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Fluorescein Prototropism within Poly(ethylene glycol)s and Their Aqueous Mixtures

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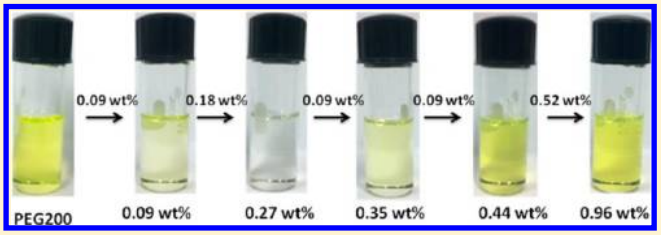
ABSTRACT: Depending on the solubilizing milieu and conditions, fluorescein may exist in one or more of its many prototropic forms [cationic, neutral (zwitterionic, quinoid, and lactone), monoanionic (phenolate and carboxylate), and dianionic]. Fluorescein prototropism is investigated in liquid poly(ethylene glycol)s (PEGs) of different average molecular weight (MW) and their aqueous mixtures using UV−vis absorbance along with static and time-resolved fluorescence spectroscopic techniques. Information regarding various prototropic forms of fluorescein in up to 30 wt % different average MW PEG-added aqueous buffers at varying pH reveals that addition of PEG causes lactonization of fluorescein in the milieu; higher the average MW of PEG, the more the lactonization is. Neat PEG200, PEG400, and PEG600 are found to support the dianionic form of fluorescein, while PEG1000 supports the neutral lactonized form. It is demonstrated that various prototropic forms of fluorescein may be generated within PEGs by addition of adequate amounts of acidic aqueous buffer. Significant bathochromic shift in absorbance and fluorescence band maxima of dianionic fluorescein as concentration of PEG200 is increased correlates well with hydrogen bond accepting basicity, hydrogen bond donating acidity, and dipolarity/polarizability of the aqueous PEG200 mixture. Interestingly, fluorescence emission from the cationic form of fluorescein is observed from dilute aqueous acidic media in the presence of high concentration of PEG200, whereas the fluorescence emission from cation in the absence of PEG200 is observed only from aqueous solutions of very high acidity (>5 M [H^+]). Excited-state intensity decay is also used to support this outcome. It is proposed that, in the presence of a small amount of acid in PEG200, a highly acidic water-rich solvation microenvironment is formed around fluorescein, which converts its dianionic form to cationic form and considerably hinders the rapid deprotonation of the excited state of the cationic form.



INTRODUCTION

The role of the solvent in chemical reactions is one of immediate and daily concern to practising chemists.[1] The majority of the solvents used in industry are volatile organic compounds (VOCs) that usually possess high vapor pressure and low water solubility. Lately, there have been efforts from both academic and industrial research communities to seek neoteric and plausible "greener" alternatives to these VOCs.[2−8] Among many such alternatives being explored currently (e.g., supercritical fluids, room-temperature ionic liquids, etc.), polymers and aqueous polymer-based media are gaining widespread acceptance.[8] As a result, investigations and applications of polymer-based media have increased manyfold. Especially, exploration and assessment of the structural and dynamical properties of molten polymers and polymer solutions have become a topic of great interest. In particular, poly(ethylene glycol)s (PEGs) are of great industrial, pharmaceutical, and biomedical importance due to their favorable physicochemical properties and high solubility in many solvents, especially water.[9−11]

Recently, several research groups including our own have demonstrated that the behavior of structurally diverse probes within PEG-based media sometimes exhibited unusual or unpredicted responses possibly due to specific solute−solvent interactions or to the presence of interesting solvent nanostructuring. In this context, PEGs may significantly alter the prototropic behavior and subsequent properties of common molecules. Toward this, we have carried out a detailed investigation of the prototropic and solvatochromic response of fluorescein, a biologically important prototropic probe, in neat PEGs of different average molecular weight (MW) and aqueous mixtures of these PEGs. Fluorescein is one of the most common fluorescence probes having high molar absorptivity and fluorescence quantum yield.[12−17] These desirable properties have rendered fluorescein one of the most useful and sensitive fluorescent labels. Fluorescein shows rich, diverse, and interesting prototropism.[18,19] As a result, the application of fluorescein requires a thorough understanding of its prototropic behavior. Depending on the conditions, one or more forms of fluorescein among the cationic, neutral (zwitterionic, quinoid, and lactone), monoanionic (phenolate and carboxylate), and dianionic may be present in the medium (Scheme 1).[18,19] The apparent protolytic constants relating the chemical activities of the cation, neutral, anion, and dianion forms are $pK_{a1} \approx 2.1$, $pK_{a2} \approx 4.3$, and $pK_{a3} \approx 6.4$, respectively, in aqueous buffer media (Scheme 2).[18,19]

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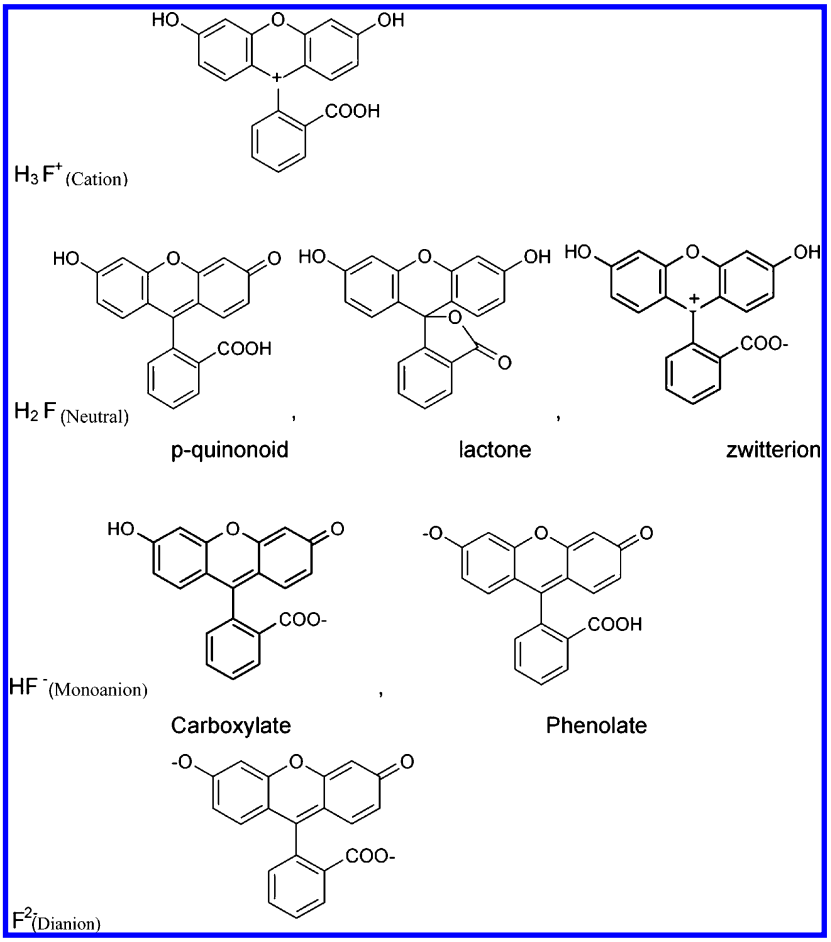
Scheme 1. Different Prototropic Forms of Fluorescein



Scheme 2. Prototropic Equilibria of Fluorescein in Aqueous Solution

$$H_3F^+ \xrightleftharpoons{pK_{a_1}\sim 2.1} H_2F \xrightleftharpoons{pK_{a_2}\sim 4.3} HF^- \xrightleftharpoons{pK_{a_3}\sim 6.4} F^{2-}$$

As mentioned earlier, in this work, we report the effect of PEG on prototropic equilibria involving various forms of fluorescein and solvatochromism of the dianionic fluorescein. Experimentally, PEGs of different average MW are systematically added to fluorescein dissolved in different pH buffer while maintaining the mixture pH, and information regarding the various prototropic forms of fluorescein in such water-rich mixtures is obtained using UV−vis molecular absorbance and static and lifetime fluorescence. Fluorescein prototropism in neat PEGs and PEG-rich media is investigated next. Solvatochromic behavior of dianionic fluorescein within aqueous PEG mixture is also investigated. It is clear from our studies that PEG has an interesting and unique effect on the prototropic behavior of fluorescein. Versatility of PEG and its aqueous mixtures as media for important reactions, such as prototropic reactions, is demonstrated.

EXPERIMENTAL SECTION

Materials. Sodium salt of fluorescein (high purity) was obtained from Acros Organics. PEGs of average MW 200 (PEG200), 400 (PEG400), 600 (PEG600), and 1000 (PEG1000) were purchased in high purity from Merck. Doubly distilled deionized water was obtained from a Millipore, Milli-Q Academic water purification system having ≥18 MΩ·cm resistivity. Ethanol (99.9%) was obtained from Merck. Sodium phosphate, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, phosphoric acid, sodium hydroxide, hydrochloric acid, and potassium chloride were purchased from Qualigens in highest purity possible.

Methods. Stock solution of fluorescein was prepared in ethanol and stored under refrigeration at 4 ± 1 °C in precleaned amber glass vials. Stock solutions of fluorescein were also prepared in PEG200, PEG400, PEG600, and PEG1000 and stored at ambient conditions. Aqueous PEG mixtures of desired pH were prepared by mixing the liquids in required amounts, and adjusting the pH, if necessary, using dilute aqueous HCl or NaOH. In some cases (for PEG1000), solutions were gently heated to 40 °C in a water bath with continuous stirring to obtain a homogeneous solution at room temperature. The required amount of the stock solution of fluorescein in ethanol was taken in the sample tube and dried by passing high-purity nitrogen gas. Buffer, PEG or aqueous mixture of PEG, as per requirement, was added to the sample tube under dry conditions to attain the desired final concentration of fluorescein. For preparation of fluorescein solutions in PEG, the stock solution in PEG was mixed with appropriate amounts of neat PEG. Buffer solutions (0.2 M) in the pH range 0.5−2.0 were prepared by proper combination of 0.2 M HCl and 0.2 M KCl. Buffer solutions (10 mM) in the pH range 2.0−13.0 were prepared by proper combinations of phosphoric acid, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, and sodium phosphate. The pH

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adjustments were done with the help of dilute aqueous HCl and dilute aqueous NaOH. Required amounts of materials were weighed using Mettler-Toledo AB104-S balance with a precision of ±0.1 mg. pH were measured and adjusted using a Cyberscan 510 pH meter. The pH meter was calibrated using pH 1.0, 4.0, 10.0 standard buffers. All measurements were taken at room temperature.

A Perkin-Elmer Lambda 35 double beam spectrophotometer with variable bandwidth was used for the acquisition of UV−vis molecular absorbance data. All the data were acquired using 1.0 cm path length quartz cuvettes. All the absorption measurements were carried out at room temperature. Fluorescence spectra were acquired on Fluorolog-3 modular spectrofluorimeter with single Czerny-Turner grating excitation and emission monochromators having 450 W Xe arc lamp as the excitation source and a photomultiplier tube (PMT) as the detector. This spectrofluorimeter was purchased from Horiba-Jobin Yvon, Inc. Fluorescence emission spectrum was recorded by keeping the excitation wavelength fixed and varying the emission wavelength. All the data were acquired using 1 cm path length quartz cuvettes. The respective solutions without fluorescein were used as blank for recording the absorbance and fluorescence spectra. Spectral response from appropriate blanks was subtracted before data analysis. All the data were collected at least in triplicate starting from sample preparation. In order to obtain fluorescence lifetime, excited-state intensity decay data were acquired in the time domain using Fluorocube time-correlated single-photon-counting (TCSPC) fluorimeter purchased from Horiba-Jobin Yvon, Inc. The fluorescein dissolved in aqueous PEG200 mixtures was excited at 405 nm using a UV-pulsed NanoLED-405 source having pulse width <1.0 ns. The emission was collected using Peltier-cooled red-sensitive TBX-04 PMT detection module at 477 and 525 nm. The temperature was controlled with Peltier (TC125) having an accuracy of ±0.3 °C and stability of ±0.03 °C. The data was collected with DAQ-MCA-3 Series (P7882) multichannel analyzer. The excited-state intensity decays were analyzed using DAS6 analysis software.

RESULTS AND DISCUSSION

Depending on the pH, various prototropic forms of fluorescein [cation (H_3F^+), neutral (H_2F), monoanion (HF^-), and dianion (F^{2-})] could be present in aqueous solution (Scheme 2). The peak in the absorbance spectrum for dianion appears at ca. 490 nm with a shoulder in the vicinity of 475 nm. Relatively weaker absorbance of monoanion is characterized by two bands at ca. 472 and ca. 453 nm of approximately the same molar absorptivity.[18,19] The neutral and cationic species show absorbance maxima in the visible region at ca. 434 nm (with a side maxima at 475 nm) and ca. 437 nm, respectively; the molar absorptivity of the transition for cationic species is significantly higher than that for the neutral species. The peak in fluorescence emission spectrum of dianion is observed at ca. 515 nm. The monoanion and quinoid species show a broader peak at 515 nm with relatively weak emission. Fluorescence emission from fluorescein cation is observed only at very high acidities (>5 M [H^+]).[20−22] The fluorescence emission from fluorescein dissolved in ≤0.2 M aqueous HCl shows features (band maxima at ca. 515 nm) corresponding to the anionic and/or the neutral form due to the well-documented fast proton transfer in the excited state from the cationic and/or the neutral fluorescein to water present in the solvation sphere of the species.[18−22]

Addition of PEG to Aqueous Solution of Fluorescein. The addition of up to 30 wt % of PEG200 to aqueous buffer solutions of fluorescein of pH 1 and pH 2, where mainly cationic and neutral species dominate, causes a rapid decrease in the molar absorptivity (Figure 1). This behavior is attributed

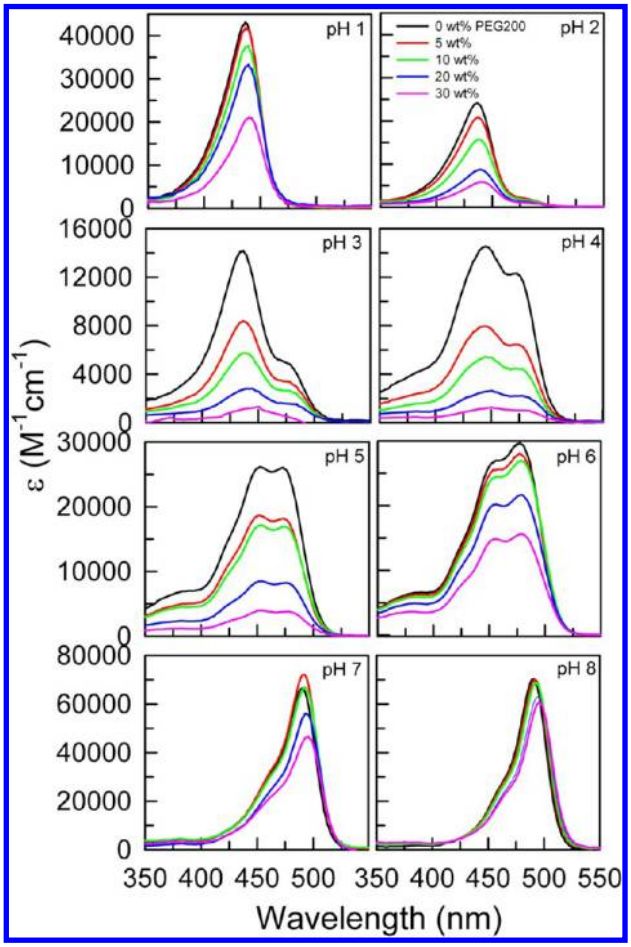

Figure 1. Absorbance spectra of fluorescein in PEG200-added aqueous mixtures at different pH at ambient conditions.

to the lactonization of the quinoid or the zwitterionic form that takes place as PEG200 is added. It has already been established that the lactone form of fluorescein does not show any absorbance in the region 350−550 nm due to the presence of sp^3 hybridization, which ruptures the conjugation of π-bonds in xanthene moiety of the fluorescein.[18,19] A decrease in the solvent hydrogen-bond-donating (HBD) ability (i.e., increased aprotic nature) is known to favor the lactonization.[23−25] The Kamlet−Taft parameter α, which represents HBD acidity of the solvent milieu, is reported to decrease as PEG200 is added to water that may result in increased aprotic nature of the medium facilitating lactonization within the solution.[26] It is clear from Figure 1 that the relative decrease in molar absorptivity is higher in the case of aqueous PEG200 mixture of pH 2 as compared to pH 1. This can be attributed to the presence of greater amount of neutral species within the pH 2 mixture, which undergoes lactonization on addition of PEG200. A bathochromic shift is also observed as PEG200 is added. Addition of PEG200 to mixture of pH 3 and pH 4, where neutral species are the dominant ones, also results in decrease in molar absorptivities; however, the decrease in molar

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absorptivity is significantly more (Figure 1). This further confirms the enhanced efficiency of the formation of lactone form of fluorescein as PEG200 is added. As we increase the pH of the aqueous PEG200 mixture further, we observe a lesser decrease in molar absorptivity upon PEG200 addition. This is easy to conceive as at pH 5 and pH 6, lesser amounts of quinoid or zwitterion forms are present as fluorescein monoanion tends to be the predominant species. For aqueous mixtures of PEG200 of pH 7 and 8, where dianion is the major species in the solution, the addition of PEG200 causes only a slight decrease in molar absorptivity accompanied by a significant bathochromic shift (Figure 1). The bathochromic shift, as reported in the literature, is a manifestation of the changes in the hydrogen-bonding (HB) power of the cybotactic milieu of fluorescein.[27,28]

Fluorescence emission spectra of fluorescein in PEG200-added aqueous mixtures of different pH were acquired next (Figure 2). At pH 1, cation is the dominant species. However,

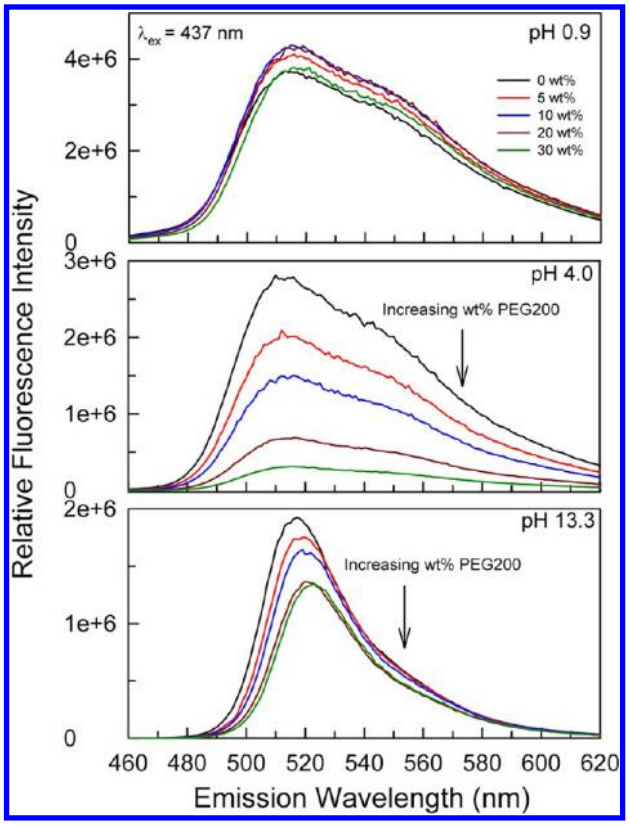

Figure 2. Fluorescence emission spectra of fluorescein (10 μM) in PEG200-added aqueous mixtures of different pH at ambient conditions.

as mentioned earlier, fluorescence emission from fluorescein cation is observed only at extreme high acidities.[20−22] Instead, excited-state proton transfer causes the cation to convert to quinoid/monoanion form. Thus, we observe a quinoid/monoanion emission peak at 515 nm for fluorescein dissolved in buffer at pH 1. Unlike the molar absorptivity of fluorescein at pH 1, the fluorescence intensity does not change much as PEG200 is added. Based on our suggestion that addition of PEG200 causes lactonization of quinoid/zwitterionic form of fluorescein, we expected a decrease in fluorescence intensity. In water or aqueous PEG200 mixture at pH 1, the fluorescence is perhaps mainly from the monoanionic species and not from the neutral species. As the concentration of cation remains almost unchanged on PEG200 addition to aqueous buffer, fluorescence intensity does not change significantly. Interestingly, for pH 4 aqueous PEG200 mixture, fluorescence intensity decreases drastically upon increased addition of PEG200 as neutral species dominate at this pH, and apparently, lactonization readily takes place (Figure 2). Similar behavior is observed for pH 13 mixture; however, the decrease in fluorescence intensity is much less due to the presence of relatively smaller concentrations of neutral species (Figure 2). Considerable bathochromic shift is again observed within pH 13 mixture on PEG200 addition.

The absorbance of fluorescein, in general, decreases on increasing the concentration of PEG200 in the aqueous mixture; fluorescence emission shows different trends at different mixture pH—in mixtures of pH 1, 5, 6, 7, and 8 fluorescence intensity increases on addition of PEG200, while it decreases within mixtures of pH 2, 3, and 4 where neutral form is the dominant species. Subsequently, fluorescence quantum yield (Φ_F) of fluorescein dissolved in 5 wt % PEG200 aqueous buffer mixture at different pH is estimated using fluorescein in 0.1 M NaOH as standard with Φ_F = 0.84.[29] The measured Φ_F are plotted as the function of the pH of the aqueous PEG200 mixture (Figure 3). Φ_F in the presence of 5 wt % PEG200

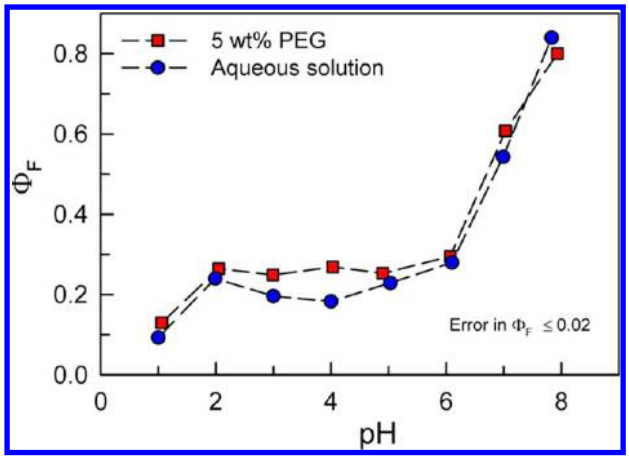

Figure 3. Variation of fluorescence quantum yield (Φ_F) with the pH of the aqueous PEG200 mixture at ambient conditions.

appears to be higher as compared to its value in aqueous solution of the same pH except for pH 8 system. The increase in Φ_F within 5 wt % PEG200 mixture with water, however, is statistically significant only for pH 3 and pH 4 systems where neutral and monoanionic species usually dominate. We infer that PEG200 addition to water may make neutral and/or monoanion forms of fluorescein more fluorescent.

We next added PEGs of different average MW to aqueous buffer solutions of fluorescein of different pH. As with PEG200, addition of 30 wt % of PEG400, PEG600, and PEG1000 to aqueous solutions of fluorescein at various pH again caused a decrease in absorbance values (Figure 4)—interestingly, the higher the average MW of the PEG added, the greater the decrease in the absorbance. Within pH 1.1 and 3.6 the dominant forms of fluorescein are cationic and neutral, respectively. The absorbance spectral features of the prototropic forms at lower pH (i.e., pH 1.1, 3.6, and 5.5 mixtures) appear to be affected more by PEG addition. The decrease in absorbance could again be attributed to the increased

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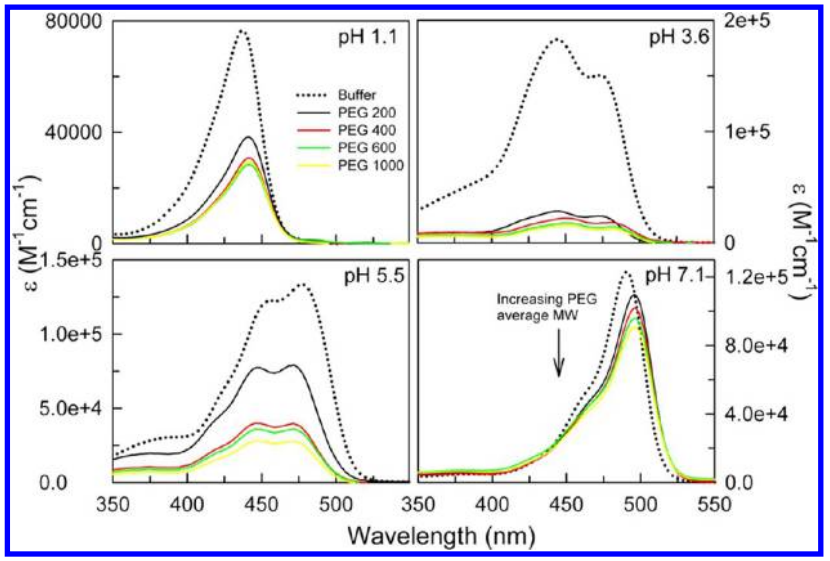

Figure 4. Absorbance spectra of 30 wt % different average MW PEG-added aqueous mixtures of fluorescein at different pH at ambient conditions.

lactonization of the zwitterionic/quinoid form or it may be due to the medium effect as viscosity, density, and refractive index of aqueous PEG mixtures increase as we increase the average MW of the added PEG.[30,31] As the average MW of PEG increases, the number of PEG molecules for the same weight % of PEG addition decreases. It is concluded that an increase in polymer chain length may increase the efficiency of fluorescein lactonization.

Fluorescein in Neat PEG and Buffer-Added PEG-Rich Mixture. Fluorescein dissolved in neat PEG200 displays absorbance spectral characteristic similar to that of its dianionic form (Figure 5), but with decreased molar absorptivity as

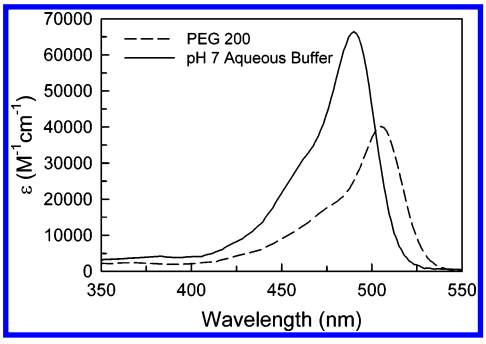

Figure 5. Absorbance spectra of fluorescein in neat PEG200 as compared to that in pH 7 aqueous buffer under ambient conditions.

compared to aqueous buffer of pH 7. However, the absorbance band is significantly bathochromically shifted. Based on the similarity of fluorescein absorbance behavior, the "apparent" pH of PEG200 can be considered to be >6. On addition of small amount of pH 1 buffer to fluorescein dissolved in PEG200, the absorbance decreases to the extent that almost no absorbance is observed in presence of 4.3 wt % pH 1 buffer (Figure 6). The addition of pH 1 buffer causes a decrease in the "apparent" pH of the mixture that subsequently results in increased amount of neutral species in its lactonized form as PEG200 is present as the component in bulk. We have already demonstrated favored lactonization by PEG200 earlier. Similar absorbance behavior is observed on addition of pH 3, pH 5, and pH 8 aqueous buffers to neat PEG200, but the decrease in the absorbance happens to a lesser extent as the pH of the added buffer is increased (Figure 6). This could easily be attributed to the fact that the pH decrease induced by the buffer of the higher pH is lesser thus resulting in lesser lactonization.

Higher amounts of pH 1 buffer addition to fluorescein dissolved in PEG200 showed a transition from dianion to monoanion to neutral to cation form (Figure 7), which further establishes the fact that the pH of PEG200 decreases on addition of pH 1 buffer. However, during this interconversion of prototropic forms the absorbance stays fairly low due to the presence of significant amounts of fluorescein in lactonized form. Fluorescence emission spectra of fluorescein also show an initial decrease in intensity followed by increase on addition of pH 1 buffer to PEG200 (data not shown). The spectra also become broad, showing a transition from anionic to cationic form. All prototropic forms of fluorescein could be generated within PEG200 by controlled addition of aqueous buffer. Buffers of pH 3 and pH 6 were also added in greater amounts to fluorescein dissolved in PEG200 and absorbance spectra were recorded (Figure 7). Similar effect as that for pH 1 buffer addition was observed. Dianionic form converts to monoanionic to neutral on pH 3 buffer addition. Addition of pH 6 buffer showed transformation from dianion to monoanion form supported by the increase in absorbance on increasing the buffer amount. Lesser the extent of lactonization of the neutral form, higher is the absorbance value.

Similar to the outcomes within PEG200, neat PEG400 and PEG600 also support the dianionic form of fluorescein (Figure 8). However, we see a bathochromic shift in fluorescein absorbance maxima as the average MW of the solubilizing PEG becomes higher. λ_{max}^{abs} of fluorescein in PEG200, PEG400, and PEG600 are 505 ± 0.5, 508 ± 0.5, and 511 ± 0.5 nm, respectively. This is accompanied by decrease in the molar absorptivities of the dianionic fluorescein within PEGs as average MW of PEG is increased. This could be due to the greater lactonization of the zwitterionic and the quinoid forms of fluorescein or due to change in properties (density/viscosity/refractive index) of the solubilizing milieu as proposed earlier.[30,31] Similar to the observation within PEG200, addition of pH 1 buffer in small amounts (up to 4.3 wt %) results in gradual decrease in absorbance of fluorescein dissolved in PEG400 and PEG600, respectively (Figure 8). As proposed

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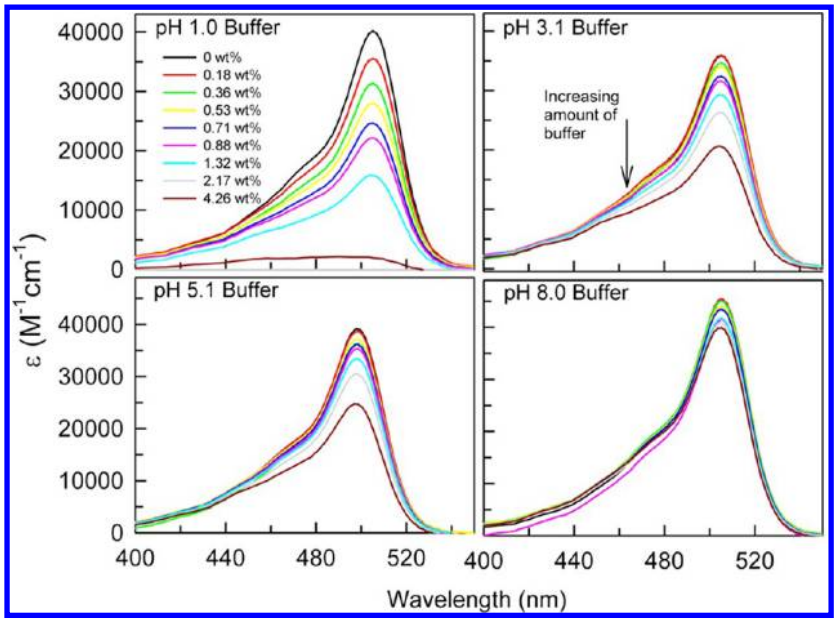

Figure 6. Absorbance spectra of fluorescein in buffer-added PEG200 mixtures at low weight % buffer at ambient conditions.

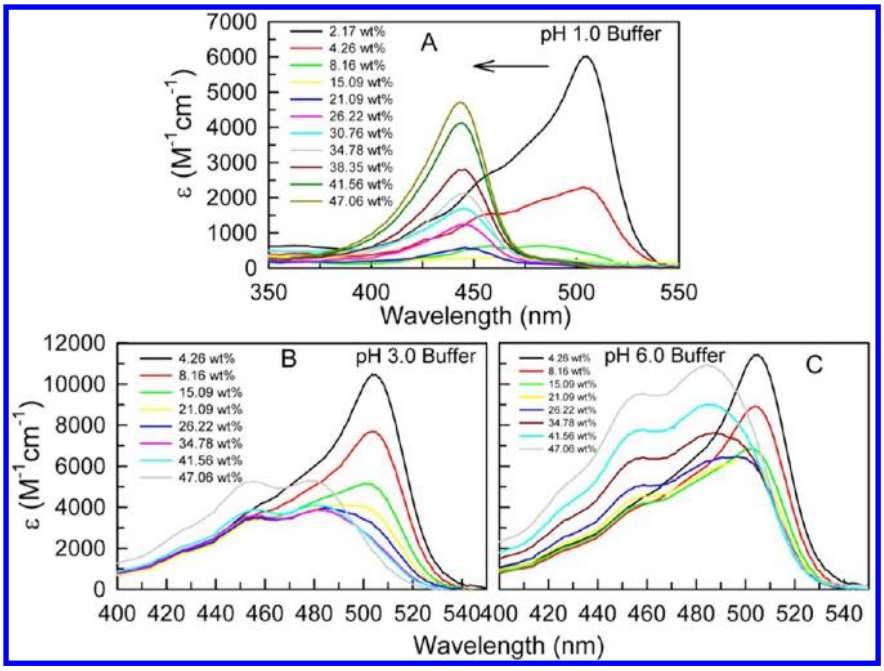

Figure 7. Absorbance spectra of fluorescein in buffer-added PEG200 mixtures at high weight % buffer at ambient conditions.

earlier, this is attributed to the decrease in the pH of the mixture which helps convert dianionic form of fluorescein to neutral/monoanionic form. PEG supports colorless lactonized form of neutral fluorescein, so almost no absorbance is seen with 4.3 wt % addition of pH 1 buffer to PEG. Interestingly, neat PEG1000 supports lactonized neutral form of fluorescein due to which almost no absorbance is shown by even as high as 1 mM fluorescein dissolved in PEG1000 (Figure 8). Addition of small amounts of pH 1 buffer to fluorescein dissolved in PEG1000 has no significant effect on the absorbance spectral profile probably because addition of buffer is not enough to bring about a significant change in pH of the mixture to change lactonized neutral form of fluorescein to the cationic form.

At 2.2 wt % pH 1 buffer added PEG200, it is the dianionic form of fluorescein which dominates, and gets converted to monoanionic to neutral to cationic form as the amount of buffer is increased due to the lowering of the pH of the medium (Figure 7). However, at 2.2 wt % pH 1 buffer added PEG400, it is the monoanionic and the lactonized neutral forms of fluorescein that dominate (Figure 8). Addition of higher amounts of buffer to PEG400 lowers the pH rendering the cationic form of fluorescein as the dominant one (Figure 9). PEG600 behaves similar to PEG200 and PEG400 on addition of pH 1 buffer. Interestingly, addition of higher amounts of buffer to PEG1000 converts the neutral form of fluorescein to cationic form as the mixture becomes more acidic (Figure 9).

Solvatochromism of Dianionic Fluorescein within Aqueous PEG200 Mixture. The dianionic form of fluorescein shows solvatochromic behavior depending on the H-bonding capability of the solubilizing milieu.[27,28] It was demonstrated

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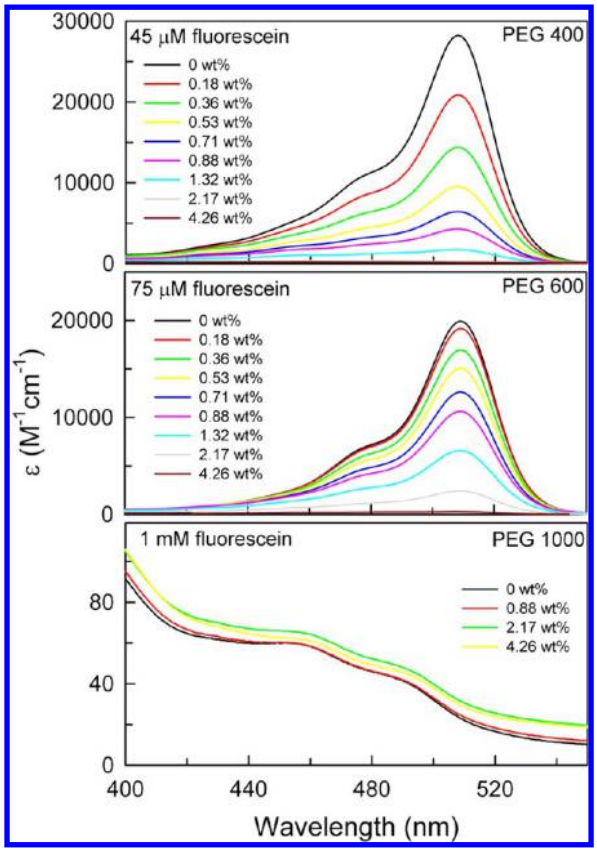

Figure 8. Absorbance spectra of fluorescein in pH 1 buffer added aqueous PEG mixtures formed with different average MW PEGs at low weight % buffer at ambient conditions.

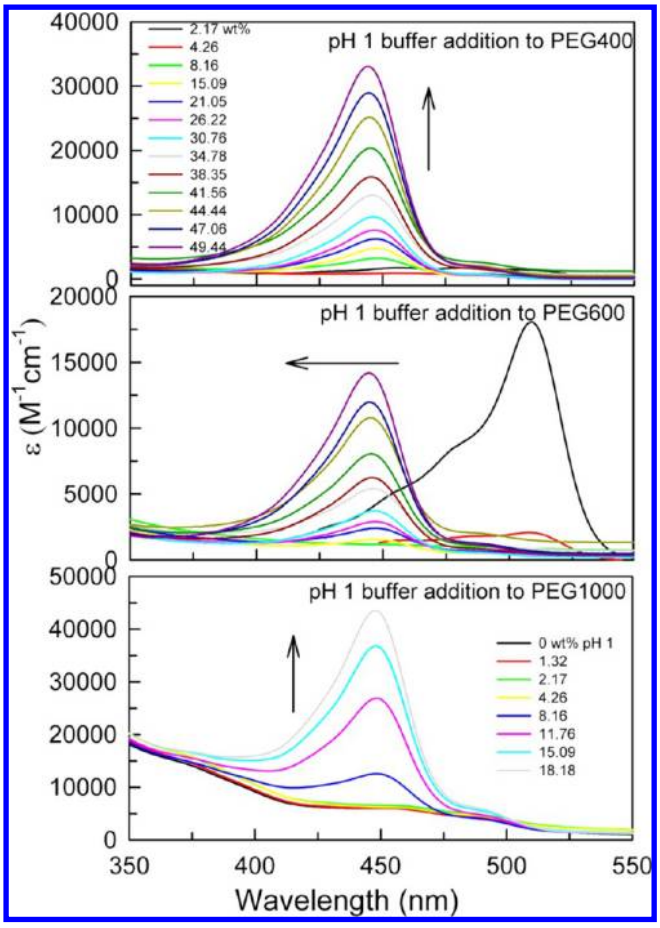

Figure 9. Absorbance spectra of fluorescein in pH 1 buffer added aqueous PEG mixtures formed with different average MW PEGs at high weight % buffer at ambient conditions.

that H-bonding between fluorescein and the solvent led to a hypsochromic shift of the absorbance as well as the fluorescence emission maxima accompanied by an increase in the fluorescence quantum yield.[27] The addition of PEG200 to fluorescein dissolved in pH 13 buffer results in several interesting outcomes. As the amount of PEG200 is increased in the solution, bathochromic shifts in both absorbance and fluorescence emission maxima (Figure 10) are accompanied by decrease in molar absorptivity and relative fluorescence intensity. The decrease in molar absorptivity appears to be a medium effect where the composition of aqueous PEG200 mixture is changing from buffer-rich to PEG200-rich. This decrease in molar absorptivity may result in decreased fluorescence intensity; contribution from a reduction in fluorescence quantum yield in the presence of PEG200 may not be ruled out. The bathochromic shift in the absorbance as well as fluorescence emission maxima is fairly significant as 90 wt % PEG200 is added to fluorescein dissolved in pH 13 buffer—shifts of 15 and 14 nm are observed in the absorbance and fluorescence emission maxima, respectively (Figure 10).

For solvatochromic probes, bathochromic or hypsochromic shifts in absorbance and fluorescence band maxima are usually observed as the physicochemical properties of the cybotactic region are changed. In order to establish correlation, if any, between the observed spectral shifts and physicochemical properties of the milieu, we obtained the Kamlet–Taft parameters π^* [dipolarity/polarizability], α [H-bond-donating (HBD) acidity], and β [H-bond-accepting (HBA) basicity] of aqueous mixtures of PEG200 from the literature.[26] As reported in the literature, the H-bonding between the fluorescein dianion and the solvent leads to the hypsochromic shift of the absorbance and fluorescence band maxima.[27,28] Sawyer and co-workers[28] stated that the magnitude of the spectral shift correlated with both α and β, whereas Martin[27] and Pandey[32] have shown the correlation to be acceptable only with α. As α and π^* of aqueous PEG200 decrease and β increases as PEG200 is added to water, we observe a bathochromic shift in both absorbance and fluorescence band maxima of fluorescein. Surprisingly, our correlation of fluorescein spectral shift with α is not so good, while it appears to be better with π^* and β (Figure 11).

Fluorescein Prototropism within Acidic Aqueous PEG200 Mixture. As mentioned earlier, the apparent protolytic constants relating the chemical activities of the cation, neutral, anion, and dianion forms in aqueous buffer are $pK_{a1} \sim 2.1$, $pK_{a2} \sim 4.3$, and $pK_{a3} \sim 6.4$. It is reported, however, that even at pH 1.5 the cationic and neutral species upon excitation get converted into the anionic form (fluorescence emission maxima ~515 nm) implying the "apparent" excited-state protolytic constant pK_{a1}^* and/or pK_{a2}^* to be considerably lower than the corresponding ground-state protolytic constant pK_{a1} and/or pK_{a2}.[18,19] It is further demonstrated that, even in 1 M aqueous HCl, fluorescence from anionic form dominates though an additional band grows around 470 nm which gets fully established only in 10 M aqueous HCl, a solution having very high acid strength.[18] This fluorescence feature corresponds to the excited cation, and it is concluded that the fluorescence

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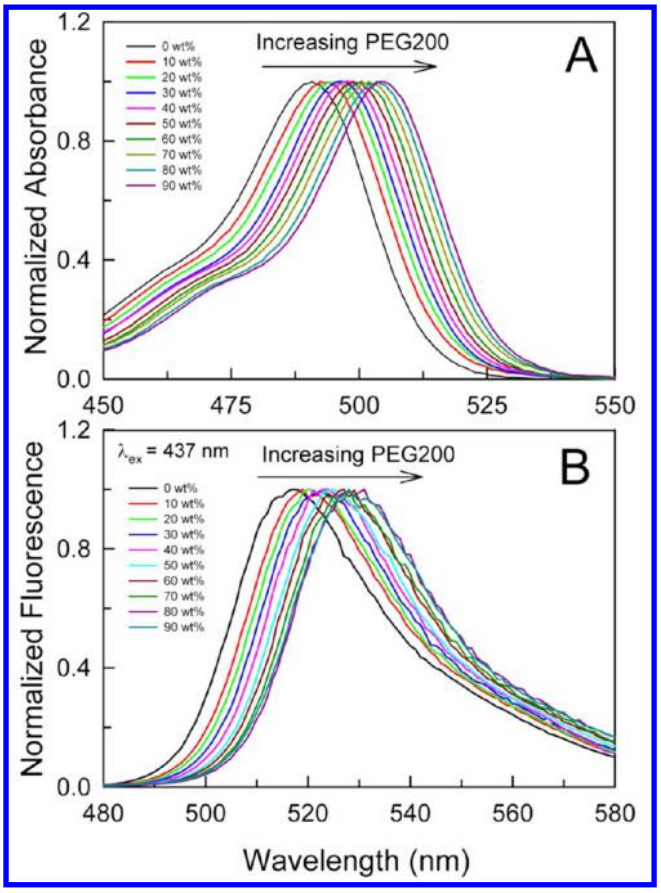

Figure 10. Normalized absorbance (panel A) and fluorescence emission spectra (panel B) of fluorescein (10 μM) dissolved in PEG200-added pH 13 buffer at ambient conditions.

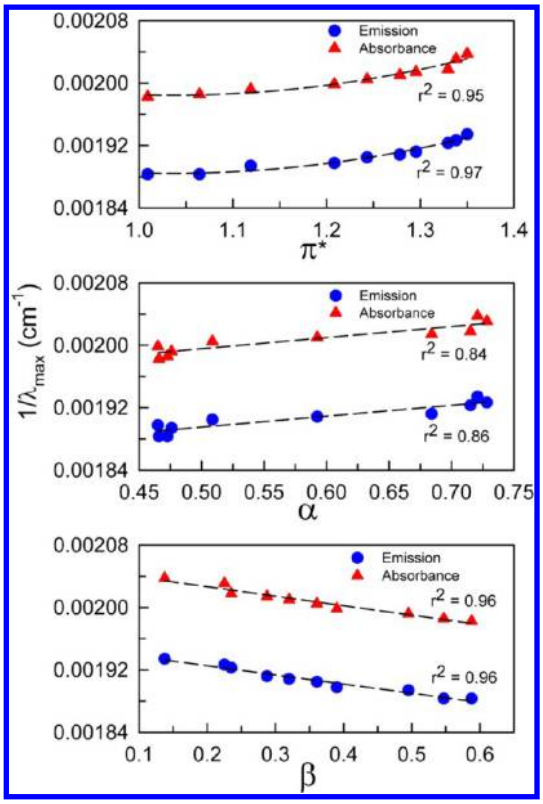

Figure 11. Correlation between absorbance/fluorescence emission maxima (λ_{max}) of fluorescein and Kamlet–Taft parameters α, β, and π^* in PEG200-added pH 13 buffer at ambient conditions.

from the cation of fluorescein can only be observed at very high acidity and that the excited neutral species has no significant fluorescence of its own under any conditions.[19] Another report emphasizes that the neutral form has identical fluorescence features to those of the monoanionic form.[18]

We observed fluorescence emission from fluorescein dissolved in 5 M aqueous HCl with maxima ca. 470 nm corresponding to emission from the cationic species. On adding PEG200, a sideband begins to develop at ~515 nm corresponding to neutral/monoanionic species (Figure 12). This is probably because PEG200, which supports the dianionic form of fluorescein, decreases the acidity of the medium due to which some of the cationic fluorescein on being excited gets converted to monoanionic/neutral form. On increasing the concentration of PEG200 in the medium, the intensity of the monoanionic/neutral emission (~515 nm) increases relative to that of the cation (~470 nm) until about 80 wt % PEG200 is added (Figure 12). Surprisingly, for PEG200 $\geq$ 80 wt %, the emission from the cation starts to increase relative to that from the monoanionic/neutral form to the extent that at 99 wt % PEG200 only fluorescein cation emission at ~478 nm is observed. It is important to mention here that the absorbance spectra of fluorescein within 5 M aqueous HCl upon PEG200 addition show the presence of only one band with maxima ca. 437 nm corresponding to the cationic species irrespective of the mixture composition (Figure 13). The molar absorptivity associated with this transition decreases and is accompanied by a bathochromic shift as PEG200 concentration is increased.

Fluorescein dissolved in lower acidity 1 M aqueous HCl solution, as expected, shows a prominent emission centered at 515 nm representing neutral and/or anionic form (vide supra). Addition of PEG200 to this mixture results in interesting outcomes (Figure 12). Very prominent fluorescence emission centered at 475 nm corresponding to emission from cation is observed as 90 wt % PEG200 is added. The emission from cation relative to the emission from monoanionic/neutral fluorescein tends to increase as up to 97 wt % PEG200 is added. Further addition of PEG200 displays fluorescence features of fluorescein corresponding to its neutral form, which is confirmed by UV–vis absorbance features as well (data not shown). This is because addition of ~1 wt % 1 M aqueous HCl to PEG200 decreases the pH of the system, changing it from >7 (in neat PEG200) to ~3–4 where the neutral colorless lactonized form of fluorescein dominates.

Fluorescence emission from fluorescein dissolved in 0.2 M aqueous HCl shows only a single band centered ~515 nm corresponding to neutral/anionic form. However, we were again able to obtain fluorescence emission corresponding to the cation (~475 nm) via addition of higher amounts of PEG200 (Figure 12). Fluorescence emission from cation is shown only for 88–92 wt % added PEG200 where the cationic form of fluorescein exists in the medium as confirmed by the absorbance spectra (Figure 13). Fluorescein dissolved in 0.01 M aqueous HCl did not show any fluorescence emission from cationic species irrespective of the concentration of PEG200 probably because 0.01 M HCl is not acidic enough to generate cationic form of fluorescein even at high PEG200 concentration (Figure 12). Figure 14 presents a summary of these outcomes as it shows an abrupt increase in fluorescence emission intensity of the cationic form relative to neutral/anionic form of

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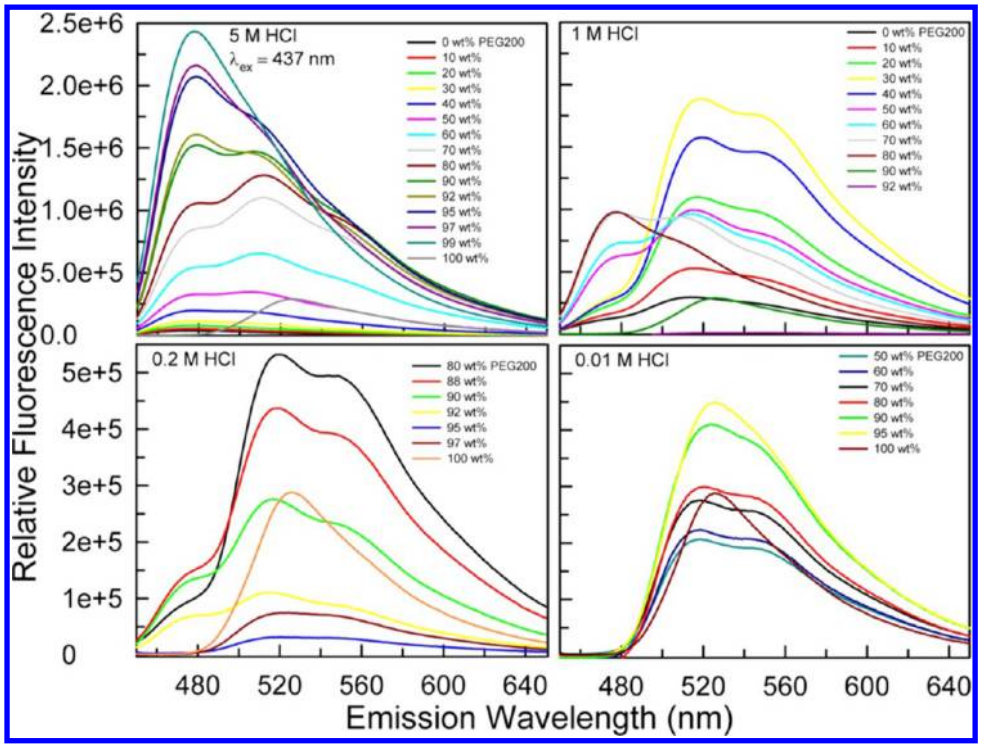

Figure 12. Effect of PEG200 addition on fluorescence emission spectra of fluorescein (10 μM) dissolved in different molarity HCl at ambient conditions.

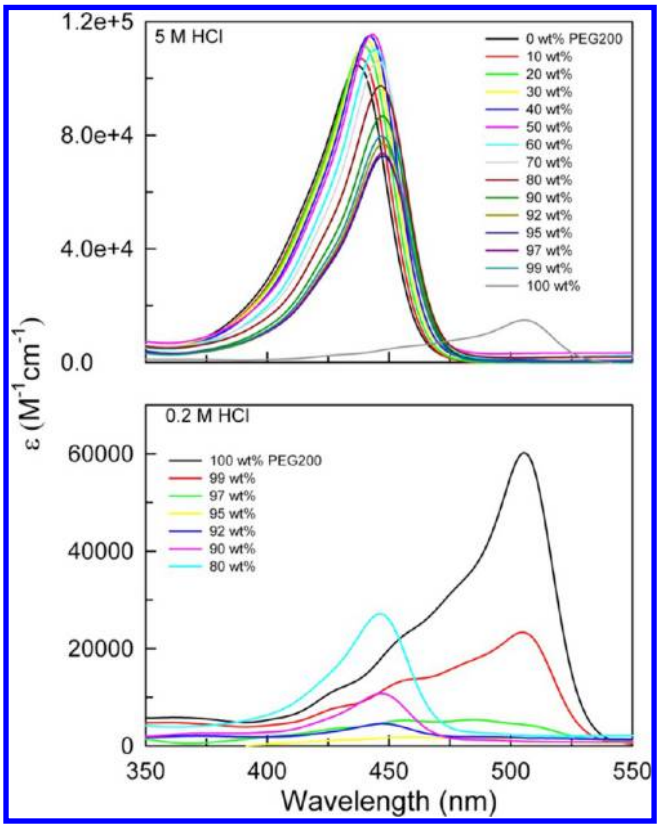

Figure 13. Effect of PEG200 addition on absorbance spectra of fluorescein dissolved in different molarity HCl at ambient conditions.

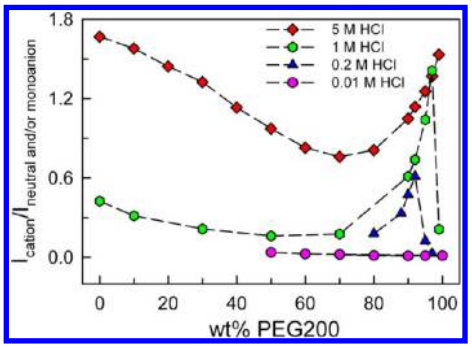

Figure 14. Effect of PEG200 addition on the ratio of the fluorescence emission intensities of the cation to the neutral/monoanionic forms of fluorescein dissolved in different molarity HCl at ambient conditions.

fluorescein in 80−99 wt % PEG200 added mixtures of 5, 1, and 0.2 M aqueous HCl. No emission from cationic form is observed within PEG200-added 0.01 M aqueous HCl mixtures.

We have observed a dramatic increase in "apparent" pK_{a1}* of fluorescein at high concentration of PEG200. PEG200 can induce fluorescence emission from fluorescein cation even from mixtures having fairly low acidity (such as 0.2 M aqueous HCl). Such unusual cationic emission of fluorescein has earlier been reported from ionic liquid [bmim][BF_4] added 0.01 and 0.2 M aqueous HCl.[22]

Photophysical Properties of Fluorescein Dissolved in PEG200 in the Presence of up to 1 wt % 5 M Aqueous HCl. As mentioned in the previous section, we observed significant changes in fluorescence emission and absorbance features as ca. 1 wt % 5 M aqueous HCl is added to fluorescein dissolved in PEG200—the fluorescence emission maxima of fluorescein shifted from ∼525 nm (corresponding to dianion) to ∼477 nm (corresponding to cation) accompanied by a 6-fold increase in fluorescence intensity. We explored further the abrupt changes in both absorption and fluorescence emission behavior of fluorescein as small amounts (0−1 wt %) of 5 M aqueous HCl is added to fluorescein dissolved in PEG200. Neat

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PEG200 supports dianionic form of fluorescein with low molar absorptivity (vide supra). On addition of ~0.1 wt % 5 M aqueous HCl to PEG200, pH of the mixture decreases, which causes the dianionic form of fluorescein to convert to monoanionic form indicated by a decrease in fluorescence emission intensity (Figure 15). Further addition (0.27 wt %) of

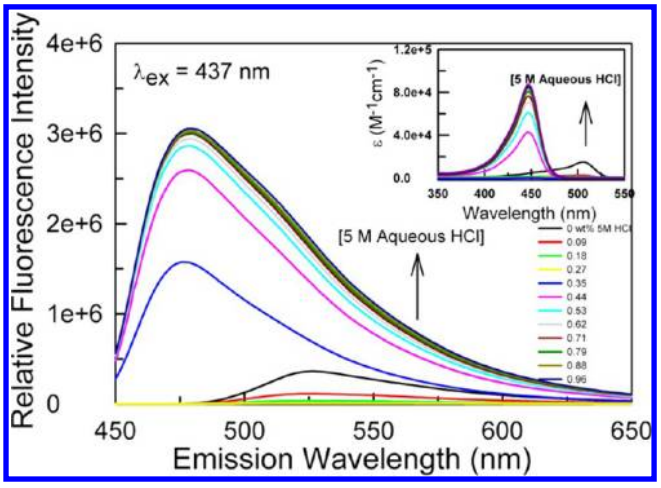

Figure 15. Fluorescence emission and absorbance (inset) spectra of fluorescein in <1 wt % 5 M aqueous HCl added PEG200 at ambient conditions.

5 M aqueous HCl converts the monoanionic form of fluorescein to colorless lactonized neutral form. Increasing the weight % of 5 M aqueous HCl from 0.27 to 0.35 wt % in PEG200 gives way to the cationic form of fluorescein with an abrupt shift in emission maxima from ~525 to ~477 nm with 150-fold increase in fluorescence intensity. The absorbance spectra of fluorescein in 5 M aqueous HCl added PEG200 also show sudden transition from neutral to cationic form on increasing the weight % of aqueous HCl from 0.35 to 0.44 (inset, Figure 15). The sudden change in both fluorescence emission and absorbance spectra of fluorescein on adding small amounts of 5 M aqueous HCl to PEG200 is surprising and, as a result, is further explored. It appears that within 5 M aqueous HCl-added PEG200-rich mixtures fluorescein exists in the highly acidic water-rich cybotactic region where rapid deprotonation of excited-state fluorescein cation is hindered due to the presence of PEG200.

Fluorescence lifetimes of dianionic (measured in 0.1 M aqueous NaOH), monoanionic (measured in pH 4.5 aqueous buffer), quinoid (measured in pH 1.6 aqueous buffer), and cationic (measured in 5 M aqueous H_2SO_4) forms of fluorescein are reported to be 4.06, 3.37, 2.97, and 3.5−4.4 ns, respectively.[18] In order to substantiate the proposition that deprotonation of excited-state fluorescein cation is hindered in the presence of PEG200, we have acquired excited-state intensity decay of fluorescein dissolved in PEG200 in the presence of 0, 0.35, 0.44, and 0.96 wt % 5 M aqueous HCl at 525 and 477 nm using 405 nm violet diode laser for excitation (Figure 16). Excited-state intensity decay data of fluorescein in mixtures having <0.35 wt % 5 M aqueous HCl in PEG200 could not be acquired as the fluorescence intensities are too low. Results of the fit of the excited-state intensity decay data to the single-exponential and double-exponential decay models are presented in Table 1. Within neat PEG200, the single-exponential decay model affords a satisfactory fit to the

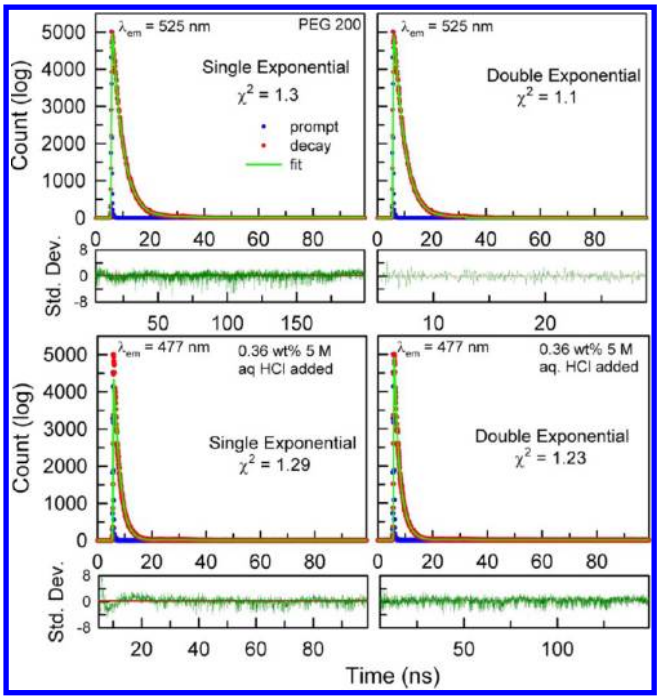

Figure 16. Excited-state intensity decay data of fluorescein (10 μM) in neat PEG200 (upper two panels) and 0.36 wt % 5 M aqueous HCl added PEG200 (lower two panels) at ambient conditions.

Table 1. Recovered Intensity Decay Parameters along with the Goodness of Fit (χ^2) for Fluorescein Dissolved in <1 wt % 5 M Aqueous HCl Added PEG200 at Ambient Conditions[a]

wt % 5 M aqueous HCl	τ_1/ns (α_1/%)	τ_2/ns (α_2/%)	χ^2
	λ_{em} = 525 nm		
0 wt % (neat PEG200)	4.0		1.3
	1.9 (11)	3.8 (89)	1.1
0.35 wt %	2.5		1.3
	1.4 (25)	2.7 (75)	1.0
0.44 wt %	2.6		1.2
	1.6 (27)	2.8 (73)	1.1
0.96 wt %	2.6		1.4
	1.82 (46)	2.9 (54)	1.1
	λ_{em} = 477 nm		
0.35 wt %	2.3		1.4
	0.8 (34)	2.4 (66)	1.1
0.44 wt %	2.4		1.2
	0.8 (32)	2.6 (68)	1.0
0.96 wt %	2.2		1.6
	0.9 (38)	2.6 (62)	1.1

[a]Errors in recovered decay times (τ's) and pre-exponential factors (α's) are <±0.2 ns and <±5%, respectively.

fluorescein excited-state intensity decay data with χ^2 = 1.3 suggesting the presence of dianionic form of fluorescein (τ = 4.06 ns). A fit to double-exponential model marginally improves the χ^2 of fitting with a major decay time of 3.8 ns, corresponding to the dianion, and a second decay time of 1.9 ns corresponding to the presence of monoanion/quinoid species. However, as 0.35 wt % 5 M aqueous HCl is added to PEG200, the fluorescence lifetime of fluorescein undergoes a dramatic change (τ changes from 4.0 to 2.5 ns as per single-exponential decay model). The fluorescence lifetime of fluorescein remains

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almost the same as the wt% of 5 M aqueous HCl is increased from 0.35 to 0.96 in PEG200. This is in accordance with the steady-state fluorescence emission data (Figure 15), where we were able to observe fluorescence emission from fluorescein cation ($\lambda_{max}^{em} \sim 477$ nm) on addition of 0.35 wt % 5 M aqueous HCl to PEG200, which persisted till 0.96 wt % 5 M aqueous HCl-added PEG200. Thus, the excited-state intensity decay data of fluorescein dissolved in 5 M aqueous HCl-added PEG200 also support the data obtained from static spectroscopic measurements. Overall, the deprotonation of excited fluorescein cation is shown to be hindered in the presence of small amounts of 5 M aqueous HCl within PEG200.

■ CONCLUSIONS

Liquid PEGs as solubilizing milieu have an interesting and unprecedented effect on the prototropic behavior of fluorescein. They cause efficient lactonization when added to buffer solutions of fluorescein: greater the average MW of PEG, greater the extent of lactonization. Neat PEG200, PEG400, and PEG600 support the dianionic form of fluorescein. On the other hand, colorless lactonized neutral form of fluorescein dominates in neat PEG1000. Other prototropic forms can be produced by the addition of appropriate acidic buffer to fluorescein solution in PEGs of different average MWs. Fluorescence emission from the cationic form of fluorescein that is only observed from aqueous solutions of extremely high acidity (>5 M $[H^+]$) could be observed from mildly acidic solutions in the presence of high concentrations of PEG200. Formation of a highly acidic water-rich solvation microenvironment around fluorescein cation at high PEG200 concentrations is proposed to be the reason for this observation.

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Notes

The authors declare no competing financial interest.

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