui**, and 6-amino-naphthalimides **5** and **6** [14]. As shown in the figure, **1**, **3** and **4** showed an emission increase in the presence of amyloid aggregates. Nevertheless, **AQui** clearly showed the best results [15].

We hypothesized that protonation of the dyes at the low working pH may be involved in the binding affinity. We quantified the acidic pK_a values, and obtained values of 2.22 ± 0.04 and 1.09 ± 0.03 for 9-methoxy-quinolimides **1** and **2**. One of the most effective dyes was the 9-amino-quinolimide **4**, which exhibited a pK_a of 1.1 ± 0.1. Moreover, amino-naphthalimides **5** and **6** showed protonation at near-neutral pH [14], being the reason of the less marked solvatochromic behaviour of these dyes and their lower efficiency for detecting protein interactions. In contrast, **AQui** exhibited a much weaker base behavior, and the pK_a was < 0.5. Therefore, most of the dyes remain neutral at the working pH 2.0, and no correlation was found between the acid-base properties of the dyes and the ability to report on amyloid aggregation.

3.2. Testing FRET pairs for the study of apoferritin aggregation

Once it was determined that the 9-amino-quinolimide **AQui** was the most sensitive quinolimide for the detection of apoferritin amyloid aggregates, we investigated the possibility of improving the detection and contrast by multiparametric FLIM by designing a suitable FRET pair in which both dyes interact with aggregates with different affinities.

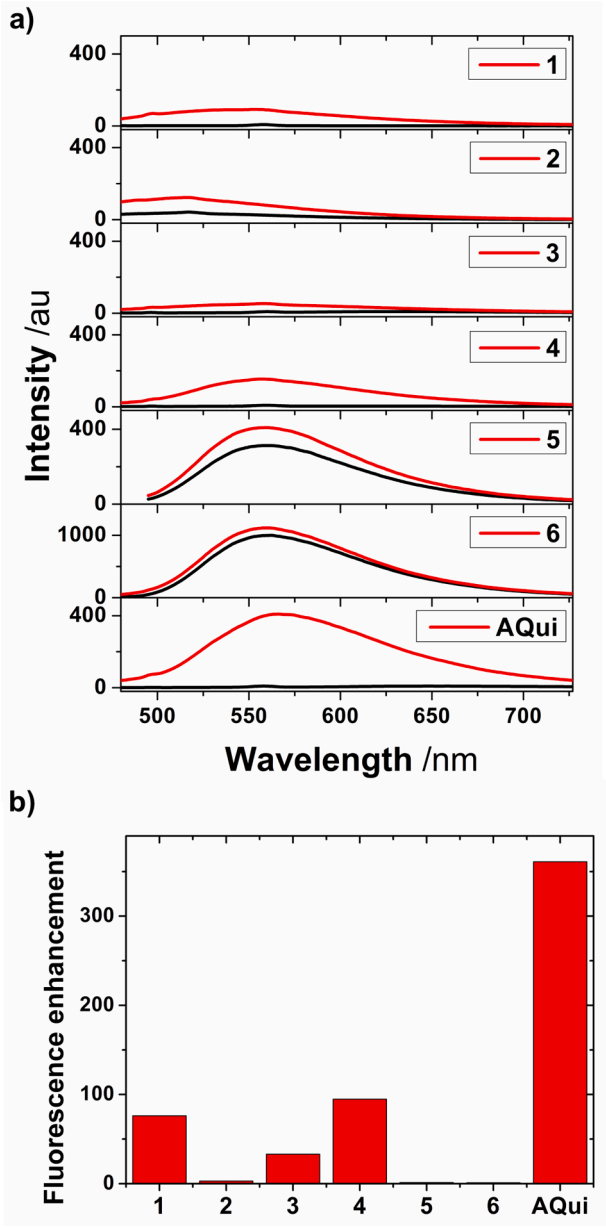

Fig. 1. a) Emission spectra of quinolimides **1**–**4** and **AQui** and naphthalimides **5** and **6** in aqueous solution at pH 2 (black line) and upon interaction with apoferritin (30 μM) incubated for 24 h under amyloidogenic conditions (red line). b) Fluorescence intensity enhancement upon interaction with apoferritin (30 μM) incubated for 24 h, measured at the maximum emission wavelength, from dyes **1**–**6** and **AQui**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Energy transfer from the donor dye to the acceptor dye within the amyloid aggregate would allow changes in both the emission intensity ratios and fluorescence lifetime of the donor (Fig. 2a).

We established two potential FRET pair candidates according to suitable spectral overlaps (Fig. 2b) and Förster's radius, R_0, values (Table 1). In the first one, we set thioflavin T (**ThT**), a well-known amyloid fibril binding dye [17,18], as the energy donor and **AQui** as the acceptor. In the second option, we considered **AQui** as the energy donor and Nile Blue A (**NBA**), a dye that is also known to bind amyloid aggregates [8], as the acceptor. Fig. 3a and b shows the results of the FRET pair **ThT** → **AQui**. Contrary to what was expected, we did not find a clear FRET increase with amyloid fibril growth. This may be caused by the fact that **ThT** only binds mature fibrils, whereas **AQui** binds even

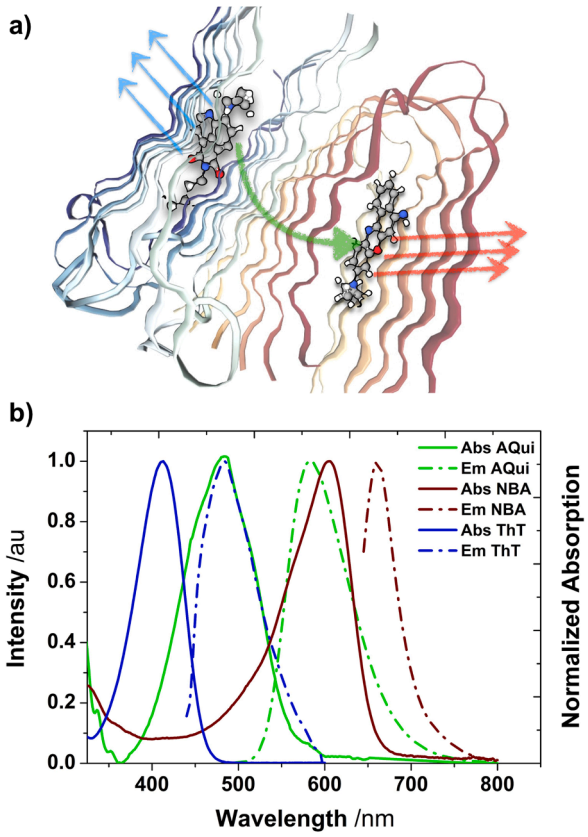

Fig. 2. a) Concept of intra-aggregate FRET between a dye pair for enhancing contrast in multiparametric FLIM. b) Absorption (solid lines) and emission (dash-dotted lines) spectra of **ThT** (blue lines; emission at λ_{ex} = 400 nm; reproduced from PhotochemCAD 3 [16]), **AQui** (green lines; emission at λ_{ex} = 485 nm) and **NBA** (brown lines; emission at λ_{ex} = 635 nm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Photophysical parameters employed to estimate R_0 values for the two selected FRET pairs.

	ϕ_D [a]	ε_A [b]/M^{-1} cm^{-1}	J [c]/10^{15} M^{-1} cm^{-1} nm^4	R_0 [d]/nm
ThT → AQui	0.43	15,550	6.71	4.05
AQui → NBA	0.20	67,000	5.57	5.07

[a] **ThT** quantum yield from references [19,20] and **AQui** quantum yield from reference [10].

[b] **AQui** molar absorptivity from reference [10] and NBA molar absorptivity from reference [16].

[c] Overlap integral.

[d] The orientation factor, κ^2, was considered equal to 2/3 for free rotation of the dyes. This may not be the case when bound to amyloid fibrils but having considered so allows comparison with other FRET pairs in the literature. The refractive index, *n*, was equal to 1.4 considering large concentrations of protein in the medium.

early-stage aggregates. Saturation of the binding sites with **AQui** during fibril growth may prevent the subsequent binding of **ThT**, and thus, the spectral changes were very small. In any case, the I_{570}/I_{480} ratio would allow aggregation kinetics to be followed (Fig. 3b), but with limited sensitivity.

In contrast, the pair **AQui → NBA** showed effective FRET within the amyloid aggregates, with much larger sensitivity (Fig. 3c and d). The spectral changes between the bands at 570 nm and 660 nm fostered the application of ratiometric imaging to detect amyloid aggregates at

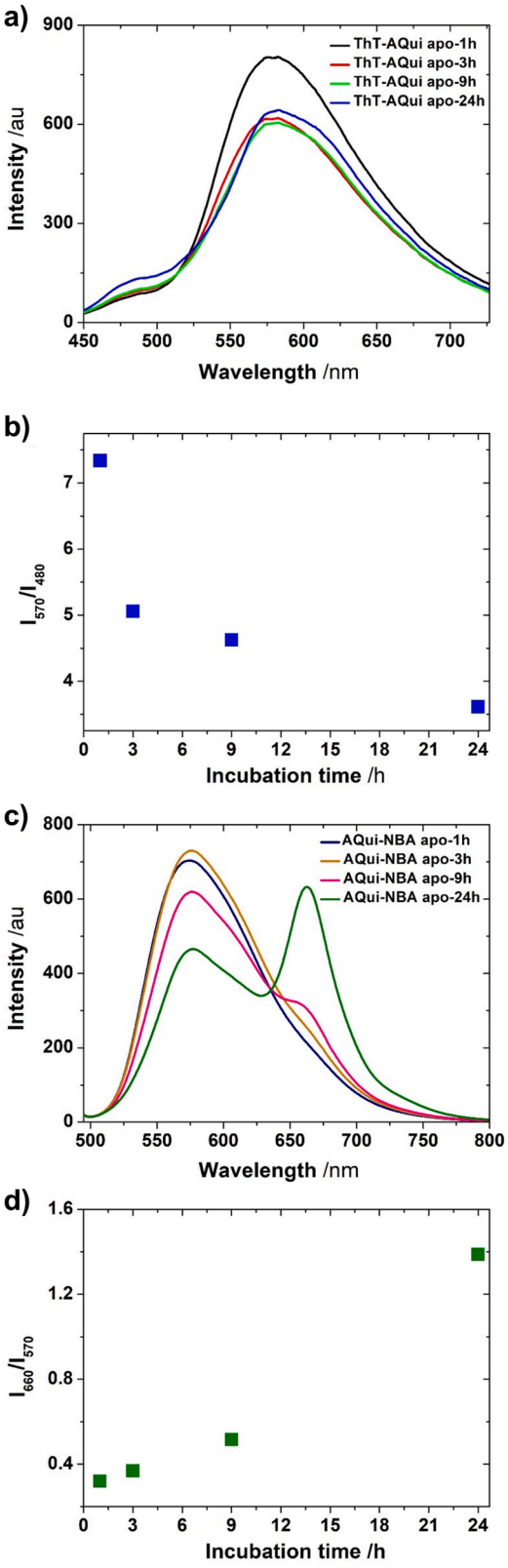

Fig. 3. a-b) Emission spectra (a) and the corresponding I_{570} / I_{480} intensity ratio (b) from apoferritin samples incubated at different times with the **ThT**:**AQui** pair (λ_{ex} = 440 nm). c-d) Emission spectra (c) and the corresponding I_{660} / I_{570} intensity ratio (d) from apoferritin samples incubated at different times with the **AQui**:**NBA** pair (λ_{ex} = 485 nm). Panel c) reprinted and minimally adapted from *Sens. Actuat. B-Chem.*, 350 (2022) 130882, A. Ruiz-Arias et al., 'A FRET pair for quantitative and superresolution imaging of amyloid fibril formation', with permission from Elsevier.

different stages. Likewise, the quenching of **AQui** due to FRET caused changes in the fluorescence lifetime that allowed us to use FLIM to follow the aggregation process. Full details of this study can be read in our previous work [9].

4. Conclusions

The appropriate combination of fluorophores may pave the way for novel studies of the critical stages in protein aggregation using advanced microscopy techniques, even for proteins of clinical relevance. Herein, we have provided additional results of the optimization of our proposed methodology.

CRediT authorship contribution statement

Álvaro Ruiz-Arias: Investigation, Formal analysis, Visualization, Writing – review & editing. **Rocío Jurado:** Investigation, Resources, Writing – review & editing. **Francisco Fueyo-González:** Investigation, Methodology, Resources, Writing – review & editing. **Rosario Herranz:** Methodology, Resources, Supervision, Writing – review & editing. **Natividad Gálvez:** Methodology, Resources, Supervision, Writing – review & editing. **Juan A. González-Vera:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition, Writing – original draft. **Angel Orte:** Conceptualization, Methodology, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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