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The interaction of amino acids with macrocyclic pH probes of pseudopeptidic nature

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The fluorescence quenching, by a series of amino acids, of pseudopeptidic compounds acting as probes for cellular acidity has been investigated. It has been found that amino acids containing electron-rich aromatic side chains like Trp or Tyr, as well as Met quench the emission of the probes mainly *via* a collisional mechanism, with Stern-Volmer constants in the 7-43 M^{-1} range, while other amino acids such as His, Val or Phe did not cause deactivation of the fluorescence. Only a minor contribution of a static quenching due to the formation of ground-state complexes has been found for Trp and Tyr, with association constants in the 9-24 M^{-1} range. For these ground-state complexes, a comparison between the macrocyclic probes and an open chain analogue reveals the existence of a moderate macrocyclic effect due to the preorganization of the probes in the more rigid structure.

Introduction

Molecular fluorescent probes for imaging cellular acidity are well known tools for researchers in the biomedical areas.[1] Due to the relationships between intracellular pH and many physiological and pathological processes, they are invaluable resources in many studies using spectroscopic equipment such as confocal microscopy or flow cytometry.[2] But some drawbacks must be circumvented before a certain fluorescent probe is considered to be suitable for microscopy or flow cytometry studies. For instance, a fluorescent sensor needs enough solubility in water or the corresponding buffer to reach appropriate concentrations, but also needs good permeability to cross the cellular membrane. Another requisite is the resistance to photobleaching caused by the high intensity of the laser sources employed in the aforementioned optical techniques. The list of drawbacks to overcome could be even longer and will depend mainly on the final application of the indicator.[3] An often overlooked issue is the possibility of fluorescence quenching of the probe (excited state) by species present in the surrounding environment. This is especially true for probes with long emission lifetimes. For example, the group of Webb has described an important quenching effect on the emission of some of the very popular Alexa® fluorophores by some amino acids.[4] Specifically, it has been found that AL488, AL555 and AL594 are strongly quenched by tryptophan and, to a minor extent, by tyrosine, histidine and methionine. The quenching by Trp of the emission of several organic dyes used in biomedical studies has also been reported for fluorescein,[5,6] oxazine MR121,[5,7] rhodamine B,[5] TMR,[5] Cy5 [5] and some bodipy[5] and ATTO fluorochromes.[5,7-9] Recently, we reported a new family of fluorescent macrocyclic probes for the intracellular analysis of pH and used them successfully in mouse macrophages and human tumoral monocytes in combination with confocal microscopy and flow cytometry.[10] In the light of the former caveat, it is clear that a proper understanding of the full potential of this kind of fluorescent probes requires a detailed study of their behavior in the presence of species of biological relevance that could act as fluorescent quenchers, in particular amino acids. The initial step for this approach must be the photophysical characterization of the corresponding probes in the presence of individual amino acids under controlled conditions.

Taking this into account, it was considered important to analyze the effect of several amino acids (Trp, Tyr, His, Met, Phe, Val) on the emission of compounds **1**-**3** (Chart 1), selected as examples of the families of pseudopeptidic fluorescent probes above mentioned. For the three probes, the operational mechanism for detecting pH changes in the medium relies on the well-known intramolecular photoinduced electron transfer (PET) process, extensively used for the development of a great number of chemosensing systems.[11] Briefly, in neutral basic medium the lone pair of electrons in the free amine is able to participate in a PET process with the first excited singlet state of the neighboring anthracene moiety, making the emission from such fluorophore less likely. Upon acidification, protonation of the

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amine groups takes place, the PET process is no longer possible and, consequently, the emission from the anthracene unit is restored. This basic mechanism has been found practical in many circumstances but the effect of high concentrations of amino acids had not been studied so far for macrocyclic pseudopeptidic pH probes. As the effects on the quenching of the fluorescence of the anthracene moiety could be based on the formation of ground-state complexes of the amino acids with the pseudopeptidic probes, the selection of the probes (**1**-**3**) allowed to analyze to the importance, in this regard, of the presence of additional polar functionalities (i.e. comparing **1** and **2**) and the effect of the preorganization (i.e. comparing **1** and **3**, macrocyclic effect) which has shown to be particularly relevant in the supramolecular properties of this kind of pseudopeptides.[12,13]

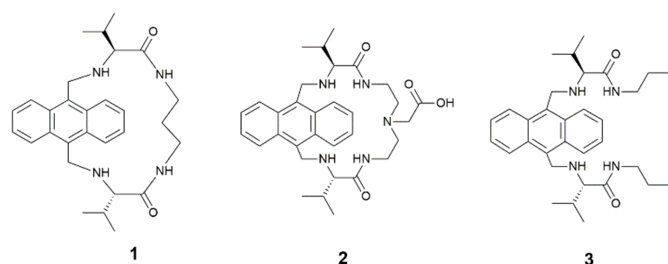

Chart 1. Chemical structure of pseudopeptidic fluorescencent probes **1**-**3**.

Results and discussion

The deactivation of the first excited singlet state of probes **1**-**3** can occur via dynamic and / or static mechanisms.[14] The static quenching implies a supramolecular association between the quencher and the emitter, whereas the dynamic one only involves a collisional deactivation of the excited state. Several mathematical models have been proposed to describe both types of quenching. The simplified expression shown in eqn (1) has been used satisfactorily by the group of Sauer to describe the fluorescence quenching of several dyes, including fluorescein, rhodamine B, TMR, Cy5, MR121 and ATTO dyes, by amino acids, including Trp, Tyr and Met.[5]

$$I_0/I = (1 + K_d \cdot [Q])(1 + K_a \cdot [Q]) \quad (1)$$

In eqn (1) the ratio of emission intensities in the absence (I_0) and in the presence (I) of a certain quencher Q is dependent on the quencher concentration ([Q]), while K_d is the dynamic quenching constant and K_a is the association constant for the formation of a ground state, non-emissive, complex. When only collisional quenching occurs, then K_a=0 and the expression can be reformulated as eqn (2).

$$I_0/I = \tau_0/\tau = 1 + K_d \cdot [Q] = 1 + k_q \cdot \tau_0 \cdot [Q] \quad (2)$$

In eqn. (2) the ratio of intensities coincides with the ratio of fluorescence lifetimes (τ_0/τ) and K_d can be equalized to $k_q \cdot \tau_0$, where k_q is the bimolecular quenching constant and τ_0 is the emission lifetime of the probe in the absence of quencher.

Molecule **1** is the prototype of the family of macrocyclic pH probes mentioned above,[10a,b] molecule **2** contains a pendant carboxylic acid group that modifies its basicity and water solubility,[10c] and molecule **3** is the open chain version,[10a,b] included in this study to evaluate the effect of a rigid structure *vs* a more flexible architecture as mentioned above.

The emission intensities of probes **1**-**3** were recorded in the presence of increasing concentrations of different amino acids. All the measurements were done at pH 3 in order to ensure that both amines in the molecule are protonated and that intramolecular PET is not operating during the quenching measurements (pK_a of all the probes is > 4.5). The emission intensity of **1**-**3** decreased upon addition of increasing amounts of Tyr, Met and specially Trp. No measurable effect was induced by Val or Phe. Surprisingly, the electron-rich His did not cause any measurable effect on the fluorescence of **1**-**3**, although a slight quenching by this amino acid has been reported for other dyes.[4] The fluorescence lifetimes of **1**-**3** were also measured in the presence of the above mentioned amino acids. A representative example of both intensity and lifetime measurements of **2** in the presence of Trp can be seen in Fig. 1.

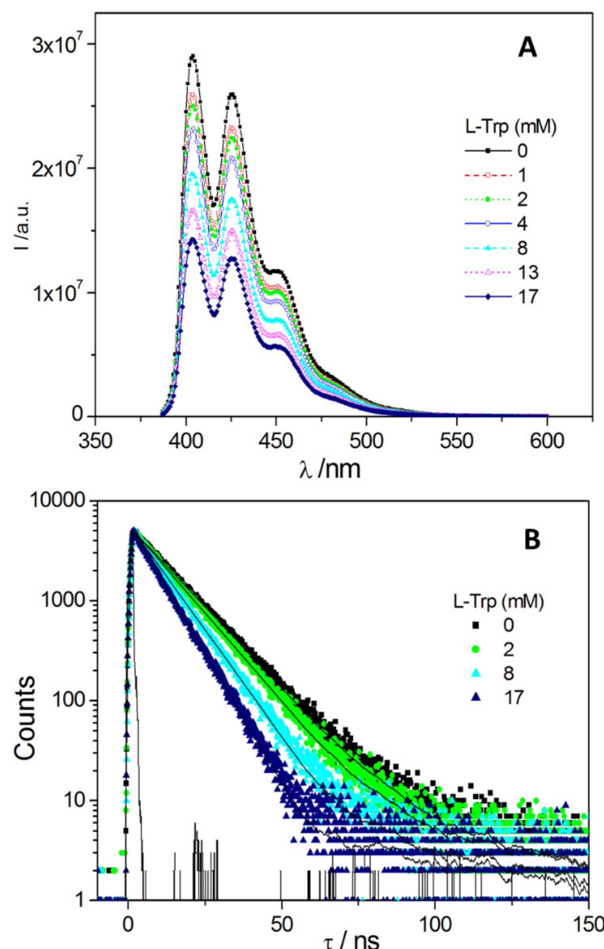

Fig. 1. Representative quenching of the fluorescence of **2** by L-Trp in aqueous solution at pH 3 (0.2% DMSO): A) steady state measurements (λ_{exc} = 374 nm); B) time-resolved measurements (λ_{exc}= 372 nm, λ_{em} = 420 nm).

A comparison of the Stern-Volmer plots for Trp using fluorescence intensity and lifetime data reveals the presence

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of different slopes (Fig. 2), suggesting that the observed quenching is a combination of static and dynamic processes. In addition, the data corresponding to the intensities cannot be fitted to a straight line: these data display a slight upward curvature, typical of a supramolecular association of emitter and quencher in the ground state.[14] On the contrary, the time-resolved data follow a clear linear tendency, which can be fitted to eqn (2), to afford the dynamic (collisional) contribution to the process. In the case of **1** quenched by L-Trp, K_d = 35.8 M^{-1}. Using this value, the association constant can be estimated by application of eqn (1), resulting in a value of K_a= 16.0 M^{-1}. Finally, the bimolecular quenching constant was calculated using the obtained value for τ_0 (13.0 ns) to yield k_q= 2.7×10^9 M^{-1}·s^{-1}. Application of this methodology to the set of the three probes **1**-**3** and the six amino acids (Trp, Tyr, Met, His, Phe, Val) gave a complete picture of the quenching effect of amino acids on this class of fluorescent probes. Table 1 gathers the data for the cases when a measurable quenching effect was detected (Trp, Tyr and Met). The rest of amino acids (His, Phe and Val) induced no change in the emission of **1**-**3** and hence the corresponding data will not be analyzed in detail.

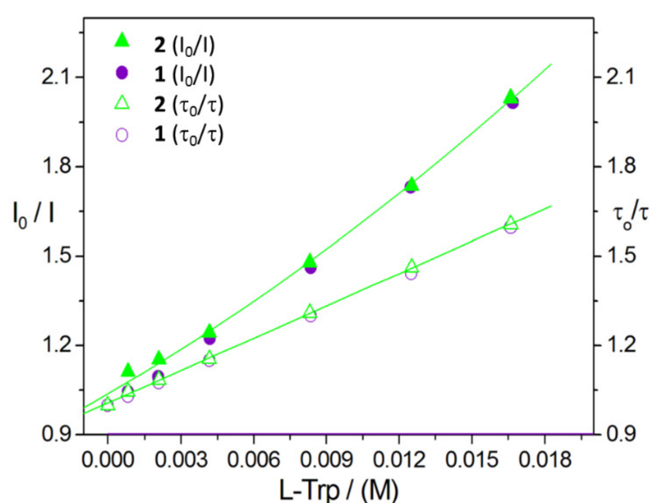

Fig. 2. Quenching of the fluorescence of **1**, and **2** by L-Trp in an aqueous solution at pH 3 (0.2 % DMSO).

Table 1. Dynamic Stern-Volmer and bimolecular dynamic quenching constants, association constants for the interaction of amino acids with **1**-**5**.

Quencher	Probe	K_d (M^{-1}) ^{a}	k_q (x10^9 M^{-1}s^{-1}) ^{a}	K_a (M^{-1}) ^{a}
Trp	**1**	35.8 (37.7)	2.7 (2.9)	16.0 (15.3)
	2	36.8 (39.7)	2.7 (3.1)	14.6 (11.4)
	3	42.6 (45.7)	3.2 (3.5)	9.4 (7.8)
	4^b	59.1 (74.1)	2.8 (3.6)	59.2 (40.7)
	5^b	74.0 (70.8)	3.3 (3.2)	32.1 (30.7)
Tyr	**1**	19.0	1.5	11.0
	2	17.8	1.4	24.4
	3	25.9	1.9	<1
Met	**1**	15.5	1.2	<1
	2	16.6	1.3	<1
	3	7.1	0.5	<1

a: in brackets D enantiomer; b: from the literature (ref 16), in H_2SO_4 1M

As it can be seen in Table 1 several conclusions can be drawn regarding the influence of the structure of the probes on the fluorescence quenching by amino acids. Trp is clearly the most notable quencher of all the studied amino acids, displaying the highest values of dynamic quenching constants (K_d between 35 and 43 M^{-1}). Tyr causes the quenching of the emission to a lower extent (K_d between 17 and 25 M^{-1}) and an even lower quenching is caused by Met (K_d between 7 and 16 M^{-1}). This could be explained taking into account that the most likely mechanism for the quenching is the intermolecular photoinduced electron transfer from the electron-rich pendant residue of the amino acid to the photoexcited anthracene fluorophore in **1**-**3**. The group of Previtali has studied carefully the fluorescence quenching of anthracene by tryptophan and other indole derivatives in several media.[15] The existence of charge transfer processes has been demonstrated unequivocally by means of transient absorption measurements as well as by application of the Rehm-Weller formalism. The bimolecular rate constants obtained by Previtali for anthracene and model compounds (indoles) and the values found for **1**-**3** and Trp lie within the same range. For instance, the reported value for the quenching of anthracene by indole is 3.3×10^9 M^{-1}s^{-1} (EtOH) and **1** is quenched by Trp at a similar rate (2.7×10^9 M^{-1}s^{-1}) in water at pH 3. This value implies that this process occurs very efficiently, approximately at one third of the diffusion controlled rate (ca. 9×10^9 M^{-1}s^{-1} according to the literature[4]). The participation of charge-transfer processes is also in agreement with the less effective quenching effect of Tyr and Met, with higher oxidation potentials than Trp. The E_{ox} for Trp is around 1 V, which makes this amino acid a common quencher of fluorescence.[16] As indicated in the introduction section, the Alexa® dye AL488 is collisionally quenched very efficiently by Trp (k_q=3.5×10^9 M^{-1}s^{-1}),[4] and many other dyes have been reported to experience an analogous influence. These include, for instance, Cy5 (k_q = 3.9×10^9 M^{-1}s^{-1}), TMR (k_q = 4.3×10^9 M^{-1} s^{-1}), fluorescein (k_q = 5.29×10^{9}M^{-1}s^{-1}) or MR121 (k_q = 4.0×10^9 M^{-1}s^{-1}).[5] Hence, the collisional quenching of **1**-**3** caused by Trp is not surprising and seems to represent a common feature for many fluorescent probes of this class. However, taking into account previous reports on the supramolecular interaction between carboxylic acids or amino acids and pseudopeptidic or related polynitrogenated receptors,[11f,13c,17,18] it was of higher interest to analyze in detail the influence of the formation of supramolecular non-fluorescent complexes between the probes and the amino acids, leading to static quenching, a much less studied phenomenon.

A general overview of data presented in Table 1 affords the general conclusion that in most cases the fluorescence quenching is dominated by the dynamic quenching phenomenon and that the association between probes and amino acids in the ground state to from a non-emissive complex usually provides a less relevant contribution. For instance, dynamic quenching by Trp is 3-4 times more effective (K_d ~ 35 - 42 M^{-1}) than the static one (K_a ~ 9 - 16 M^{-1}). A comparison of the macrocyclic probes **1** and **2** affords the

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conclusion that the presence of the additional amino and carboxylate functional moieties in **2**, which could modify the intermolecular interactions with the host, has a minor influence on the dynamic fluorescence quenching by Trp (< 3 %) and this structural change seems not to produce any significant enhancement in the association constant (14.6 M^{-1} *vs* 16.0 M^{-1}). A more important difference, however, can be noticed when **1** is compared to the related open chain derivative **3**. Although the dynamic quenching is higher for the open chain analogue (42.6 M^{-1} *vs* 35.8 M^{-1}), the association constant is about 35% lower for **3**. This seems to reflect the lower preorganization of the open chain derivative in comparison with the closed one. Examples of this macrocyclic effect have been reported in the literature for stronger complexes[17] but not for fluorescent pH probes to the best of our knowledge. For comparison purposes, Table 1 also includes the data obtained for a pair of pseudopeptidic acridine molecules described previously, the macrocycle **4** and the open chain analogue **5** (Chart 2).[18] In this case the K_a for the association of **4** with Trp is higher (59.2 M^{-1}) than the values described in this work for **1**-**3**. The non-macrocyclic compound **5** displays an association constant with Trp 46% lower (32.1 M^{-1}) than the cyclic counterpart **4**, thus confirming the importance of the preorganization of the receptor as described above for the association at the ground state.

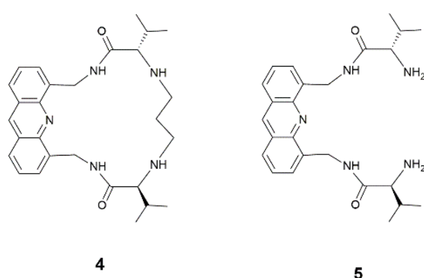

Chart 2. Chemical structure of pseudopeptidic acridine derivatives **4** and **5**.

The analysis of data in Table 1 for Tyr provides some remarkable observations. First of all, Tyr behaves as a less efficient dynamic quencher than Trp, with K_d values that are approximately one half of those for Trp and, as a consequence, the relative contribution of the association at the ground state is higher for **1** and **2**, being $K_a > K_d$ for **2**. The role of preorganization for defining the association at the ground state is even more pronounced in the case of Tyr. As it can be seen in Table 1, the interaction of **3** with Tyr is entirely collisional (up to the limit of the analysis, estimated at $K_a < 1$ M^{-1}). However, **1** associates to Tyr with $K_a = 11.0$ M^{-1} and, more remarkably, **2** associates to Tyr even stronger, with $K_a = 24.4$ M^{-1}. The enhanced interaction of Tyr with the multifunctional receptor **2** containing additional polar and H-bonding motifs, is in good agreement with the involvement of the phenolic side chain in specific supramolecular interactions recently observed in the selective recognition of Tyr peptides by pseudopeptidic cages.[19]

When Met is used as a quencher, the dynamic quenching is significantly reduced, but the most notable observation is that the association is negligible with the three pseudopeptides (K_a < 1 M^{-1}). This fact highlights the importance of aromatic-aromatic interactions, in supramolecular species formed by amino acids and peptides with receptors containing polycyclic aromatic regions, in particular in the case of Trp and Tyr.[17] The complexation between probes here presented can be considered as examples of low binding. Other cases of weak complexation have been reported for the association between Trp and organic dyes: for instance fluorescein (K_a 16 M^{-1}),[5] Alexa® 488 (15.1 M^{-1}),[4] RB (9.3 M^{-1}),[5] TMR (14.0 M^{-1}),[5] and ATTO590 (15.0 M^{-1})[5]. Only few cases of medium complexation are described for MR121 (96.0M^{-1}), ATTO655 (206.0 M^{-1}) and ATTO 680 (144 M^{-1}).[5]

Table 1 also shows the values corresponding to the quenching of the fluorescence of pseudopeptides **1** – **5** by the D-enantiomer of Trp. demonstrating a rather low degree of enantioselectivity in the association with the L-enantiomer.

As mentioned above, the trends detected for the dynamic quenching of a given receptor by the different amino acids, should reflect the respective values of their oxidation potentials. In this regard, although the exact values reported can slightly vary with the experimental method used for the determination, values of 1.00 V and 0.96 V *vs* NHE have been recently defined for Trp and Tyr respectively,[20] while values of 1.2-1.5 V have been estimated for Met.[21] However, it is important to note that those generally accepted values correspond to the ones obtained at pH 7.0 and 25ºC and oxidation potentials for amino acids experiment a significant dependence with pH, usually increasing for lower pH values, though this dependence shows a different slope for each amino acid.[20b,21a,22] This is particularly relevant for the Trp/Tyr couple as the relative order of their oxidation potentials is reversed at acidic pH values. In the acidic regions considered in our experimental design, it has been estimated that the values of the oxidation potentials for Trp and Tyr (pH 2.0) are 1.15 and 1.22 V respectively,[20b] which agrees well with the dynamic quenching results.

It is also of interest to analyze the relevance of the observed formation of ground-state fluorescent complexes from the point of view of the potential applicability of this family of compounds as probes for biological imaging. This can be estimated by calculating the degree of complexation of the probe under certain concentrations of biological relevance, taking into account the association constants. The concentration of Trp in plasma has been reported in the micromolar range, depending on the measured species (free Trp around 6 μM or total Trp around 30-100 μM).[23] A 49% complexation would be obtained for a solution being 10 μM in the probe and 100 μM in Trp only if K is at least 10^4 M^{-1}. If K is reduced to 10^3 M^{-1} then the complexation degree drops to 9%, and for K_a = 100 M^{-1} the complexation is limited to 1%. Therefore, it seems quite unlikely that constants like those here reported for the association between **1**-**3** and Trp (or Tyr) would cause any appreciable effect on the biological measurements carried out using those probes by means of

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confocal microscopy or flow cytometry. For a situation of very high concentration of Trp the binding seems to affect very slightly the pK_a at which the sensor switches from the *off* to the *on* state. Fig. 3 shows the titration curve of probe **1** (2 μM) in the presence of 20 mM Trp, a concentration at least 200 times higher than the normal levels of this amino acid in physiological media (curve b). For those concentrations the complexation degree is only 16%. As it can be seen in Fig. 3, the absolute intensity is half of the one for the titration curve without Trp (curve a), but the pK_a values calculated from such curves are almost identical (5.1 in the absence and 5.2 in the presence of Trp).

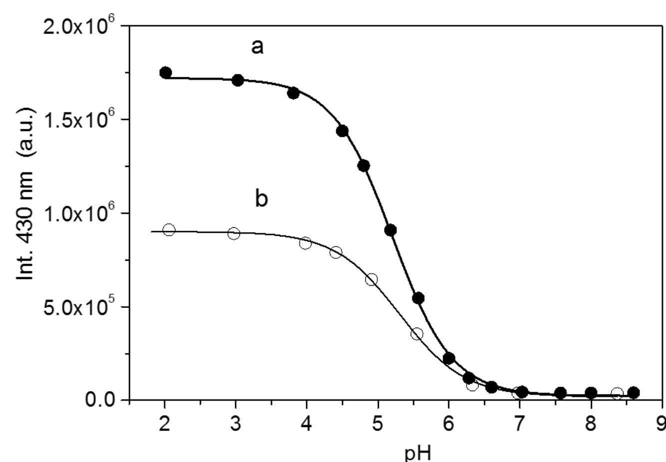

Fig. 3. Intensity of the maximum emission of **1** (2 μM) in aqueous solution (0.2% DMSO) as a function of the pH in the absence (a) and in the presence of 20 mM Trp (b); λ_{exc} = 374 nm.

Experimental

Chemicals and starting materials

L-tryptophan, L-tyrosine, L-methionine, L-histidine, L-valine, L-phenylalanine, L-glutamic acid, D-tryptophan, D-tyrosine, D-methionine, D-histidine, D-valine and D-phenylalanine, were purchased from Aldrich and used without further purification unless otherwise stated.

Steady-state fluorescence spectroscopy: Steady-state fluorescence spectra were recorded in a Spex Fluorog 3–11 equipped with a 450 W xenon lamp. Fluorescence spectra were recorded in the front face mode. All these samples were measured in aerated conditions otherwise stated.

Time-resolved fluorescence spectroscopy: Time-resolved fluorescence measurements were done with the technique of TCSPC in an IBH–5000U. Samples were excited with an IBH 372 nm NanoLED with a FWHM of 1.3 ns at repetition rate of 100 kHz. Data were fitted to the appropriate exponential model after deconvolution of the instrument response function by an iterative deconvolution technique, using the IBH DAS6 fluorescence decay analysis software, where reduced χ^2 and weighted residuals serve as parameters for goodness of fit. All the samples were measured in aerated conditions otherwise stated.

Sample preparation for photophysical characterization and quenching experiments: Stock solutions of compounds **1-3**, were prepared in DMSO at a 1 mM concentration. The samples of the compounds were diluted in H_2O to a final concentration of 2 μM. The pH was adjusted by adding aliquots of HCl and NaOH at different concentrations. Different concentrations of amino acids were prepared to investigate the fluorescence quenching of molecules **1**-**3**.

Conclusions

In summary, the fluorescence quenching of macrocyclic probes **1**-**3** by the amino acids Trp, Tyr, Met, His, Val and Phe has been investigated. It has been found that electron-rich amino acids like Trp, Tyr and Met quench the emission of probes mainly *via* a collisional mechanism, with Stern-Volmer constants in the 7 - 43 M^{-1} range, being Trp the species with a stronger quenching effect. Static quenching due to the formation of ground-state complexes has been found only for Trp and Tyr, with very low association constants, in the 9 - 24 M^{-1} range. This binding does not represent any drawback for the use of the studied probes in confocal microscopy or flow cytometry, given the typical concentrations of the probes and the amino acids (micromolar) used under normal conditions. A comparison between the macrocyclic probes **1**, **2** and the open chain analogue **3** reveals a stronger ground-state binding in the case of macrocyclic compound **1** and **2** due to the preorganization of these probes with a rigid structure (macrocyclic effect).

The study here reported is conducted on a minimalistic model system, i.e., it is a basic research on a group of fluorescent sensors under ideal conditions. It must be noted that a stronger quenching is not ruled out under real biological conditions due to the association of the dicationic probes (pH<4) to proteins with negative net charge and containing electron-rich amino acids. This scenario will deserve a comprehensive study in the future.

Acknowledgements

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