THE JOURNAL OF PHYSICAL CHEMISTRY A

Article

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Photophysics of a Live-Cell-Marker, Red Silicon-Substituted Xanthene Dye

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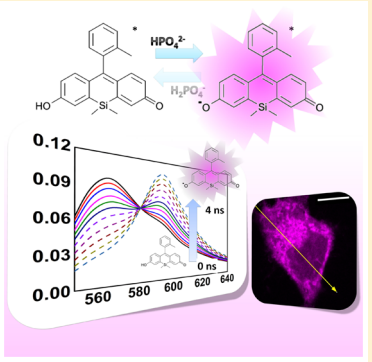
Supporting Information

ABSTRACT: Dyes with near-red emission are of great interest because of their undoubted advantages for use as probes in living cells. In-depth knowledge of their photophysics is essential for employment of such dyes. In this article, the photophysical behavior of a new silicon-substituted xanthene, 7-hydroxy-5,5-dimethyl-10-(*o*-tolyl)-dibenzo[*b*,*e*]silin-3(5*H*)-one (**2-Me TM**), was explored by means absorption, steady-state, and time-resolved fluorescence. First, the near-neutral pH, ground-state acidity constant of the dye, $pK_{N\text{-}A}$, was determined by absorbance and steady-state fluorescence at very low buffer concentrations. Next, we determined whether the addition of phosphate buffer promoted the excited-state proton-transfer (ESPT) reaction among the neutral and anion form of **2-Me TM** in aqueous solutions at near-neutral pH. For this analysis, both the steady-state fluorescence method and time-resolved emission spectroscopy (TRES) were employed. The TRES experiments demonstrated a remarkably favored conversion of the neutral form to the anion form. Then, the values of the excited-state rate constants were determined by global analysis of the fluorescence decay traces recorded as a function of pH, and buffer concentration. The revealed kinetic parameters were consistent with the TRES results, exhibiting a higher rate constant for deprotonation than for protonation, which implies an unusual low value of the excited-state acidity constant $pK^*_{N\text{-}A}$ and therefore an enhanced photoacid behavior of the neutral form. Finally, we determined whether **2-Me TM** could be used as a sensor inside live cells by measuring the intensity profile of the probe in different cellular compartments of HeLa 229 cells.



1. INTRODUCTION

The family of fluorescein derivatives has been widely employed for use as fluorescent probes because of their high water solubility and favorable spectral parameters, including the high molar extinction coefficient of the dianion form at a wavelength of 490 nm, which matches the 488 nm spectral line of the argon ion laser, and their high fluorescence quantum yield.[1,2] Fluorescein in aqueous solution can exist in four different prototropic forms, depending on the pH. At near-neutral pH values, the two predominant forms are the dianion and monoanion, each with different molar extinction coefficients, and fluorescence quantum yields.[3] The spectral changes associated with the monoanion-dianion transition in fluorescein, and its derivative 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, BCECF, have been employed for measuring pH values in biological systems.[4−6]

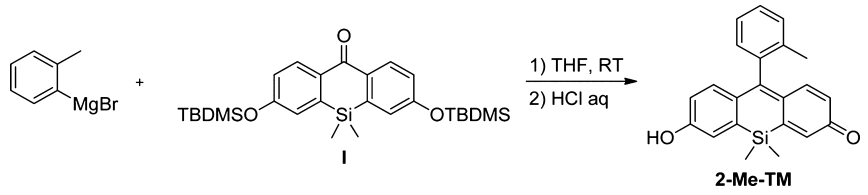
Various fluorescein derivatives, in which methyl, methoxy, or *tert*-butyl groups have been introduced into the benzene moiety, have been synthesized in the past decade.[7,8] These dyes have the additional advantage of providing a single anion species in aqueous solution, and some behave as on/off fluorescent probes. Among the latter, 9-[1-(2-methyl-4-methoxyphenyl)]-6-hydroxy-3*H*-xanthen-3-one (**2-Me-4-OMe TG**) and 9-[1-(4-*tert*-butyl-2-methoxyphenyl)]-6-hydroxy-3*H*-xanthen-3-one (Granada Green, **GG**), which both emit green fluorescence, have been used as fluorescent indicators of phosphate ions in living cells in fluorescence lifetime imaging microscopy (FLIM).[9,10] Although the FLIM methodology is adequate for removing the contribution of the autofluorescence of cells and biological fluids, it is also interesting to explore dyes that emit in other color regions for their use in bioimaging. Since the first red-emission silaanthracene fluorophore (TMDHS) was synthesized by Fu et al.,[11] fluorescent probes both silicon-substituted rhodamines[12,13] and silicon-substituted fluoresceins[14−16] have been synthesized and developed as labeling and bioimaging reagents. The interested reader can be directed to a topical minireview covering recent progress on silicon-substituted xanthene dyes.[17] In this context, one significant dye silicon-substituted xanthene, the 7-hydroxy-5,5-dimethyl-10-(*o*-tolyl)dibenzo[*b*,*e*]silin-3(5*H*)-one (**2-Me TM**)[18] was recently synthesized. This dye exhibits absorption and emission wavelengths that are approximately 90 nm longer than those of fluorescein derivatives.

As is recognized, the use of fluorescent probes as these silicon-substituted xanthene derivatives as sensors, requires a full understanding of the complex photophysics of the dyes. It

Received: August 13, 2015
Revised: October 13, 2015

DOI: 10.1021/acs.jpca.5b07898
J. Phys. Chem. A XXXX, XXX, XXX−XXX

Scheme 1. Synthesis of the 2-Me TM Derivative



has been well established that fluorescein derivatives have the ability to undergo ESPT reactions, thereby rapidly interconverting between two protonated forms in the presence of an appropriate proton donor−acceptor. In particular, the phosphate buffer acts as the right proton donor−acceptor, stimulating the ESPT reaction in fluorescein[2] or BCECF,[6] whereas a lower-pK_a buffer, as is acetate buffer, stimulates ESPT reactions in 2′,7′-difluorofluorescein (Oregon Green 488, **OG488**).[19,20] Because the presence of the ESPT reactions influences the fluorescent decay from excited prototropic forms, exhibiting complex kinetics, and decay times that depend on both the pH and buffer concentration, it is necessary to know in depth the photophysics of these new silicon-substituted xanthene derivatives in what is referred to as the excited-state dynamics of the molecular forms present at near physiological pH values. Nevertheless, the presence of ESPT reactions could be advantageous because in these reactions, the decay times are tuned by the phosphate buffer concentration at a determined pH, thus sensing the phosphate concentration of either a complex solution or living cells.[9,10]

The time-correlated single-photon counting (TCSPC)[21] technique supplies fluorescence data from which both the time-resolved emission spectra (TRES) and other relevant photophysical parameters of the system can be obtained. TRES are fluorescence spectra obtained at distinct times in the course of the fluorescence decay. To perform TRES and determine the appropriate relaxation model to describe the photophysical system, a tridimensional fluorescence decay surface that represents the fluorescence intensity at all wavelengths and times during the fluorescence decay is measured. Thus, the reconstructed emission is defined in terms of both the spectral and time resolution.[22] Once it is demonstrated that the spectral family corresponds to a system in two excited states, a suitable model of ESPT reaction in the company of a proton donor/acceptor should be proposed to determine the significant photophysical parameters.[2] To resolve the model, a multidimensional fluorescence decay data surface under various excitation (λ_{ex}) and emission (λ_{em}) wavelengths, buffer concentration, C^B, and pH is measured. Finally, to achieve an accurate estimation of the parameters, the fluorescence decay traces should be analyzed in terms of their decay times (τ_i) and associated amplitudes (p_i) by global analysis, in which the τ_i values are linked parameters in all the decay traces collected at various excitation and emission wavelengths from the same sample.[23] A full study of the variation of the decay times with pH and buffer concentration enables determination of the underlying rate constants (k_{ij}) that define the excited-state kinetics.[24] Once the constants are known, both the steady-state fluorescence intensity and fluorescence decay time values could be calculated at any pH, and buffer concentration.[2,20]

In this paper, aqueous solutions of **2-Me TM** were investigated via absorption, steady-state and time-resolved emission spectroscopy. The ground-state equilibrium between the neutral and anionic forms of **2-Me TM** was examined, and the p$K_{N\text{-}A}$ was measured by means absorption and fluorometric titrations. Next, both steady-state and TRES methodologies were employed to reveal the buffer-mediated two-excited-state photophysics system. Finally, the pertinent kinetic ESPT reaction model and its dynamics were resolved in detail by collecting the fluorescence decay traces, and determining the fundamental kinetic rate constants that describe the dynamic behavior of the system. As a final point, the applicability of **2-Me TM** for bioimaging was determined using HeLa 229 cells by collecting the emitted near-red signal with a fluorescence microscope system.

2. EXPERIMENTAL METHODS

2.1. Materials and Preparation of Solutions. For the preparation of phosphate buffer solutions, $NaH_2PO_4{\cdot}H_2O$ and $Na_2HPO_4{\cdot}7H_2O$ (both Fluka, puriss p.a.) were used. To adjust the pH of the unbuffered aqueous solutions, NaOH 0.01 M (platelets, Sigma-Aldrich, Spain) and $HClO_4$ 0.01 M (Sigma-Aldrich) both spectroscopic grade were employed. Milli-Q water was used as solvent. The chemicals were used as received.

2-Me TM was synthesized with a good yield (85%) by the addition of commercially available *o*-tolylmagnesium bromide to 3,7-bis((*tert*-butyldimethylsilyl)oxy)-5,5-dimethyldibenzo-[*b*,*e*]silin-10(5*H*)-one **I** and subsequent dehydration with aqueous hydrochloric acid (Scheme 1). The corresponding TBDMS-derivative **I** was prepared by following the protocol described by Best et al.[25] The purity of **2-Me TM** was confirmed by ^{1}H NMR and ^{13}C NMR (data and figures in the Supporting Information).

A stock solution of **2-Me TM** (10^{-4} M) in diluted NaOH was prepared. From this stock solution, the required concentrations of **2-Me TM** at the suitable pH and phosphate concentrations were prepared. For fluorescence measurements, solutions with absorbance at λ_{ex} less than 0.1were employed. All the solutions were freshly prepared.

For the pK_a calculation using the absorbance or steady-state fluorescence experiments, 11 solutions of **2-Me TM** (5×10^{-5} M or 5×10^{-6} M, respectively) in 0.05 M phosphate buffer with pH values in the range 6.18−10.14 were prepared. For the TRES experiments, eight solutions of **2-Me TM** (5×10^{-6} M) in 0.7 M phosphate buffer with pH values in the range 3.97−8.72 were prepared, and each solution was excited at either 485 or 530 nm to preferentially excite the neutral or anion form. The decay traces from each solution at each excitation wavelength were registered at each 5 nm in the emission wavelength range from 550 to 640 nm. To construct the multidimensional fluorescence decay surface to determine the fundamental kinetic parameters, 11 unbuffered solutions and 44 phosphate buffered solutions (C^B = 0.05, 0.15, 0.30, and 0.6 M), all in the pH range 4.0−9.5, were prepared.

HeLa 229 (ECACC 86090201) cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/mL penicillin, and 0.1 μg/mL streptomycin in a humidified 5% CO_2

DOI: 10.1021/acs.jpca.5b07898
J. Phys. Chem. A XXXX, XXX, XXX−XXX

incubator. For the fluorescence imaging microscopy experiments, HeLa 229 cells were seeded onto 20 mm diameter coverslips in 6-well plates, then transferred to the MicroTime 200 fluorescence microscope system, and washed with Krebs–Ringer–phosphate (KRP) buffer before addition of the working solutions. The KRP buffer was prepared at pH 7.4 and contained the following: NaCl 118 mM, KCl 5 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1.2 mM, KH_2PO_4 1.2 mM, and Hepes 30 mM (all chemicals from Sigma-Aldrich). The working solutions were prepared using KRP buffer and **2-Me TM** (10^{-7} M).

2.2. Instrumentation. Absorption and steady-state fluorescence spectra were recorded using a PerkinElmer Lambda 650 UV/vis spectrophotometer, and a JASCO FP-6500 spectrofluorometer, respectively, both equipped with a temperature controller. All measurements were recorded at 20 °C using 10 × 10 mm cuvettes. The pH of the solutions was measured immediately after recording each spectrum.

Fluorescence decay traces were recorded via the time-correlated single photon counting (TCSPC)[21,26] method using a FluoTime 200 fluorometer (PicoQuant GmbH, Germany). The excitation source consisted of either an LDH-485 or LDH-530 pulsed laser (PicoQuant) with 88 ps pulse width. The rate of pulse repetition was 20 MHz. Both detection polarizer set at the magic angle and detection monochromator are located before the photomultiplier detector. The fluorescence decay traces were collected in 1320 channels with a time increment of 36 ps/channel. The instrument response functions (using LUDOX as scatter) and sample decays were recorded until they reached 2 × 10^4 counts on the peak channel. In the TRES experiments, when the count rate was very low in the extreme portion of the emission wavelength range chosen (550 to 640 nm), the decay traces were collected for 5 min to achieve a count number sufficient for analysis.

Confocal fluorescence microscopy was carried out on a MicroTime 200 instrument (PicoQuant), using the aforementioned LDH-530 pulsed laser as the excitation source. The laser was directed into the specimen using a dichroic mirror (Z532RDC, Chroma) and the oil immersion objective (1.4 NA, 100×) of an inverted microscope (IX-71, Olympus). The emitted fluorescence was filtered by a cutoff filter (550LP, AHF/Chroma) and focused onto a 75 μm pinhole. The detection filter was a FB600-40 bandpass filter (Thorlabs), and the fluorescence was detected using a single-photon avalanche diode (SPCM-AQR 14, PerkinElmer). Photon counting, imaging reconstruction, and data acquisition were performed with a TimeHarp 200 TCSPC module (PicoQuant). Raster scanned images were recorded with a 512 × 512 pixel resolution. The fluorescence images were analyzed using an open-source platform for biological-image analysis.[27]

2.3. Fluorescence Decay Traces Analysis. The fluorescence decay traces for TRES were individually analyzed using exponential models by means of the FluoFit software package (PicoQuant). The impulse response curves, collected either with the same number of counts at the peak or in a discrete period of time when the emission intensity was low, were corrected and then normalized to a steady-state emission spectrum recorded on the same instrument.

The decay traces of samples at the same pH, buffer concentration, and excitation wavelength were globally analyzed using the FluoFit package. The decay times were linked parameters, whereas the pre-exponential factors were kept as local adjustable parameters.

3. RESULTS AND DISCUSSION

3.1. Absorption Measurements and Ground-State Equilibria of 2-Me TM. The visible absorption spectra of aqueous solutions of **2-Me TM** (5 × 10^{-5} M) in a pH range between −0.40 and +10.14 were recorded at very low ionic strength (0.01 M phosphate buffer). These spectra exhibited pH-induced transitions due to ground-state proton-transfer reactions dictated by the ground-state pK_a values. Figure 1A

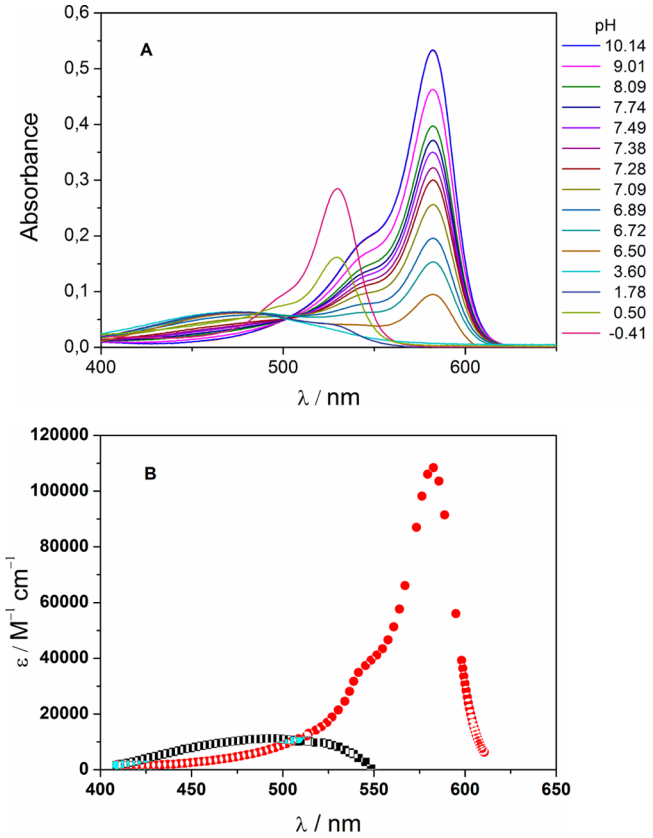


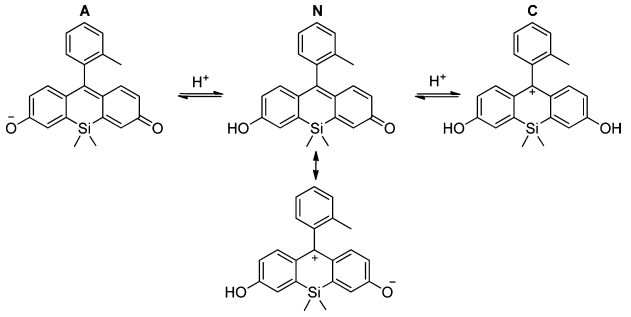
Figure 1. (A) Absorption spectra from 5 × 10^{-6} M aqueous solutions of **2-Me TM** in the pH range between −0.4 and +10.14. (B) Recovered molar absorption coefficients versus wavelength for the neutral (black) and anionic (red) form of **2-Me TM**.

shows two isosbestic points, one occurring at approximately 505 nm with moderate acid or basic pH values, and the other, at low pH values, arises at 485 nm. The similarity of the chemical structure of **2-Me TM** to those of 2-OMe-5-Me TG and 2-Me-4-OMe TG[28,29] and the presence of two isosbestic points suggest that the aqueous solutions of **2-Me TM** present three absorbing protonated species, the cation (C), neutral (N), and anion (A), and two pK_a describing the proton transfers (Scheme 2). The N and A forms are the only relevant species at near-neutral pH values.

As shown in Figure 1A, at a basic pH, the spectrum is basically composed of a band centered at 585 nm and a shoulder at approximately 550 nm, which is identical to the spectrum observed in NaOH (1 M). With decreasing pH, the shoulder disappears and the band that peaks at 585 nm is blue-shifted, exhibiting a little pronounced maximum and a very broad profile. At a pH of approximately 4.00, the spectrum shape is a wide band with a diffuse maximum at approximately 475 nm. The absorption spectra at pH values ranging between 6.50 and 10.14 exhibit only one transition at near-neutral pH,

DOI: 10.1021/acs.jpca.5b07898
J. Phys. Chem. A XXXX, XXX, XXX−XXX

Scheme 2. Prototropic Forms of 2-Me TM: A (anion), N (neutral), and C (cation)[a]



[a]The neutral form is represented by two resonance structures.

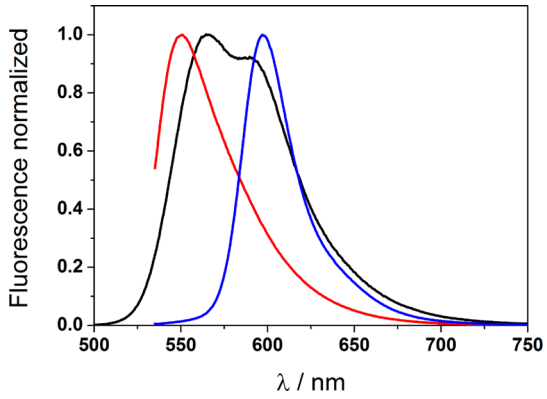


Figure 2. Normalized fluorescence spectral profiles of the three prototropic forms of **2-Me TM**: anion (blue), neutral (black), and cation (red).

which is characterized by the ground-state constant $K_{N\text{-}A}$. The recovered spectra in this pH range, and their appointment of them to both anion and neutral prototropic forms, are concordant with those recently reported.[15] At pH values of less than 6.50, in the pH range −0.4 to +3.60, a wide band with a diffuse maximum at approximately 475 nm was red-shifted as a new band arises, peaking at 530 nm. In that pH range, another isosbestic point at 485 nm can be obviously distinguished, thus indicating that another species is involved in this other acid−base equilibrium, characterized by the ground-state constant K_{C-N}. The absorption spectrum of the cation form was confirmed using a 2.0 M $HClO_4$ aqueous solution, and it exhibited a peak at 530 nm. Thus, the experimental visible absorption spectra of **2-Me TM** aqueous solutions in the pH range between −0.4 and +10.14 is consistent with the ionic equilibria between the prototopic forms represented in Scheme 2.

The absorbance of the aqueous solutions of **2-Me TM** depends on the pH according to the acid−base equilibrium theory and Beer's law. Because the only pK_a of interest for biological applications is that which the dye presents at near−neutral pH, the selected absorption spectra at pH values ranging between 6.50 and 10.14 were analyzed. The nonlinear global fitting of the complete surface of A vs pH and λ_{abs} to Beer's law and the equations of the acid−base equilibria (Supporting Information eqs S1−S3) allows us to determinate the molar absorption coefficients $\varepsilon_i(\lambda_{abs})$ and pK_{N-A}.[3] In the fitting, the pK_{N-A} was linked over the whole surface, whereas $\varepsilon_i(\lambda_{abs})$ was a locally adjustable parameter at each wavelength for each species. The absorbance data surface was composed of 11 pH values and 224 λ_{abs} values between 400 and 624 nm at each pH. Beer's law for two protonated species provided the best fit to the experimental absorption data. Plots of the some individual absorbance values vs pH at different wavelengths along with the curves generated from the fitting are shown in Figure S3 (Supporting Information). In the fitting process, the recovered parameters were independent of the values initially assigned to these parameters. Figure 1B shows the estimated values of the molar absorption coefficients of the neutral (ε_N) and anionic forms (ε_A) at low phosphate buffer concentration (0.01 M). The recovered pK_{N-A} value was 7.31 ± 0.03.

3.2. Steady-State Emission Spectra. In Figure 2, the three different emission spectral profiles that correspond to the three prototropic forms are shown. In contrast with the other xanthene dye, the cation exhibited no photoacid behavior (Figure S4).[20,29] Nevertheless, the spectral profile of the cation form shown in Figure 2 was recorded in a 2.0 M $HClO_4$ aqueous solution.

The ground-state acid−base equilibrium constants can also be obtained by fluorometric titrations whenever there is constant intensity of excitation, lower absorbance of 0.100, and negligible rates of ESPT reactions. Consequently, the steady-state fluorescence spectra of **2-Me TM** solutions in the pH range from 6.18 to 9.00 were recorded at two excitation wavelengths (485 and 580 nm). To minimize the rate of possible ESPT reactions mediated by the presence of a proton donor/acceptor, we used phosphate (0.005 M) as a buffer.[30] Only the A and N emission spectral shapes were noticed in that pH range. The recovered pK_{N-A} value among the neutral and anion species of **2-Me TM** was 7.41 ± 0.02. Examples of the emission spectra and fitting of the fundamental equations to the experimental data are shown in Figures S5 and S6 in the Supporting Information.

The pK_a values from the absorbance and emission measurements were consistent and slightly greater than the previously reported value of 6.8, although this latest value was determined in the presence of phosphate buffer (0.1 M) and 1% dimethyl sulfoxide (DMSO).[18]

3.3. Buffer-Mediated ESPT Reactions. Xanthene derivatives undergo fast ESPT reactions when an appropriate proton donor/acceptor is present in solution at an adequate concentration. These reactions may radically alter the result of steady-state or time-resolved fluorescence experiments. **2-Me TM** can undergo such reactions when facilitated by the presence of phosphate buffer, and we study these reactions here.

The steady-state fluorescence spectra from aqueous 5×10^{-6} M solutions of **2-Me TM** at pH 6.10 in the presence of different concentrations of phosphate buffer in the range 0.010−0.75 M were recorded from 500 to 700 nm using an excitation wavelength of 485 nm. The observed spectra are shown in Figure 3. This figure shows that with these experimental conditions, an increase in the buffer concentration resulted in both a red shift and an increasing intensity of the emission band, which peaked at 600 nm. Moreover, a concomitant decrease in the emission band that peaks at 560 nm, which is attributable to the neutral species, is also observed. The above results reveal an ESPT reaction stimulated by the phosphate buffer. Indeed, the 485 nm excitation wavelength with the pH of 6.10 preferentially excites the much-less-fluorescent **2-Me TM** neutral form. Then, the buffer-mediated ESPT reaction

DOI: 10.1021/acs.jpca.5b07898
J. Phys. Chem. A XXXX, XXX, XXX−XXX

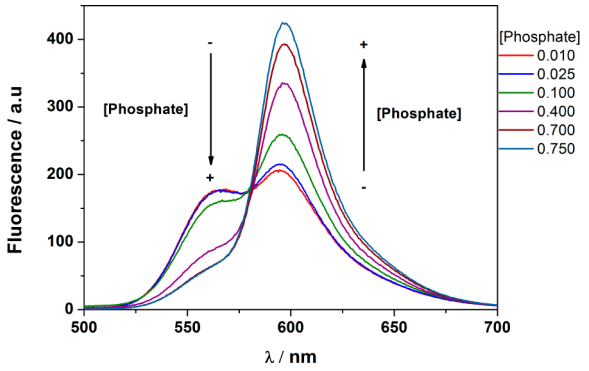


Figure 3. Steady-state emission spectra from 5×10^{-6} M **2-Me TM** aqueous solutions at a pH of 6.10 and different phosphate buffer concentrations ranging from 0.010 to 0.75 M (λ_{ex} = 485 nm).

quickly happens during the lifetime of the excited-state system, thus forming the much more fluorescent anion form and causing variation in the spectral profile and fluorescence intensity.

To observe the dynamic properties of the fluorescent emission, we also collected the fluorescence decay traces from the same solutions employed in the previous experiment. The excitation wavelength was 485 nm, and the emission wavelength was 610 nm, which preferentially detects the anionic species. The results are shown in Figure S7, in which only four decay traces are shown for clarity. It is apparent from the figure that increasing the phosphate concentration caused an increase in the fluorescence decay time. This result is consistent with the idea that the neutral species (which was excited preferentially in this experiment) undergoes an ESPT reaction during the lifetime of the excited state, which is influenced by the concentration of phosphate buffer. In the ESPT reaction, a portion of the excited neutral species becomes an excited anion, and if the reaction occurs sufficiently quickly, both species decay simultaneously. As previously mentioned, the reaction rate depends on the phosphate concentration; therefore, at a higher buffer concentration, the reaction will be faster, and the corresponding amount of the anion, which is dictated by the $pK^*_{N\text{-}A}$ and pH of the experiment, will be formed earlier. Thus, the subsequent fluorescence decay corresponds to the two coupled species. If the neutral/anion relationship corresponding to the pH of the experiment is achieved faster, the coupled fluorescence of the system will be detected for longer times.

3.4. TRES. The ESPT reaction mediated by phosphate should exhibit a double-exponential decay behavior, with decay times independent of the wavelength of emission but dependent on the forward and reverse rates of the ESPT reaction.[2] Therefore, the fluorescence spectra observed at distinct times during the fluorescence decay (TRES) can provide detailed information about the photophysical two-excited-state system. To accomplish this goal, we performed TRES with decay traces collected from aqueous solutions of **2-Me TM** in 0.7 M phosphate buffer and at eight pH values in the range 4.00−8.70, and using the excitation wavelengths of 485 and 530 nm to preferentially excite either the neutral or the anionic species, respectively. Emission in the wavelength range 550−640 nm was recorded in 5 nm steps. The complete data matrix is composed of 304 decay traces and can be described by a three-dimensional surface, $I(\lambda_{ex},\lambda_{em},t)$, that represents the fluorescence intensity at all excitation and emission wavelengths and times during the fluorescence decay. All of the decay traces could be adjusted using biexponential functions. It should be mentioned that for the emission wavelengths corresponding to the ends of the experimental range, the decay traces had low fluorescent signals. Therefore, the decay traces were recorded for different collection times, and hence the pre-exponential coefficients at each wavelength were corrected by the collection time and then normalized to the same count number in the decay. Thus, the decay traces were proportional to the steady-state fluorescence intensity. The pre-exponential factors were also corrected for the different sensitivity of the detector using a factor that related the total emission during the decay ($p_1 \times \tau_1 + p_2 \times \tau_2$) to the steady-state-intensity emission.

Figure 4 shows the TRES generated at pH 5.90 and both λ_{ex} = 485 and 530 nm. The TRES are represented by a plot of the

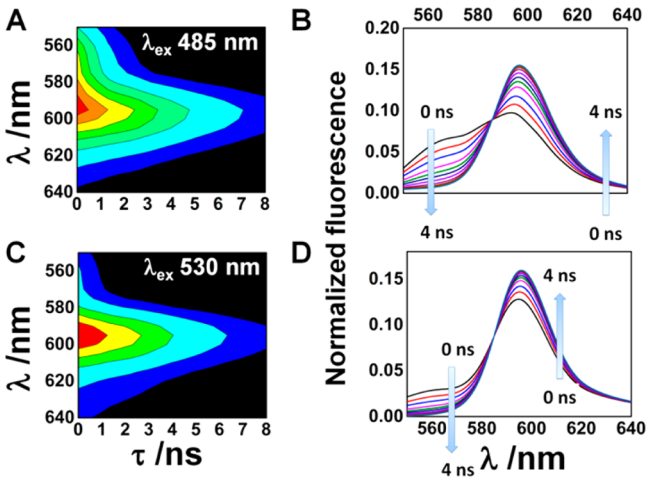


Figure 4. Contours of fluorescence intensity of **2-Me TM** after a δ-pulse excitation as a function of time and λ_{em} in 0.7 M phosphate buffer at pH 5.90 (A, λ_{ex} 485 nm; C, λ_{ex} 530 nm). Corresponding normalized emission spectra at different times after the excitation pulse (B, λ_{ex} 485 nm; D, λ_{ex} 530 nm).

fluorescence intensity level as a function of time and emission wavelength (Figure 4A,C), along with the normalized emission spectra at different times after the excitation pulse in the range of 0−4 ns (Figure 4B,D).

The spectrum profile at a shorter time after the pulse in Figure 4B reflects a large amount of the neutral species, which was preferentially excited under the experimental conditions employed (λ_{ex} = 485 mm). With increasing emission time, the spectral profile was gradually red-shifted, thus reflecting the successive greater abundance of the anionic species. Finally, after a longer time, the profile remained virtually unchanged because the neutral/anion relationship dictated by the pK_a^* and pH of the experiment was achieved. When the anion species was preferentially excited at a wavelength of 530 nm (and the same pH of 5.90), the initial spectral profile exhibited a small contribution of the neutral species. Remarkably, the spectral profiles also reflect a successive greater abundance in the anionic species with increasing emission time (Figure 4D), thereby demonstrating that the $pK^*_{N\text{-}A}$ of the excited state is significantly less than the pH of the experiment. Different fluorescent spectral shapes were obtained by determining the spectra at each excitation wavelength using several pH values. In the Supporting Information (Figures S8 and S9), different illustrative examples of plots of fluorescence intensity versus time and emission wavelength, along with the normalized

DOI: 10.1021/acs.jpca.5b07898
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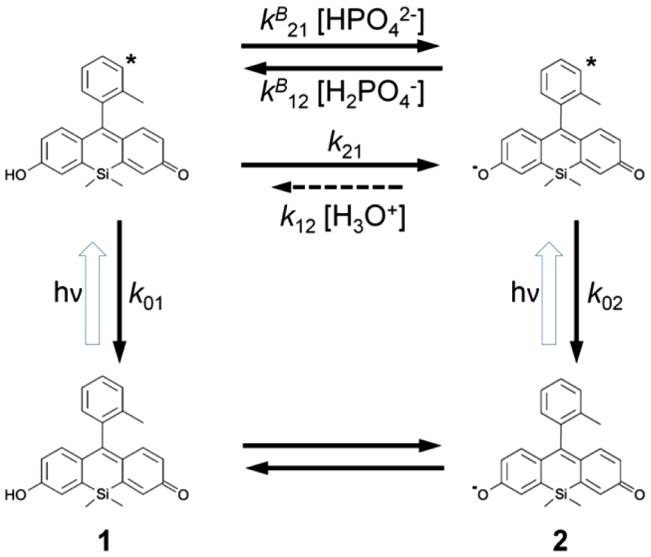
emission spectra at different times of emission, are shown. In brief, the spectral profiles were consistent with the phosphate-mediated ESPT reaction between the neutral and anionic species of **2-Me TM**. Moreover, the TRES experiments demonstrated a preferred conversion of the neutral form (defined by the shoulder at 560 nm) to the anion form (which exhibited an emission maximum at 590 nm). This finding indicates that buffer-mediated deprotonation of the neutral form is the main process, whereas buffer-mediated protonation of the anion form is a much slower process.

3.5. Kinetic and Spectral Parameters of the Phosphate-Mediated ESPT Reaction. Once experimental evidence of the photophysical two-excited-state system was obtained, a multidimensional fluorescence decay surface was used to determine the kinetic parameters of the system in the excited state, gaining insights into the kinetic behavior. Scheme 3 shows the reactions involved in the ESPT model along with the emission for the two species of **2-Me TM** present at near-neutral pH.

Scheme 3. Kinetic Model of Ground- and Excited-State Proton-Transfer Reactions of Aqueous 2-Me TM Added of Phosphate Buffer



In Scheme 3, species **1** and **2** correspond to the neutral and anionic forms of **2-Me TM**, respectively. Following excitation by a fast δ-pulse of light, the excited-state species **1*** and **2*** are generated. These excited species can decay through fluorescence (F) and nonradiative (NR) processes. The composite rate constants for these processes are denoted by k_{01} ($=k_{F1} + k_{NR1}$) and k_{02} ($=k_{F2} + k_{NR2}$). k_{21} denotes the rate constant for the dissociation of **1*** into **2*** and H^+. Because in the experimental pH range, $[H^+]$ is sufficiently small, the rate of association of **2*** + $H^+ \rightarrow$ **1*** (k_{12}) can be considered negligible. The ground-state proton exchange reaction of **2-Me TM** is described by the acidity constant $K_{N\text{-}A}$. The acidity constant of the buffer is denoted by $K_a^B = [HPO_4^{2-}][H^+]/[H_2PO_4^-]$. The rate constant k^B_{21} characterizes the reaction of **1*** with HPO_4^{2-} to form **2*** and $H_2PO_4^-$ in the excited state. The reverse reaction of **2*** and $H_2PO_4^-$ that yields **1*** and HPO_4^{2-} is designated by the rate constant k^B_{12}.

The theory and methods of solving buffer-mediated ESPT reactions are well-established[2,20,24,31] (see also the Supporting Information). To achieve a unique determination of the rate constants, fluorescence decay traces under different experimental conditions (i.e., the absence or presence of diverse phosphate buffer concentrations, different values of pH, and λ_{em}) were recorded, obtaining a multidimensional decay trace surface.[24]

The decay traces were analyzed in terms of the decay times, and associated pre-exponential factors, by means global analyses of traces recorded at the same pH and C^B but at different λ_{ex} and λ_{em}. The decay times were linked for decay traces collected with these characteristics, whereas the pre-exponentials were locally adjustable parameters.

Without phosphate buffer, the decay times were biexponential with positive pre-exponential factors, and independent of both the emission and excitation wavelengths and the pH. Therefore, ESPT does not happen in the absence of buffer. Global analysis of 66 curves corresponding to $C^B = 0$ M in the pH range between 4.50 and 10.26 at $\lambda_{ex} = 485$ and 530 nm and $\lambda_{em} = 560$, 580, and 600 nm provided consistent decay time estimates: $\tau_1 = 0.990 \pm 0.003$ ns and $\tau_2 = 3.78 \pm 0.03$ ns ($\chi_g^2 = 1.15$). At pH values greater than 8, only τ_2 was present. At these pH values, the reprotonation reaction in the excited state is very slow because of the low concentration of protons and does not compete with the radiative constant. Therefore, this decay time unambiguously defines the value for k_{02}. Nevertheless, the shorter lifetime τ_1 consists of the sum of the excited-state dissociation rate constant, k_{21}, and the radiative constant of the neutral form, k_{01}. From global analyses of the decay traces, we determined that $k_{02} = 2.64 \times 10^8$ s^{-1} and $k_{01} + k_{21} = 1.08 \times 10^9$ s^{-1}.

To obtain the ESPT rate constants k^B_{21} and k^B_{12}, 366 decay traces were collected in the pH range 4.00−10.26 with different phosphate buffer concentrations (0, 0.15, 0.3, and 0.6 M) at two λ_{ex} (485 and 530 nm) and three λ_{em} (560, 580, and 600 nm) values. The theoretical equations (eqs S8 and S9 in the Supporting Information) were fit with the decay times previously determined to obtain the rate constants. Figure 5 shows the decay times determined, along with the fitted curves ($\chi_g^2 = 1.18$). Global analysis provided all of the rate constants of Scheme 3 (Table 1). During the model fitting, the value of k_{21} was always close to 0 and exhibited a very large associated

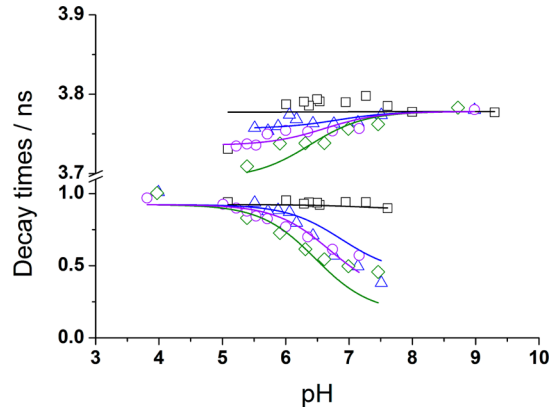


Figure 5. Global fitting (solid lines) of the theoretical equations (eqs S8−S11 in the Supporting Information) to the decay times at different buffer concentrations (0 M, □; 0.15 M, △; 0.3 M, ○; 0.6 M, ◇) and pH values. Note the change in scale in the two sections of the ordinate axis.

DOI: 10.1021/acs.jpca.5b07898
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Table 1. Rate Constant Values for the Phosphate-Mediated ESPT Reaction of 2-Me TM

rate constant	value
k_{01}/s^{-1}	$1.08(\pm0.01) \times 10^{9}$
k_{02}/s^{-1}	$2.646(\pm0.009) \times 10^{8}$
$k_{12}^{B}/M^{-1}\ s^{-1}$	$1.02\ (\pm0.43) \times 10^{7}$
$k_{21}^{B}/M^{-1}\ s^{-1}$	$7.88\ (\pm0.66) \times 10^{9}$

error. Hence, we considered the spontaneous excited-state deprotonation negligible.

The relative values of the rate constants k_{21}^{B} and k_{12}^{B} indicate that buffer-mediated deprotonation of the neutral state is 2 orders of magnitude faster that the excited-state phosphate-assisted protonation of the anion form. This is in perfect agreement with the TRES results, which mainly exhibited excited-state transformation to the anion form. Finally, the rate constants k_{21}^{B} and k_{12}^{B}, along with the known value of K_a^{B}, can provide the excited-state $pK^*_{N\text{-}A}$. Using 7.2 as the pK_a^{B}, the previously determined rate constants, and eq S10 (Supporting Information), we obtained a value of 4.31 for the $pK^*_{N\text{-}A}$. This value indicates that **2-Me TM** is a stronger photoacid than the green xanthene derivatives, in which the excited-state pK^* did not vary substantially with respect to the ground-state equilibrium constant.[2,20,29,30]

3.6. Fluorescence Imaging Microscopy of 2-Me TM in HeLa Cells. Water solubility and membrane permeability are crucial properties for a fluorescent sensor that functions inside live cells. Some xanthene-based dyes have exhibited good penetration in cells, and they could be used as intracellular sensors.[9,10] In fact, **2-Me TM** βgal was applied to visualize β-galactosidase activity in HEK293 cells.[18] To test the penetration and accumulation of **2-Me TM** in the various locations of the cell interior, measurements on live HeLa 229 cells were performed. We monitored the loading (1×10^{-7} M) of the probe in the intracellular compartments. Immediately after the loading, confocal fluorescence imaging was performed. As shown in Figure 6A–D, the intracellular accumulation of **2-Me TM** suggests spontaneous, fast, and good membrane penetration without a need for any external molecular assistance. In addition, in contrast with the pattern observed for previous xanthene-based dyes,[9,10] characteristic fluorescence of **2-Me TM** was also observed in the interior of the nucleus. To examine this new accumulation pattern, we measured the intensity profile of the probe in different cellular compartments inside HeLa 229 cells. Parts E–H of Figure 6 show the profile of the fluorescence intensity signal, which corresponds to the arrows in Figure 6A–D, respectively. In every cell examined, the cytosol and nuclei exhibited a similar intensity of fluorescence. This result suggests that good nuclear membrane penetration of **2-Me TM** allows homogeneous and analogous accumulation of the probe in the cytosol and nuclei. Moreover, the probe exhibited strong accumulation in different cytoplasmic structures. This noticeable signal will permit isolation, differential treatment, and tracking or study of these structures independent of the cytosol/nucleus fluorescence.

Therefore, on the basis of the fluorescence intensity upon excitation of the cells at 530 nm during dye loading, it can be established that **2-Me TM** readily accumulates inside cytoplasmic structures and less effectively but homogeneously accumulates in the cytosol and nucleus.

4. CONCLUSIONS

To investigate the use of the new silicon-substituted xanthene derivative **2-Me TM** as a sensor, we have performed a study of the photophysics of the dye. Three absorbing and emitting species have been identified, and the acidity constant of the dye at near-neutral pH was determined using both absorbance and steady-state fluorescence methods. Using steady-state and time-resolved fluorescence experiments, we demonstrated that **2-Me TM** undergoes a phosphate-mediated ESPT reaction and rapidly interconverts between the neutral and anion species. TRES experiments demonstrated a preferred conversion of the neutral form to the anion form, thereby indicating that the buffer-mediated deprotonation of the neutral form is the main process, whereas buffer-mediated protonation of the anion form is a much slower process. The kinetic parameters determined were consistent with the TRES results, thus indicating a higher rate constant for deprotonation than protonation, which

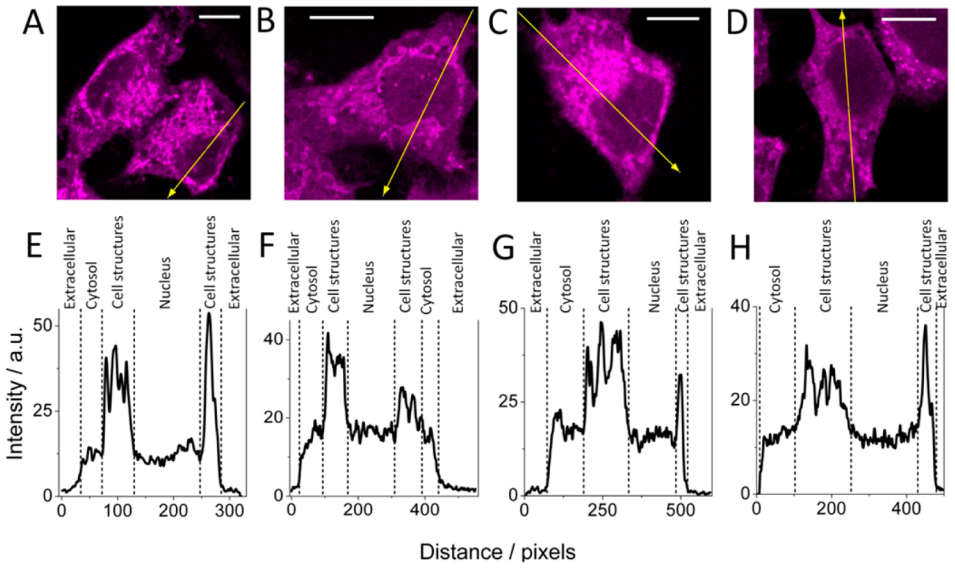

Figure 6. (A)–(D) Illustrative fluorescence images of HeLa 229 cells with **2-Me TM** (10^{-7} M). (E)–(H) Intensity profiles of the arrows from Figures 6A–D, respectively. Scale bars represent 10 μm.

DOI: 10.1021/acs.jpca.5b07898
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implies a very low value of $pK^*_{N\text{-}A}$. Finally, we used the dye inside cells to examine its future application as a cell sensor. Excitation of the cells at 530 nm indicated that **2-Me TM** accumulates visibly inside cytoplasmic structures and less effectively but homogeneously accumulates in the cytosol and nucleus.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpca.5b07898.

^{1}H and ^{13}C NMR data together with a copy of both spectra (Figures S1 and S2); analysis of the absorbance vs pH; analysis of the experimental fluorescence vs pH; Figures S3−S9 of absorbance and fluorescence vs pH traces, absorbance and emission spectra, fluorsecence decay traces, contours of fluorescence instensity; and evaluation of kinetic parameters (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by MINECO (project CTQ2014-56370-R), and MINECO (project CTQ2014-53598).

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