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A Multisensing Emissive Pyrimidine

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Abstract

Fluorescent nucleoside analogs, commonly used to explore nucleic acid dynamics, recognition and damage, frequently respond to a single environmental parameter. Here we address the development of chromophores that can simultaneously probe more than one environmental factor while having each associated with a unique spectroscopic signature. We demonstrate that an isomorphous emissive pyridine–modified 2-deoxy-uridine **1**, containing multiple sensory elements, responds to changes in acidity, viscosity, and polarity. Protonation of the pyridine moiety (pK_a 4.4) leads to enhanced emission ($\lambda_{em} = 388$ nm) and red-shifted absorption spectra ($\lambda_{abs} = 319$ nm), suggesting the formation of an intramolecular hydrogen bond with the neighboring pyrimidine carbonyl. This “locked” conformation can also be mimicked by increasing solvent viscosity resulting in a stark enhancement of emission quantum yield. Finally, increasing solvent polarity substantially impacts the chromophore’s Stokes shift [from 5.8×10^3 cm^{−1} at $E_T(30) = 36.4$ kcal/mol to 9.3×10^3 cm^{−1} at $E_T(30) = 63.1$ kcal/mol]. Opposite effect is seen for the impact of solvent polarity of the protonated form. The characteristic photophysical signature induced by each parameter facilitates the exploration of these environmental factors both individually and simultaneously.

Keywords

nucleosides; fluorescent probe; acidity; viscosity; polarity

Introduction

The combination of fluorescence spectroscopy and appropriately designed fluorescent probes is a powerful tool for the study of biomolecules and their interaction with their immediate surroundings and exogenous ligands.¹ A caveat associated with this technique is that any fluorescent probe, due to its distinct molecular structure, could perturb the biomolecular architecture and its assembly, potentially tainting the read-out. Of key importance for the design of “ideal” probes is therefore to minimize such potential structural and functional perturbations. This has led to the development of chromophores that show high structural resemblance to the native biomolecular building blocks they mimic, a feature commonly referred to as isomorphism.^{1,2}

Modified nucleosides can be considered isomorphous if they permit WC duplex formation, implying that their hydrogen bonding face stays intact, and the modified DNA helix remains largely unperturbed. To meet these requirements several classes of nucleoside-based probes, carrying modifications anchored to the 5 or the 5 and 6 positions of the pyrimidines, have

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been developed, placing the modification in the relatively accommodating major groove.¹ These nucleosides have been successfully implemented in detecting DNA and RNA lesions as well as in probing nucleic acids structure and ligand binding.^{1–9}

Although diverse motifs and applications have been reported,^{10–15} the responsiveness and utility of fluorescent nucleosides is fundamentally rooted in their sensitivity toward basic factors including environmental polarity, viscosity, and acidity. Most probes predominantly respond to one specific perturbation, which ideally results either in emission intensity changes or wavelength shifts.¹ Is it possible to simultaneously probe more than one environmental factor while having each associated with a unique spectroscopic signature? The first challenge, introducing sensitivity to distinct environmental changes, can potentially be addressed by logical structural and electronic modifications known to impart specific photophysical features. The second, ensuring that each perturbation yields a distinct photophysical response, is much more complex and, with the limited understanding of the correlation between molecular structure and spectroscopic properties, largely empirical. Herein we report on a multisensing fluorescent nucleoside analog with the ability to simultaneously probe acidity, viscosity, and polarity, where each environmental factor yields an individual spectral signature.

The modified 2'-deoxyuridine (**1**),¹⁶ is an emissive isomorphous pyrimidine comprised of multiple sensory elements (Figure 1). The pyridine nitrogen represents a distinct basic site,¹⁷ which is likely to impart sensitivity to pH.^{18–21} Furthermore, the protonated pyridyl nitrogen could intramolecularly hydrogen bond to the pyrimidine's O4, favoring co-planarization and enhanced conjugation of the two π -systems (Figure 1A), both likely to result in strong spectroscopic changes. Comparable rigidification could be non-covalently induced by locking the molecular rotor — the pyridyl linked with a single bond to the pyrimidine core — by increasing environmental viscosity while maintaining constant pH (Figure 1B).^{22,23} Finally, similarly to other 5-modified uridine analogs, the distinct electronic polarization of **1**, in either the ground or excited state, is likely to impart sensitivity to polarity (Figure 1C).^{2,7,23,24}

Results and Discussion

Sensing acidity

Both the absorption and emission spectra of **1** in aqueous buffers of different pHs reveal a remarkable sensitivity to pH (Figure 2A). A single isosbestic point at 295 nm in the ground state absorption spectra, shared by the high-energy absorption of the neutral form and the significantly red shifted absorption band of the protonated form (~ 30 nm or ~ 3600 cm^{-1}), suggests a two-state equilibrium. Excitation at this wavelength gives rise to a blue emission peaking at ~ 384 nm that shows little change in its maximum but does reveal a strong intensity loss upon increasing pH.²⁶ Plotting the normalized emission intensity versus pH gives a characteristic titration curve yielding a $\text{p}K_{\text{a}}$ value of 4.42 (standard error of the mean: 0.09) (Figure 2B).²⁷

The lower basicity compared to unmodified pyridine ($\text{p}K_{\text{a}} = 5.14$)^{18,19} can be attributed to the electron withdrawing effect of the electron deficient pyrimidine. Interestingly, it is similar to the value reported for 2-phenylpyridine ($\text{p}K_{\text{a}} = 4.5$),²⁸ suggesting the interplay of two opposing effects and slight stabilization of the protonated state of **1**. The latter is likely due to the postulated H-bonding (Figure 1A), which also promotes planarization and rigidification of the extended chromophore, explaining the red-shift of the absorption maximum and, at least in part, the observed fluorescence enhancement. The formation of an intramolecular hydrogen bond is also in accordance with modeling of **1** and its protonated form (Figure 3). In addition to a deviation from planarity between the two π -systems,

electrostatic repulsion causes the pyridine-nitrogen to point away from the pyrimidine carbonyl in the neutral form. The nucleobase conformation in the modeled structure (Figure 3B) resembles the one seen in the crystal structure (Figure 3A), adding to the confidence in the modeled protonated form (Figure 3C). A calculated distance of 1.72 Å between the proton and the pyrimidine carbonyl suggests a strong hydrogen bond. Moreover, an intramolecular hydrogen bond is also supported by almost complete planarization of the two π -systems, revealed by the small dihedral angle (Figure 3C). Crystal structures of a neutral and protonated molecule containing related pyridine fragments in proximity to carbonyl moieties have been reported.²⁹ Similar to our observations, the pyridine nitrogen points away from the carbonyl in the neutral form. Furthermore, the reported structure revealed a short distance between the proton and the carbonyl (1.80 Å) upon protonation which is in concurrence with our proposal for enhanced planarization between the two ring systems.²⁹

Sensing viscosity

The sensitivity of neutral **1** toward viscosity was studied in methanol ($\eta = 0.58$ cp), glycerol ($\eta = 1317$ cp) and binary mixtures thereof. Binary mixtures are commonly used to alter sample viscosity and explore free rotation in molecular rotors.^{22,23} Binary mixtures, however, might color the observations due to changes in solvent specific interactions. Hence, to fundamentally analyze the rotor behavior, temperature-induced viscosity changes are also employed.^{32–34} It is evident that viscosity changes only marginally influence the absorption spectra of **1** (Figure 4A). In contrast, increasing viscosity results in a stark enhancement of emission intensity.³⁵ The viscosity (η) of the methanol–glycerol mixtures can be calculated,³⁶ and subsequently used to construct a plot of $\log(\text{PL}_{\text{int}})$ vs. $\log \eta$ (Figure 4B), according to the Förster and Hoffmann relationship. The linear correlation obtained is characteristic of a chromophore with a molecular rotor element.³³

Sensing polarity

Sensitivity toward environmental polarity is typically reflected by significant changes in the chromophore's Stokes shifts ($\nu_{\text{abs}} - \nu_{\text{em}}$). The absorption and emission behavior of **1** were therefore studied in dioxane ($E_{\text{T}}(30) = 36.0$ kcal/mol), water ($E_{\text{T}}(30) = 63.1$ kcal/mol), and binary mixtures of the two (Figure 5A), an established approach to control sample polarity.³⁷ The Stokes shifts were calculated and plotted against the sample's $E_{\text{T}}(30)$ value,³⁸ a microenvironmental polarity parameter that typically shows good correlation with observed changes in Stokes shift.³⁷ The correlation obtained unambiguously reveals the sensitivity of **1** toward its environmental polarity (Figure 5B). The slope obtained, representing the sensitivity to polarity, is comparable to slopes obtained for established polarity sensitive chromophores such as dansyl, prodan, and a furan modified uridine.^{7,37}

Multisensing

The results presented above illustrate that **1** is capable of probing changes in acidity (Figure 2), viscosity (Figure 4), and polarity (Figure 5). Of special importance is to explore whether it is possible to 'isolate' a specific spectral feature or a combination of features that can be attributed to a change in one or more of the above environmental parameters. To this end, the absorption (Figure 6A) and emission (Figure 6B) characteristics of **1** were studied in three samples: methanol, glycerol, and a 1:1 ($v:v$) binary mixture of the two. These samples impose minor changes in polarity but major changes in viscosity. To explore the impact of protonation, the same samples were studied at neutral pH and under highly acidic conditions.³⁹ As seen before (Figure 4A), changing the viscosity under neutral conditions has minor impact on the absorption intensity of **1**, with a significant intensification of the emission signal while minimally affecting its maximum (Figure 6, and Table 1). Acidification of the same samples show a profound redshift (~ 30 nm, or ~ 3000 cm^{-1}) and

intensification ($\sim \times 2$) of the absorption maximum with a concomitant additional fluorescence enhancement and a red-shifted maximum (Figure 6 and Table 1). These observations are in agreement with the observations above showing the influence of the isolated parameters (Figures 2A and 4A).⁴⁰

Similarly to the analysis described above, concerning the influence of viscosity as a single parameter (Figure 4B), a double logarithmic plot of fluorescence intensity vs. sample viscosity demonstrates the difference of sensitivity to viscosity in a neutral and acidic media (Figure 7A). The slope obtained for neutral conditions matches our previous findings (Figure 4B), but is less steep for the acidified samples (Figure 7A), indicating loss of sensitivity for the latter. The sensitivity to polarity is revealed by a plot of the Stokes shift vs. the sample polarity (Figure 7B). Both lines reveal (show?) a steeper slope indicative of enhanced sensitivity compared to the slopes obtained when polarity was examined as an isolated parameter. It must be noted, however, that in the multisensing experiment (Figure 7B) the viscosity range used is large while the polarity window is rather small as compared to the range used above (Figure 5B). Regardless, responsiveness toward polarity is evident for both neutral and acidic conditions albeit with opposite slopes.⁴³

Both the viscosity and the pH study indicate that planarization of **1**, by either hampered rotation or protonation, respectively, results in fluorescence enhancement. Protonation in viscous media, however, has an added effect resulting in even higher fluorescence intensities (Figure 6B). This suggests that protonation reduces another non-radiative decay pathway, possibly one that results from photoinduced electron transfer.⁴¹ Most interestingly, both the neutral and the protonated forms of **1** show changes in Stokes shift ($\nu_{\text{abs}} - \nu_{\text{em}}$) reflecting inherent sensitivity to polarity, but with opposite spectral shifts. It is therefore possible to simultaneously visualize changes in polarity in concurrence with changes in viscosity and acidity, as summarized in Table 2.

Conclusion

Our results establish that the pyridine containing pyrimidine **1** is sensitive toward pH changes, as well as viscosity and polarity changes (Figures 2, 4, and 5) with a distinct spectral signature for each parameter. The spectroscopic features revealed by pH changes are in agreement with the postulated chromophore planarization due to intramolecular hydrogen bonding upon protonation of **1** (Figure 1). The emission intensification upon increasing viscosity reveals the molecular rotor behavior of **1**. Additional amplification of the fluorescence signal in acidic media of high viscosity indicates that protonation also diminishes non-radiative decay pathways. Finally, the sensitivity of **1** to pH and viscosity is accompanied by a distinct susceptibility to polarity. The conjugation of a pyridine moiety to the pyrimidine core thus results in a multi-sensory platform, which is amenable to additional structural modification with further tunability of the photophysical characteristics and their individual responsiveness to environmental factors.

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25. For clarity, the 2'-deoxy-D-ribose has been substituted for a methyl group in Figure 1C. HOMO and LUMO have been calculated using Spartan after semi-empirical (AM1) energy minimization.
26. Excitation spectra (see SI) for every sample pH reveal that emission originates primarily from the absorption band at ~325 nm, establishing that **1** becomes significantly fluorescent only upon protonation.
27. The tunability of the pK_a is illustrated with nucleoside **S2** (see SI), bearing a methyl group on the pyridyl moiety. From the almost identical spectroscopic response, a pK_a of 4.98 is determined in accordance with the mild donating character of the methyl group in ortho position of the pyridine nitrogen.
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30. Neutral and protonated **1** have been modeled using Spartan's molecular mechanics, MMFFaq force field calculations.
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39. To ensure protonation, a negligible volume of 4M HCl in dioxane is added to the cuvette to obtain a molar HCl concentration in an excess of ~18000 equivalents with respect to **1**.
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43. The slope under neutral conditions shows the same trend but poorly compares to the slope depicted in Figure 5B, which can be attributed to the small polarity window used in Figure 6B.

Figure 1. Proposed structural consequences for 5-(pyridin-2-yl)-2'-deoxy-uridine (**1**), of A) protonation, resulting in the putative intramolecular hydrogen bond, B) viscosity. C) HOMO and LUMO surfaces of **1** indicating potential responsiveness to polarity.²⁵

Figure 2.

A) Absorption (dashed) and emission (solid) spectra of **1** in 8 buffers ranging from pH 8.44 ($\Phi = 0.002$) to 2.18 ($\Phi = 0.019$) at a nucleoside concentration of $1.36 \times 10^{-5} \text{ M}$. Legend: highest pH (solid circles), lowest pH (open circles), and intermediate pH values (grey lines). The emission spectra were recorded after excitation at 295 nm (isosbestic point with an O.D. of ~ 0.1). B) Plot of normalized PL intensity of **1** vs. pH (solid circles representing three independent measurements) and a fit (grey line, $R^2 = 0.99353$). The dashed lines show the graphical determination of the averaged pK_a value, the number reflects the fit-based calculated value.

Figure 3.

A) Crystal structure of **1**, B) modeled structure of **1**, and C) modeled structure of protonated **1**.³⁰ For clarity, in all structures the 2'-deoxy-D-ribose has been replaced by a methyl group.³¹

Figure 4.

A) Absorption (dashed lines) and fluorescence (solid lines) spectra of **1** in methanol (open circles), glycerol (solid circles), and binary mixtures (grey lines); Emission spectra are recorded after excitation at 295 nm at 20°C. To prevent photo bleaching in the most viscous samples, a smaller wavelength range around the maximum is measured. B) $\log$ fluorescence intensity vs. $\log$ viscosity (solid circles) and a linearization of the data points (grey line, slope = 0.449, $R^2 = 0.99586$).

Figure 5.

A) Absorption (dashed lines) and fluorescence (solid lines) spectra of **1** in dioxane (black line, solid circles) and water (black line open circles) and binary mixtures (grey lines); emission spectra are recorded after excitation at 295 nm at 20°C. B) A plot of the Stokes shift vs. $E_T(30)$ (solid circles) and a linear fit (grey line, slope = 131, $R^2 = 0.96287$).

Figure 6. Absorption (A) and fluorescence (B) spectra of **1** in media of different viscosity. Key: methanol (open circles), glycerol (solid triangles) and a 50:50 v% mixture (solid circles), neutral and acidic samples are represented by grey and black lines, respectively. All emission spectra are recorded after excitation at 295 nm at 20°C.

Figure 7.

A) A plot of $\log (PL_{int})$ vs. $\log \eta$, and B) a plot of the Stokes shift vs. $E_T(30)$ of neutral (solid circles), acidic samples (open circles), and linear fits (grey lines).

Table 1Selected spectral properties of **1** under neutral and acidic conditions.^a

	Methanol	50:50	Glycerol
η	0.583	49.05	1317
E_T(30)	55.4	56.3	57.0
Neutral			
Abs_{max}	298.3	296.3	294.8
PL_{max}	363.8	367.5	375.0
Stokes shift	6037	6543	7259
PL_{int}	0.04	0.21	1.01
Acidic			
Abs_{max}	325.5	326.3	327.0
PL_{max}	399.0	389.0	380.2
Stokes shift	5644	4943	4282
PL_{int}	0.65	1.21	3.18

^aExperiments are performed in duplicate, emission maxima are corrected values for proper Stokes shift ($\nu_{\text{abs}} - \nu_{\text{em}}$) calculation.^{41,42} The values represent averages with an error <3.5% and <15% for the Stokes shift and PL_{int}, respectively. E_T(30) is reported in kcal/mol, η in cp, Abs_{max} and PL_{max} in nm, Stokes shift in cm⁻¹, and PL_{int} in $\times 10^6$ cps

Table 2

Qualitative spectral responses toward viscosity, acidity, and polarity.

Parameter	Absorption		Emission	
	Secondary	$\lambda_{\max}$ shifts	O.D. changes	$\lambda_{\max}$ changes intensity changes
Acidity	Low η ^[a]	large	large	small stark
	High η ^[a]	large	large	small stark ^[d]
Viscosity	Neutral pH ^[a]	negligible	small	small ^[e] stark
	Acidic pH ^[a]	negligible	small	moderate ^[e] stark
Polarity	Neutral pH ^[b]	increasing polarity → larger Stokes shift		
	Neutral pH ^[c]			
	Acidic pH ^[c]			

^[a] Small polarity variations.

^[b] Low viscosity.

^[c] Large viscosity changes.

^[d] Acidification induces an additional intensification to viscosity induced fluorescence enhancement.

^[e] Most likely reflecting small differences in sample polarity.