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A Study for the Cause of Ferulic Acid-Induced Quenching of Tyrosine Fluorescence and Whether it is a Reliable Marker of Intermolecular Interactions or Not

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Abstract Intrinsic fluorescence of peptides and proteins is extensively used to monitor their specific interactions with several natural and synthetic molecules known to have wide-ranging beneficial or detrimental effects on health. A consequence of these interactions would be a significant decrease of the fluorescence emission intensity of Tyrosine (Tyr) and/or Tryptophan (Trp) residues in the protein due to structural rearrangements of proteic microenvironment. However fluorescence quenching can be also caused by "trivial" artefacts. In this study we examined the effect of Ferulic acid (FA) on Tyr fluorescence. FA is a natural antioxidant suggested to bind to and to modify the structural properties of several proteins thus altering their biological activities. Fluorescence spectroscopy experiments on Tyr and on proteins containing Tyr and no Trp like beta amyloid peptides and Insulin were performed. Our results suggest that Tyr fluorescence loss can mainly result from an inner filter effect rather than from specific interactions with FA.

Keywords Ferulic acid · Fluorescence quenching · Inner filter effect · Tyrosine

Abbreviations
FA   Ferulic acid
Tyr Tyrosine
Trp Tryptophan
Aβ   Beta Amyloid
Ins Insuline

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Introduction

The study of the fluorescence emission of biological molecules provides information on quite a number of their intrinsic properties as well as a variety of structural and functional features of macromolecules which they are associated with [1].

Intrinsic protein fluorescence, originating from their aromatic amino acids phenylalanine, tyrosine (Tyr), and tryptophan (Trp), is a sensitive technique that is nowadays exploited in studying the structural, physicochemical, and functional properties of proteins [2–5]. As phenylalanine is weakly fluorescent, its fluorescence is observed only in the absence of both tyrosine and tryptophan. Intramolecular or intermolecular aromatic and/or hydrophobic interactions can affect the fluorescence emission spectrum as well as the fluorescence quantum yield and lifetime of the aromatic amino acids. Consequently, fluorescence measurements can provide important information on the nature of the interactions in protein self assembly phenomena [5, 6] as well as in protein-ligand complexes [7, 8].

In the last years considerable interest has been devoted to the role of the major phenolic phytochemical components of fruits, vegetables, beverages and grains as dietary antioxidants. Many small phenolic compounds, in fact, are known to have wide-ranging effects on health for preventing several diseases. One of the major properties of phenolic compounds is their ability to interact with peptides and proteins thus modifying their structural properties and altering their biological activities and to act against amyloid-induced neurotoxicity [9, 10].

Among these compounds, with different chemical structures and characters, widespread in leaves, roots and especially fruits of the plants, of particular interest is ferulic acid

(FA), a powerful anti-oxidant present in fruit cell walls [11]. Several studies have been conducted using fluorescence spectroscopy techniques and the static quenching of protein intrinsic fluorescence in the presence of FA at high concentrations has been considered a proof of specific molecular interactions between FA and the proteic backbone [12, 13].

As clearly discussed by Marco van der Weert and Lorenzo Stella [14, 15], the use of this apparently simple methodology can lead to several errors in the data interpretation. In fact fluorescence quenching may be also due to phenomena unrelated to protein-ligand binding as collisional quenching and inner filter effect which have to be taken into account and corrected [1, 16]. More detailed critical analysis of the potential pitfalls is addressed in an interesting review of these Authors [14].

The inner filter effect is caused by the absorption of light at the excitation and emission wavelength by the molecules in solution. For example, when a compound is added to the protein solution and its optical density at excitation wavelength is high, the light reaching the protein intrinsic fluorophores is partly absorbed and shielded by it, so that emitted fluorescence is reduced. Moreover, if the compound absorption at emission wavelength is significant, less emitted radiations from the protein is detected (secondary absorption). It is important to note that it can be impossible to correct the inner filter effect, which, in some cases, represents the main cause of fluorescence quenching.

In this study we examined Tyr fluorescence intensity in order to investigate the effect of FA on neurotoxic peptides, beta amyloid (Aβ), each containing one Tyr residue and no tryptophan. As Tyr fluorescence emission spectrum is centred around the FA absorption maximum, the possibility of a decrease of Tyr fluorescence due to a "trivial" secondary absorption by FA rather than to a

quenching induced by the specific interaction between FA and Tyr residues in Aβ peptides was therefore hypothesized. To clarify this problem, two sets of control experiments were performed: one on Tyr - FA and another one using a protein that does not contain tryptophan, but only four Tyr residues, insulin (Ins). In both cases, FA concentration was varied whereas Tyr and Ins concentrations were kept constant. This work can be considered an experimental case-study contributing to better elucidate and apply the fluorescence methodology.

Materials and Methods

Beta Amyloid (Aβ) peptides (1–40) were purchased from Biopeptide Co. (San Diego, USA) and prepared according to Fezoui et al. [17].

Tyrosine from Fluka, ferulic acid and insulin from Sigma Chemical Co. were used without further purification. All samples were dissolved in 0.1 M NaCl water solutions.

Absorption and steady state fluorescence measurements were performed at room temperature with a Jasco V-550 Spectrophotometer and a HORIBA FluoroMax Luminescence Spectrometer, respectively.

Results and Discussion

Carrying on our study of the effects of small natural molecules on aggregation processes of neurotoxic peptides, Aβ, each containing one Tyr residue and no tryptophan [18, 19], we planned to exploit Tyr fluorescence emission to investigate the effect of FA on Aβ fibrillogenesis. In our experiments (Aβ/Tyr concentration=75 μM), a drastic effect of

Fig. 1 Fluorescence emission spectra (excitation wavelength= 270 nm) of 75 μM Aβ in NaCl solution with 330 μM FA (———), the same sample diluted 1:1 with NaCl solution (- - - - -) and 75 μM Aβ without FA(− − −)
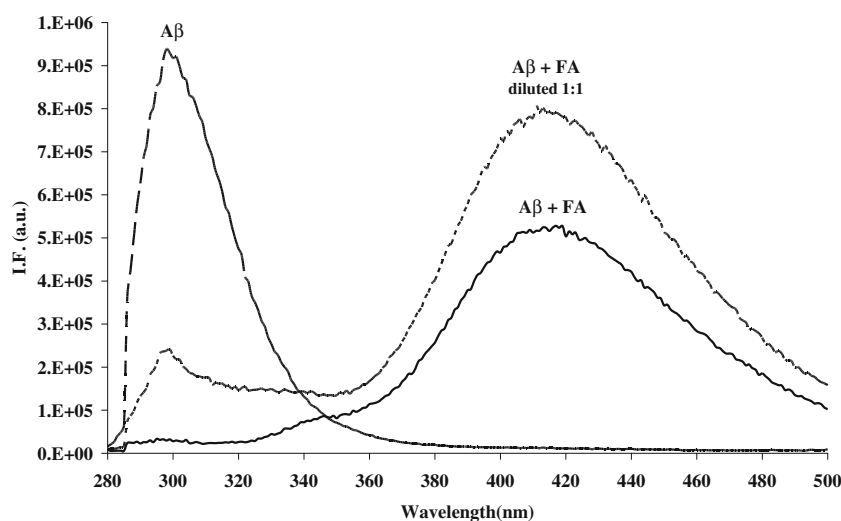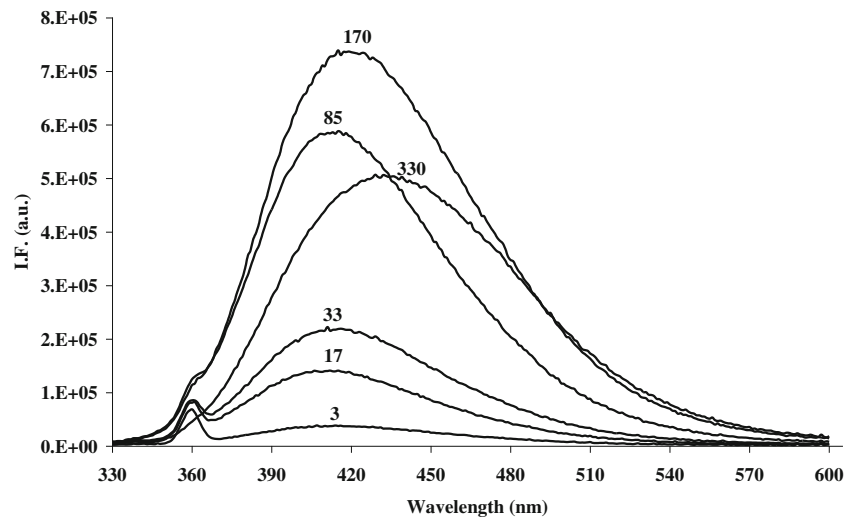
Fig. 2 Fluorescence emission spectra (excitation wavelength =320 nm) of FA in NaCl solution at different FA concentrations (0–330 μM)

FA on Tyr fluorescence was indeed observed (Fig. 1), in agreement with data reported in the literature [12, 13]. In order to be sure that the quenching was due to a real binding of FA to peptides, as suggested by Juan Kang and coauthors in the case of human serum albumin (HSA) [12], the sample was diluted 1:1 with NaCl solution and a increase of Tyr emission around 300 nm was observed. The ratio between the fluorescence intensity at 420 nm (FA emission maximum) and 300 nm (Tyr emission maximum) decreased from a value of 18.06 to 4.14 after dilution indicating that a "concentration quenching" effect could occur. To clarify this hypothesis the fluorescence spectra of FA in NaCl solution at different concentrations were measured. As observed in Fig. 2, the unequivocal "concentration quenching" of FA fluorescence at 420 nm, exciting at 320 nm, suggested the conceivable occurrence of an "inner filter effect" for FA

concentrations over 170 μM [1, 16]: the absorption at 320 nm was so high that less exciting light reached the centre of the sample and thus the fluorescence intensity of the fluorophore was reduced in a non-linear manner. Moreover, at such high concentrations self-absorption of the emitted fluorescence caused a significant red shift of the emission maximum (4–5 nm for 170 μM and 17 nm for 330 μM).

As Tyr fluorescence emission spectrum is centred around the FA absorption maximum (Fig. 3), a re-absorption of Tyr fluorescence around 300 nm by FA has to be taken into account. As a matter of fact, as shown in Fig. 4a, the fluorescence intensity emitted around 300 nm by 75 μM Tyr in NaCl solutions, exciting at 270 nm in the presence of different FA concentrations clearly decreased upon increasing FA concentration from 0 to 330 μM. Parallel to this effect on Tyr fluorescence, exciting at 320 nm a concentration-quenching

Fig. 3 Fluorescence emission spectrum of 75 μM Tyr in NaCl solution, exciting at 270 nm (*dotted line*); optical absorption spectrum of FA 33 μM in NaCl solution (*continuous line*)
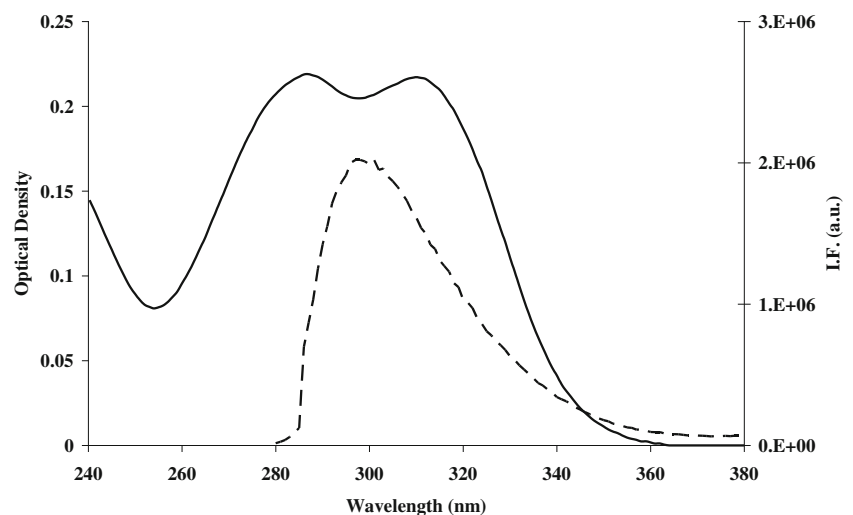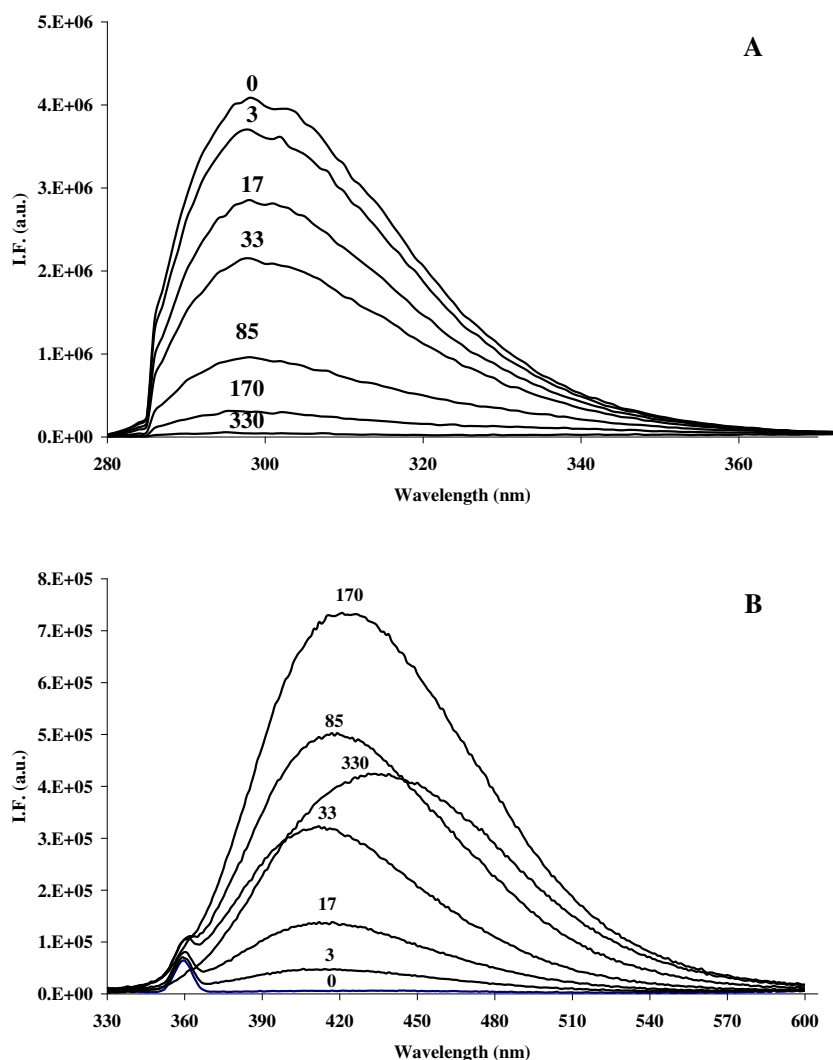
Fig. 4 Fluorescence emission spectra of 75 μM Tyr in NaCl solution in the presence of different FA concentrations (0–330 μM). **a** Tyr emission, excitation wavelength=270 nm; **b** FA emission, excitation wavelength=320 nm

of FA fluorescence around 420 nm was detected (Fig. 4b), associated with a red-shift of the emission maximum, as observed also in the case of pure FA solutions (cfr. Fig. 2).

It is worthwhile noting that the "pure" inner filter effect on FA fluorescence at 420 nm exciting at 320 nm declined for FA concentration below 170 μM (Figs. 2 and 4b), whereas the extent of Tyr fluorescence re-absorption by FA at 300 nm was significant at all the FA concentrations used (Fig. 4a). Moreover, because of FA high optical densities also at the excitation wavelength of Tyr fluorescence, 270 nm (cfr. Fig. 3), the emission signal at 300 nm could be further lowered by an "outer-filter effect" by FA that reduced the number of Tyr exciting photons.

As Aβ is an unstable short peptide prone to aggregate giving rise to fibrillar structures, in order to avoid possible spurious effects which may affect intrinsic Tyr fluorescence during fibrillogenesis, a quite different protein containing Tyr and no Trp residues, Insulin (Ins), was studied.

Fluorescence experiments on 19 μM Ins (also in this case Tyr concentration is 75 μM) in the presence of different FA concentrations were performed (Fig. 5). As in the case of the "simple" system Tyr – FA, in which "quenching" effects could not be attributed to any interaction of FA with any macromolecular structure, Tyr emission around 300 nm clearly decreased upon increasing FA concentration from 0 to 330 μM (Fig. 5a), and a concentration-quenching of FA fluorescence around 420 nm was observed associated with a red-shift of the emission maximum (Fig. 5b). Also for the Ins-FA system the "pure" inner filter effect on FA fluorescence at 420 nm dropped down for FA concentration below 170 μM (Fig. 5b), whereas re-absorption by FA of Tyr fluorescence took place at all FA concentrations (Fig. 5a).

To estimate the contribution of the inner filter effect to the fluorescence quenching of both Tyr and Ins, we corrected their fluorescence spectra in which FA

Fig. 5 Fluorescence emission spectra of 19 µM Ins (75 µM Tyr) in NaCl solution in the presence of different FA concentrations(0–330 µM). **a** Tyr emission, excitation wavelength=270 nm; **b** FA emission, excitation wavelength=320 nm
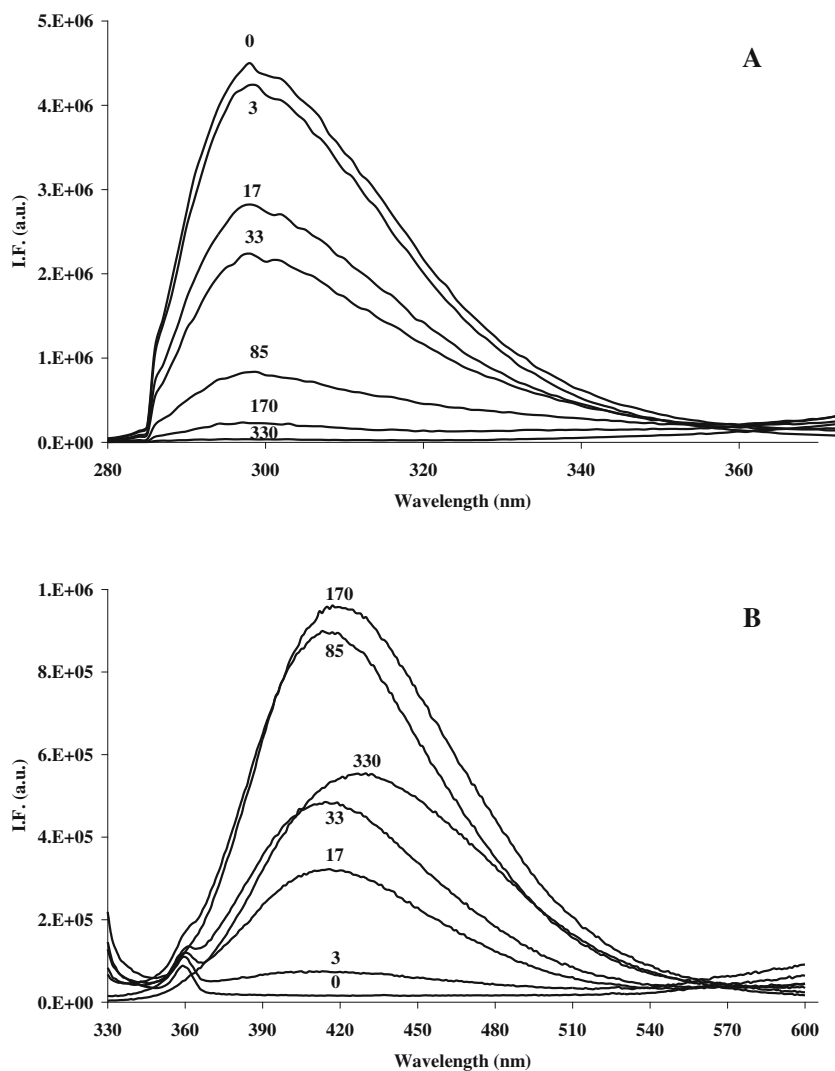
concentration was chosen such that its absorbance was quite low, 33 µM; according to the formula [16]:

$$F_m = F_c \cdot 10^{-\frac{A_{exc}d_{exc}}{2} - \frac{A_{em}d_{em}}{2}}$$

Where F_m is the measured fluorescence, F_c the correct fluorescence without inner filter effect, A_{exc} and A_{em} the absorption value at excitation and emission wavelength, respectively and d_{exc} and d_{em} the cuvette pathlength (cm) in the excitation and emission direction respectively. As it is possible to note in Fig. 6, the corrected spectra of both Tyr and Ins in the presence of 33 µM FA completely overlapped those one without FA indicating that inner filter effect played a central role in reducing fluorescence.

Altogether these results suggest that before concluding that Tyr fluorescence quenching is a reliable marker of molecular interactions of FA (and possibly other phenolic acids) with proteic backbones, other "trivial" effects have to be excluded or at least pondered. At high concentrations, in fact, FA high optical density at 320 nm is responsible of inner filter effects that decrease the intensity of the excitation at the point of observation thus reducing FA fluorescence. At the same time, secondary absorption by FA of Tyr fluorescence around 300 nm (see Fig. 2) can cause a remarkable drop of Tyr fluorescence. Significant overlap between the absorption and emission bands, moreover, may cause attenuation of the blue edge and a red-shift of the emission spectrum. These effects are inherent problems of a wide variety of fluorimetric procedures [20–23] when optical densities are relatively high (over 0.1, in general) and must therefore be appraised.

As the measurement of intrinsic Tyr fluorescence quenching of peptides and proteins is a biophysical methodology extensively used to monitor specific interactions of anti-oxidants like ferulic acid with proteic backbones, this work represents a warning in order to take into account and/or to avoid "trivial" artefacts.

Fig. 6 Fluorescence emission spectra (exciting at 270 nm) of **a** 75 μM Tyr in NaCl solution alone (0), with 33 μM FA (33) and corrected (33c); **b** 19 μM Ins (75 μM Tyr) in NaCl solution alone (0), with 33 μM FA (33) and corrected (33c)
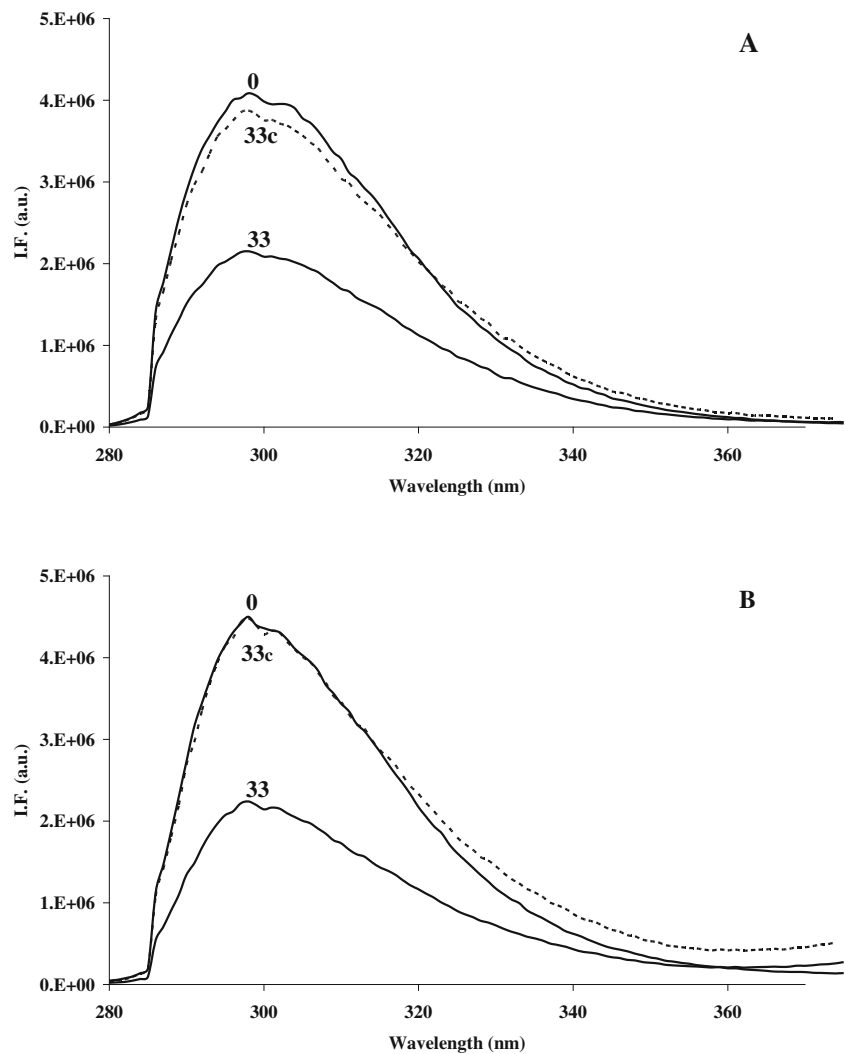
Conclusions

In conclusion, in the case of Aβ peptides which we are working on, and possibly other proteins and peptides, Tyr fluorescence loss is mainly due to a re-absorption of Tyr emission around 300 nm by FA rather than to a specific interaction of FA with the protein structure. In general, fluorescence quenching can well be the result of different interactions and processes, like energy transfer, molecular rearrangements, excited state reactions, collisional quenching and ground state complex formation, but can also be simply due to "trivial" artefacts that may affect the data and their analysis.

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