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Fluorescence lifetime based pH sensing using Resorufin.

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ABSTRACT

Accurate, non-contact pH sensing is of particular importance in the biological and clinical sciences. Fluorescence lifetime based pH sensing is potentially more useful than intensity based methods because of the reduced sensitivity to excitation source intensity variations, scattering effects, and photobleaching. In this work, we investigate the variation of fluorescence lifetime with pH for resorufin. The intensity averaged lifetime ($\bar{\tau}$) of resorufin sodium salt in 0.1M phosphate buffer shows an increase of > 3 ns over the 2 – 10 pH range, with 90% of the signal change occurring between pH 4 and 8. The fluorescence is not quenched by chloride or oxygen and was unaffected by the ionic strength of the buffer. Resorufin is relatively insoluble in non-alkaline phosphate buffered solutions, but $\bar{\tau}$ was estimated to increase by ~2 ns between pH 6 and 8. Resorufin and its sodium salt were both incorporated into sol-gels by either acid or base hydrolysis of tetra-methoxysilane (TMOS). Various surfactants were also added to the sol-gels in an attempt to optimise the fluorescence properties and pH sensitivity of the dyes, and to prevent cracking. The sols were then cast from petri-dishes or dip-coated onto acrylic and glass slides. The dyes retained their pH sensitivity, with $\bar{\tau}$ showing an increase of approximately 2 ns over the pH range 6 – 8. However, leaching of the dye is observed at higher pH and attempt to minimise dye leaching and sol-gel cracking, poly(vinyl alcohol) (PVA) was cross-linked to the silica gel to form a more flexible matrix.

Keywords: Fluorescence, lifetime, pH, sensors, sol-gel, resorufin.

1. INTRODUCTION:

Accurate, non-contact pH sensing is of particular importance in the biological and clinical sciences and optical methods have significant advantages over more traditional electrochemical methods methods. These advantages include: reduced sensitivity to electrical interference, rapid response times, miniaturised fibre-optic probes.[1] Measuring pH by fluorescence-based methods is well established for both imaging and sensing applications, and offers significant advantages over other optical methods due to its high sensitivity, high specificity, and wide range of indicator dyes.[2] Fluorescence lifetime based techniques have several advantages over more traditional fluorescence intensity methods which are susceptible to changes in excitation light intensity, to photobleaching, and to variation in light scattering and absorption of the sample. [3]

In lifetime based pH sensing a number of different approaches have been demonstrated: 1) indicator acid and base forms have different lifetimes, 2) a mixture of fluorophores only one of which is sensitive to pH, resulting in a change in the measured lifetime due to a change in the fractional contributions,[4] and 3) Fluorescence Resonant Energy Transfer (FRET). [5] The most common sensing method is that based on acid/base forms of the indicator having different lifetimes, examples of which are: carboxy SNAFL-1 for intracellular microscopy based pH measurements,[6] fibre optic based systems using carboxy-SNAFL-2, [7] and carboxy SNARF-6.[8, 9] In each case the indicator dye or the dye/matrix sensing layer should have an apparent pKa* (excited state pKa) close to the desired pH sensing range.

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Resorufin in 0.1 M citrate-phosphate buffer had been previously studied using phase-modulation measurements and 442 nm laser excitation which had shown that there was a relatively large lifetime change with pH over the pH 4 to 8 range.[7] This dye, which can be efficiently excited using inexpensive blue or green LED light sources, is therefore a good candidate for use as a lifetime based pH sensor. There have been few reports on the use of resorufin as a lifetime sensor or regarding its immobilisation into any supporting matrix suitable for pH sensing. We have therefore decided to study the effects of immobilising resorufin in a number of different sol-gels and modified sol-gels cross-linked with Polyvinylalcohol (PVA).[10] To adjust the pH sensing range of resorufin doped sol-gels we also investigated the addition of surfactants to the sol-gels. This has been shown to be a facile method of adjusting the apparent pKa of immobilised indicators and should help to produce sensors with the optimum pH range for biological and clinical applications.[11, 12] Our goal was to determine whether or not a suitable pH sensing scheme could be developed using 460 nm LED excitation and sol-gel matrices.

2. EXPERIMENTAL:

2.1 Apparatus and Procedure:

UV-Visible spectra were recorded with a Shimadzu UV-1601 UV-visible spectrophotometer, and steady state fluorescence spectra were measured using a Perkin Elmer LS 50B luminescence spectrometer. Fluorescence lifetimes were recorded using a Time Correlated Single Photon Counting (TCSPC) system that was assembled in-house using modular components. The excitation source was a 460 nm pulsed LED and the emission wavelength was selected by means of interference filters.[13, 14] The Instrument Response Function (IRF) was obtained from a non-fluorescing suspension of alumina in water held in either 1 or 10 mm pathlength quartz cells and was assumed to be wavelength independent. Lifetimes were obtained by deconvolution of the decay curves using the FluoFit software program (PicoQuant GmbH, Germany). All lifetimes were fit to a χ^2 value of less than 1.2 and with a residuals trace that was fully symmetrical about the zero axis. The average lifetimes quoted throughout are the intensity averaged lifetime, defined as $\bar{\tau} = \Sigma\alpha_i\tau_i^2 / \Sigma\alpha_i\tau_i$.

2.2 Materials:

Resorufin, resorufin sodium salt, tetra-methoxysilane (TMOS), polyvinylalcohol (PVA), and all buffer materials were Analar grade, obtained from Sigma-Aldrich, and were used without any further purification. The resorufin-doped sol-gels were made up using the sodium salt derivative as this is more soluble in both non-alkaline aqueous and methanolic solutions (Table 1).[15]

Matrix	pH	water:TMOS ratio (R)	Surfactant	Morphology	Remarks
S1	Acid	4.18	None	Dip-coated	Dye precipitated out at low pH during the aging process, not studied.
S2	Neutral	7.8	None	Monolith	Largely crack free monoliths, ~1.5 mm thick and <4 cm diameter.
S3	Neutral	7.8	Triton X100	Monolith	Largely crack free monoliths, ~1.5 mm thick and <4 cm diameter.
S4	Base	4.15	None	Monolith	Cracking was more extensive than for S2/S3
S5	Base	4.15	Triton X100	Monolith	Fragmented on exposure of the monolith to aqueous solution, not studied.

Table 1: Chemical characteristics for the resorufin doped sol-gel and TMOS-PVA matrices.

The properties of the final sol-gel material depend on, among other factors, the water concentration, and pH. Changes in the water:TMOS ratio (R value) and/or pH during fabrication affects the porosity of the final sol-gel. The aim was to investigate if changing sol-gel properties could alter the pH sensitivity of immobilised resorufin. We also modified the pH response of the resorufin doped sol-gels by the addition of Triton X-100 surfactant (S3/S5). Addition of surfactants to sol-gels has been shown to shift the ground state pKa of entrapped indicators with a resulting shift in the absorption based pH response curves. [11,12] A similar effect would be expected for the excited state pKa (pKa*), allowing the tuning of the fluorescence lifetime pH response curves.

Resorufin doped PVA:TMOS copolymers (TP1 and TP2) were synthesised by slightly modifying the method of Cajlakovic et al. [10] TP1 and TP2 were identical to the L2 and L3 schemes outlined in Ref. 10. The only significant difference in the method is that resorufin was added in 1 cm^3 aliquots (0.01M resorufin sodium salt in methanol) to the PVA/TMOS/EtOH/HCl mixtures. Once the copolymers had been dip-coated (~0.25 μm thick) onto the activated glass slides they were conditioned in pH 3 phosphate buffer for several days. No leaching of resorufin from the copolymers was observed at this pH. The final concentration of resorufin in the films was not calculated but is probably less than 10^{-3} M. We also attempted to produce a sol-gel with a lower PVA content (identical to L1 in Ref. 10) but this was found to gel too quickly for dipcoating. This effect may be due to the presence of resorufin itself.[15]

3. RESULTS AND DISCUSSION

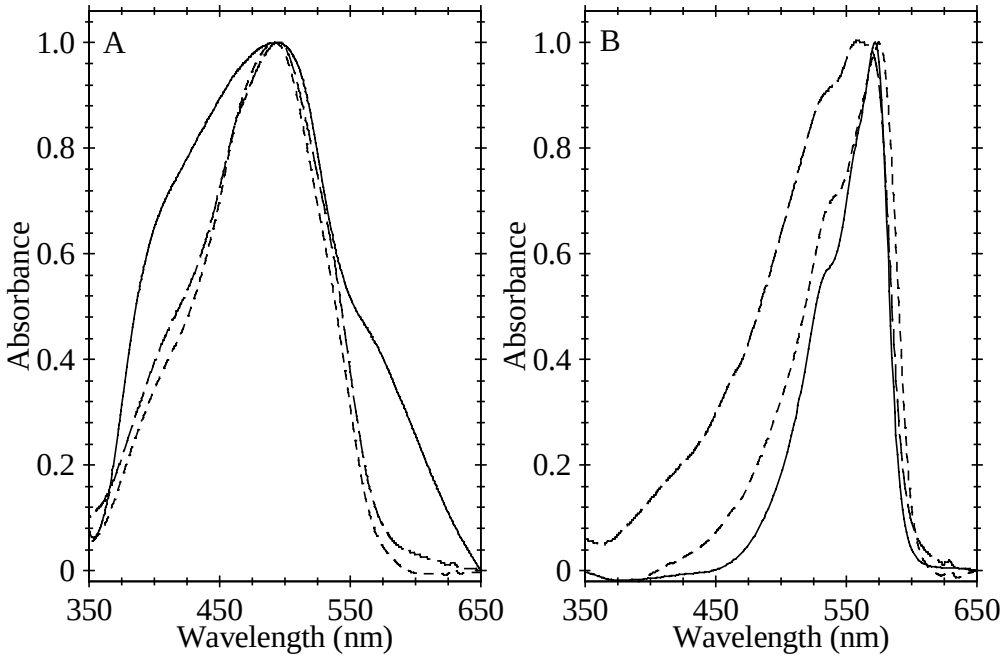

Figure 1: Absorption spectra of resorufin in solution (—), sol-gel scheme S2 (– – –) and scheme S3 (-----), at low and high pH. (A) Low pH case: resorufin in 0.9M HNO_3 solution, and in S2 and S3 at pH 3.1 and 3.6 respectively (0.1M phosphate buffer). (B) High pH case: resorufin in 0.9M NaOH solution; in S2 (pH 9.0, 0.1 M phosphate buffer); and S3 (pH 9.0, 0.1 M phosphate buffer). All spectra were normalised at their wavelength of maximum absorbance.

3.1 Absorption and Fluorescence emission spectra:

The absorption spectra of resorufin in solution, and when doped in sol-gel (S2 / S3), are strongly affected by changes in pH (Fig. 1), with the spectra recorded at low pH[†] being weaker than those at high pH. The protonated form of resorufin (RH) has an absorption maximum at 492 nm, which is the same when loaded into the S2 and S3 sol-gels (Fig. 1A). S4 sol-gels had the same absorption spectra as S2.[15] The only significant difference on loading into sol-gel is the disappearance of the shoulder at ~ 600 nm which is due to the change in environment. The resorufin anion (R^-) in alkaline solution has a maximum absorption at 572 nm, with a shorter wavelength shoulder around 535 nm, which is in good agreement with previous studies [16]. At high pH ~9, immobilisation of resorufin in the pure sol-gel (S2) results in a hypsochromic shift in the absorption maximum to 560 nm when compared to the resorufin anion (R^-) in solution (Fig. 1B). This again is probably due to changes in the polarity of the microenvironment of the dye, shifting the pKa to higher pH, a similar effect was also observed with aminofluorescein-doped sol-gels.[12] R^- in S2 also absorbs over a wider wavelength range than R^- in solution, and this coupled with the fact that the fluorescence decay is bi-exponential (Section 3.2) indicates the presence of RH in S2 at pH 9. The surfactant modified sol-gel (S3) has a very similar absorption spectrum to that of R^- in solution indicating that the surfactant has counteracted part of the pKa change induced by incorporation into sol-gel. At high pH (>12), however, resorufin also undergoes a series of reactions, which eventually results in the formation of a colourless, non-fluorescent solution, which could be the colourless hydroresorufin derivative. [17] All the measurements made in this study were done with freshly made solutions to avoid any interference from these side reactions.

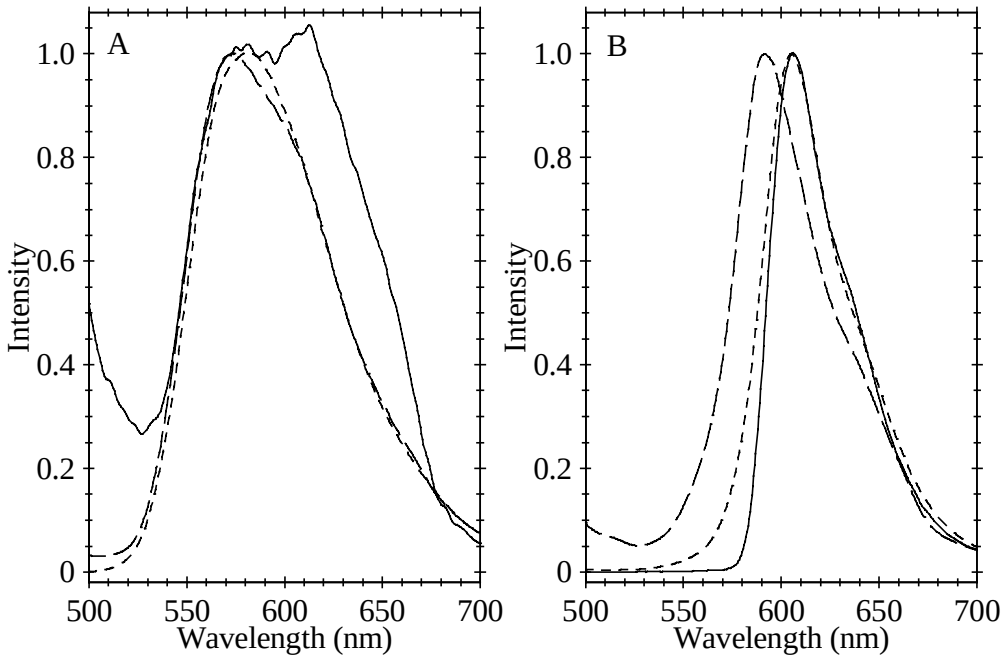

Figure 2: Steady state emission spectra of resorufin in solution, sol-gel scheme 2 (S2), and scheme 3 (S3). (A) Solution pH 2.6 (——), S2 pH 3.1 (– – –), S3 pH 3.6 (-----). (B) Solution pH 9.0 (——), S2 pH 9.0 (– – –), S3 pH 9.0 (-----). Spectra were normalised at their emission maxima except RH in solution, which was normalised at 573 nm. Spectra were recorded in 0.1M phosphate buffer using 460 nm excitation.

[†] For all sol-gels, accurate absorption spectra could not be not recorded below pH 3 due to very low absorption intensity.

R^- in alkaline solution has a relatively intense fluorescence emission spectrum with a maximum at 604 nm, while the RH fluorescence is much broader and weaker with a maximum at 612 nm and a second, shorter wavelength peak around 565 nm (Fig. 2). RH in S2 and S4 (not shown) fluoresces over a similar range to RH in solution (Fig. 2A) and exhibits a single well-resolved maximum at ~573 nm. This is blue shifted from that of the dye in solution, although the latter is very noisy due to extremely low emission intensity. The emission from RH in S2 is an order of magnitude more intense than that in solution. Addition of TX-100 to the sol-gel matrix (S3) has little effect on the steady state emission of RH.

As pH increases the emission from sol-gel immobilised resorufin becomes more intense. Immobilisation of the dye in pure sol-gel (S2) causes a hypsochromic shift of 15 nm in the emission spectrum of R^- (Fig. 2B). In the S3 sol-gel, the addition of the TX-100 causes a bathochromic shift (relative to S2) and R^- emission in S3 is practically identical to the solution case.

3.2 Fluorescence lifetimes:

The fluorescence lifetime of R^-, in 0.1 M phosphate buffer at pH 12.2, is a single exponential with a lifetime of 3.3 (550 nm) or 3.4 (600 & 650 nm) ns depending on the emission wavelength. At low pH[¥] (~2.6) the situation is more complex, with there being a marked dependence on the emission wavelength at which the lifetime is measured. At an emission wavelength of 550 nm the decay is a single exponential with a lifetime of 0.3 ns, while at longer wavelengths the decay becomes bi-exponential with an average lifetime of 0.67 ns at 600 nm and 0.55 ns at 650 nm, indicating the presence of some unprotonated anion. Plotting this lifetime change over the pH range 2.6 to 12.2 (Fig. 3) shows that the decay changes from a bi-exponential to a single exponential above pH 8, as the contribution of the protonated species diminishes.

In fitting this experimental data, the short lifetime (τ_1 due to RH) was kept constant at 0.3 ns while the remaining parameters (relative amplitudes and τ_2) were allowed to vary. At the low pH between 2.6 and 4.0, the lifetime (τ_2) of the longer-lived component undergoes an initial sharp increase, before remaining relatively constant up to pH 8. Above pH 8, the decay fits to a single exponential, indicating that R^- is the sole emitting species, and fluorescence from RH is no longer a contributing factor. Resorufin exhibits similar decay characteristics at 632 and 650 nm to those observed at 600 nm with the decays being bi-exponential below pH 8, and mono-exponential above pH 8. The relative amplitudes of each decay component also vary in a similar manner to those recorded at 600 nm. In each case, the change in average lifetime ($\bar{\tau}$) with pH is similar, with an increase of ~3 ns over the pH range 2.6 – 9.[15]

The decay characteristics of resorufin in 0.1M phosphate buffer are somewhat different at 550 nm to the other wavelengths investigated. There is a greater contribution from RH at higher pH values. For example, at pH 8.1, at emission wavelength 550 nm, the contribution from RH, accounts for 30% of the overall decay, while at the other wavelengths the contribution is negligible. This is to be expected, as examination of the steady state emission spectrum (Fig. 2) shows the fluorescence intensity of R^- is extremely weak at 550 nm in comparison to that at 600, 632, and 650 nm. As a result, at 550 nm $\bar{\tau}$ shows greater sensitivity at higher pH values (Fig. 3). Again, there is an increase of ~ 3 ns in $\bar{\tau}$ between pH 2.6 and 10, but over 80% of this change (2.6 ns) occurs over the 5 – 8 pH range. Unfortunately, the pH response is best over the 5 – 6 range, with only 1.3 ns change in $\bar{\tau}$ between pH 6 and 8. The fluorescence lifetime of phosphate buffered solutions of resorufin were also found to be unaffected by the presence of dissolved oxygen, chloride, or changes in buffer molarity (0.05 to 0.15 M) over the pH range 5.5 to 8.5.

[¥] The low fluorescence intensity of RH at pH < 2.6 prevented the measurement of accurate lifetimes.

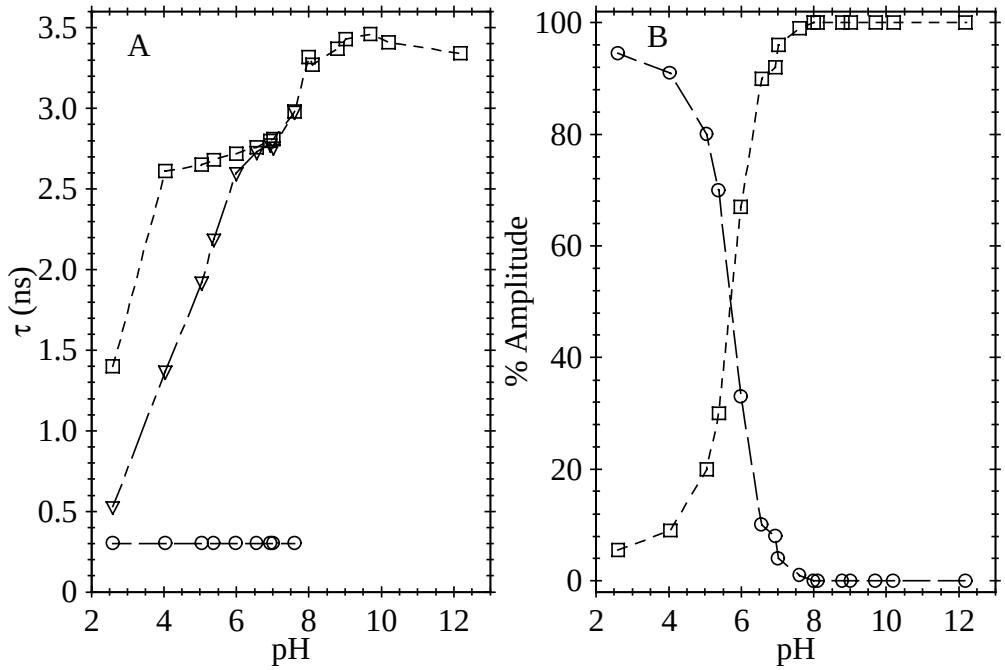

Figure 3: Resorufin in 0.1m phosphate buffer. (A) τ_1 (o), τ_2 (□), and $\bar{\tau}$ (∇) plotted as a function of pH. Emission wavelength 600 nm. (B) % Amplitude of τ_1 (o) and τ_2 (□) plotted as a function of pH.

Resorufin doped sol-gel matrices (S2/S3/S4) also show pH dependence (Fig. 4). At an emission wavelength of 600 nm, the effect of pH on the fluorescence lifetime for S2 and S4 are very similar (Fig. 4A), indicating that increased pH and decreased water content during sol-gel fabrication therefore has no significant effect on the pH response curve of sol-gel immobilised resorufin. There is, however, a significant difference between S2/S4 and S3, indicating that the surfactant has a significant influence on the pKa* of resorufin. This same effect is seen at emission wavelengths of 550 nm (Fig. 4C) and 650 nm (Fig. 4D). At 550 nm, however, the change in $\bar{\tau}$ with pH is much reduced since at this emission wavelength the longer lifetime R^- species only has a very weak fluorescence and as such a smaller contribution to the overall fluorescence.

Inclusion of resorufin into sol-gel matrices results in a number of changes to the average lifetime versus pH curves. The first point of note is that the fluorescence lifetime is considerably reduced to ~2.3 to 2.5 ns as opposed to ~3.5 ns in solution at pH 9 (Fig 4A). The difference in the lifetime vs. pH curves between the sol-gels and resorufin in solution is due to the acidic microenvironment within the sol-gels. Immobilisation of indicator dyes in sol-gel affects both the excited state acid-base equilibrium and apparent excited state pKa (pKa*). In solution, the approximate pKa* (calculated from the point at which the contribution of each lifetime component is equal) is ~5.7, while in S2/S4 it is ~ 6.8 and in S3 it occurs at ~ 6.4 (Fig 3B & 4B). More accurate values for pKa* cannot be obtained from the sol-gel materials because of extensive leaching at high pH (>9.0). In S2, at pH 9.0, the contribution from RH is ~36%, while at similar pH in solution the dye exists solely as R^- (Fig. 3 & 4). Due to the increased pKa*, and the relatively greater contribution from the shorter lifetime RH, $\bar{\tau}$ is much reduced in the sol-gel as opposed to solution. Entrapment also results in $\bar{\tau}$ having a lower sensitivity to pH, as evidenced by the smaller change of 1.48 ns compared to 2.6 ns over the pH range 3 – 9. This decreased pH sensitivity and lengthening of the pH response curve has been reported elsewhere for other sol-gel immobilised indicators.

Addition of surfactants to sol-gels has been shown to shift the ground state pKa of entrapped indicators and thus provide a convenient method for fine-tuning the response of optical sensors.[11,12] In the S3 case, the addition of non-ionic TX-100 to

the sol-gel causes the apparent pKa* to shift to lower pH. TX-100 is non-ionic and therefore, any pKa* change is not due to ionic interactions between resorufin and the surfactant, but instead must be due to molecular reorientation of the resorufin into more compatible domains within the matrix. The small decrease in pKa* (~0.4 pH units) is probably due to R^- anions being located in more hydrophobic domains created within the S3 sol-gel by the non-ionic TX-100. As such, more acidic conditions are required for protonation and the pH response curve was shifted to lower pH values. The S3 system has a slightly larger change in $\bar{\tau}$ over the 3 – 9 range (Fig. 4A), but there is only a small ~0.5 ns increase in the range pH 6 - 8 which makes it unsuitable for biological applications. .

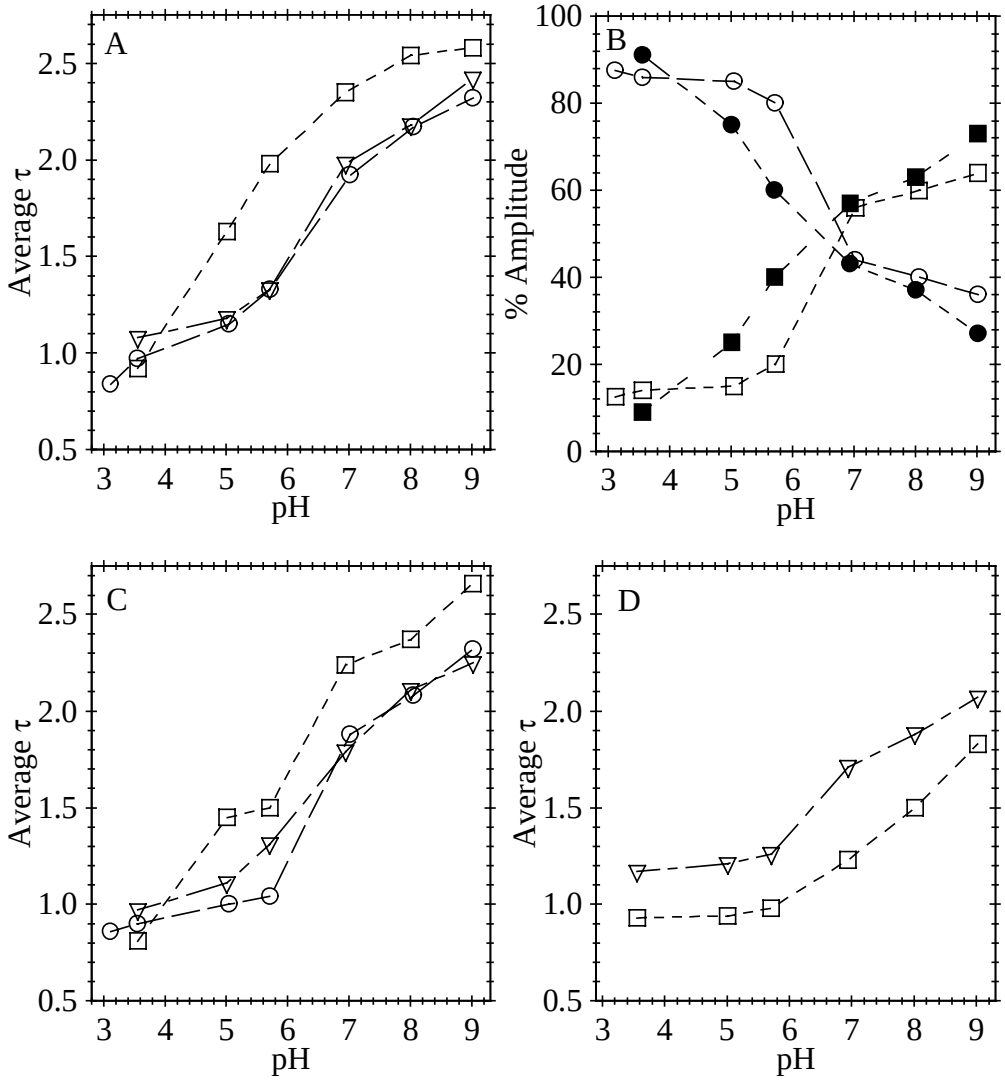

Figure 4: (A&C&D) Average fluorescence lifetime ($\bar{\tau}$) variation with pH for resorufin doped sol-gels: S2 (o), S3 (□) and S4 (∇), at emission wavelengths of (A) 600 nm, (C) 650 nm, and (D) 550 nm. (B) Percentage amplitudes of τ_1 and τ_2 at emission wavelength 600 nm: (o) τ_1 in S2, (□) τ_2 in S2, (●) τ_1 in S3 and (■) τ_2 in S3. All measurements made using 460 nm excitation in 0.1M phosphate buffers.

3.3 Resorufin in TMOS-PVA copolymers.

TP1 shows a ~0.8 ns change in $\bar{\tau}$ over the pH 5.8 to 8.0 range (Fig. 5) with the decay being bi-exponential in the range pH 5.8 – 7 and, above pH 8, the decay becomes mono-exponential with only the anion being present. Lifetimes could not be measured below pH ~5.5 because the fluorescence emission from the ~0.25 μm thick films was too weak.
In TP2, the fluorescence decay is mono-exponential with a constant lifetime of ~2.7 ns over the pH 5.8 to 8 at both emission wavelengths. We surmise that, as the PVA content increases, the matrix becomes more lipophilic and hydrophobic, resulting in the pKa* being shifted to lower pH. For both TP1 and TP2 it was not possible to make accurate lifetime measurements above ~pH 8.0 because of extensive leaching.

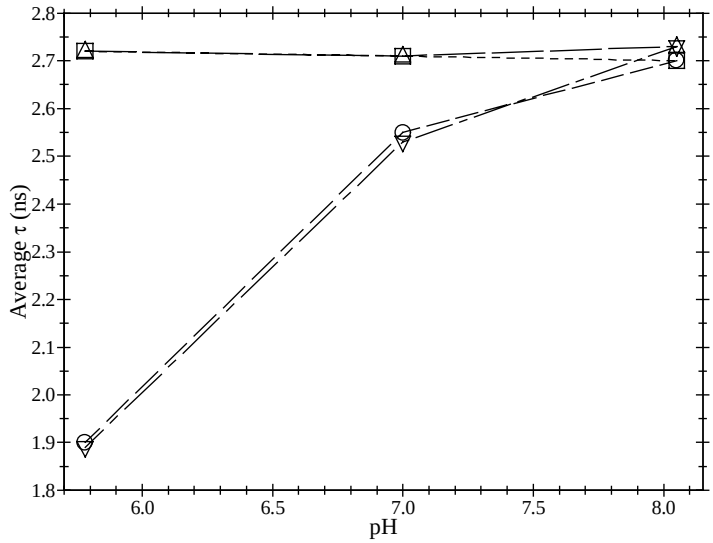

Figure 5: Average lifetime versus pH for resorufin doped TP1 at 600 nm (o) and 650 nm (∇), and TP2 at 660 nm (Δ) and 650 nm (□). Lifetimes were recorded in 0.1M phosphate buffer with 460 nm excitation.

4. CONCLUSIONS:

Resorufin in aqueous solution shows potential for use as a lifetime based pH probe since it is has a relatively large lifetime change of ~3.5 ns ($\bar{\tau}$ at 600 nm) over pH 2 to 10, is not quenched by oxygen or chloride, and is unaffected by changes in buffer concentration. Furthermore, it can be excited using inexpensive 460 nm LED light sources. The pH sensing range of resorufin can be shifted slightly to different pH ranges by measuring the lifetime at different emission wavelengths. Emission wavelength 550 nm gave the best response over the desired 6 – 8 pH range, although the optimum sensing range at this emission wavelength is between pH 5 - 6. At longer emission wavelengths, 600 and 650 nm, the best sensing range with the largest average lifetime change is between pH 2 – 6.

Resorufin can be introduced into sol-gels and retains its lifetime based pH sensitivity. However, there is a decrease in the absolute change in $\bar{\tau}$ with pH, and a shift in the apparent pKa* which results in the flattening of the pH response curve, reducing its effectiveness as a pH sensor. The apparent pKa* and pH sensing range can be shifted by addition of detergent TX-100 to the sol-gel, and therefore the pH response can be tuned to a particular pH range. Unfortunately, all the sol-gel and TMOS-PVA copolymers supports investigated here were all susceptible to extensive leaching particularly at alkaline

pH. We are continuing to investigate the photophysical properties of resorufin in different solid matrices with a goal of preventing leaching and optimising pH response.

5. ACKNOWLEDGEMENTS:

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