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pH Fluorescent Probes: Chlorinated Fluoresceins

Feng-Yan Ge · Li-Gong Chen

Abstract A series of regiospecific chlorinated fluoresceins have been synthesized by the reaction of the regiospecific chlorinated resorcinols with chlorinated phthalic anhydride. The regioisomers were successfully separated by chromatography. The photophysical properties of the obtained chlorinated fluoresceins were examined and found their absorption and emission maxima at long wavelength with high fluorescence quantum yield. Especially, pH-dependent properties of chlorinated fluoresceins have been studied in detail. These compounds show strongly pH-sensitive range of 3.5–7.0, and have lower pK_a values than fluorescein. Furthermore, their fluorescent intensity could reach the maximum in the physiological environment of pH range 6.8–7.4. Due to higher fluorescence quantum yield and lower pK_a values, chlorinated fluoresceins will be expected to be used as excellent pH fluorescent probes for pH measurement of the acidic cell.

Keywords Fluorescent probe · Chlorinated fluorescein · Synthesis · Fluorescent properties · pH-sensitive properties

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Introduction

The measurement of the pH of biological fluids is an important aspect of a variety of assays [1–3]. The use of fluorescence-based techniques may increase the sensitivity of measurements and allow the measurement of intracellular pH in single cells [4, 5]. Therefore, as pH probe, it should allow the greatest sensitivity to small changes in pH of the medium, the equilibrium constant between the acidic and basic forms of the probe should be near the pH of the selected assay medium.

Fluorescein is the most commonly used labels in biological assays because of its solubility in aqueous buffers and high fluorescence quantum yield [6]. Because its fluorescence properties strongly depend on pH, fluorescein has been described as pH fluorescent probe. Unfortunately, for all its advantages, fluorescein also possesses significant disadvantages [7, 8]. For example, fluorescein allows pH to be measured between 5.0 to 8.5, but the pH range of the acidic cell is from 4.0 to 6.0; Thus, accurate pH determination to the acidic cell can not be achieved. Additionally, the fluorescent intensity of fluorescein could reach its maximum only at pH>8.5 [9]. But at the physiological pH range, a considerable population of fluorescein is in the protonated, nonfluorescent form. Its fluorescent properties can't be shown better. Therefore, it is essential to design and synthesize novel fluorescein derivatives as pH probes suitable for the assay in the acidic cell.

Over the years, a variety of fluorescein-based pH probes have been described, including carboxyfluorescein, fluorescein-derivatised polythiophene, fluorescein sulfonic acid and seminaphthofluorescein derivatives [10–12]. However, these fluorescein derivatives could not resolve the above problems. To our knowledge, the selective substitution of chlorine for aromatic hydrogen in organic compounds

results in profound changed in their photophysical properties [13]. Therefore, it is very important to study the fluorescein derivatives substituted by chlorine. However, the preparation and photophysical properties of chlorinated fluoresceins have not been reported systemically. We set out to determine if chlorination of fluorescein would help achieve our objective.

Therefore, a series of chlorinated fluorescein (as Fig. 1) were synthesized and separated by chromatography with the eluent of concentrated ammonia/alcohol in this paper. And their photophysical properties were characterized by absorption spectroscopy and fluorescent spectroscopy. In particular, the relationships between fluorescence properties and pH were reported in detail.

Experiments

Preparation of 2′,7′-dichlorofluorescein (1)

4-Chloro-resorcinol (7.23g, 0.050 mol) and phthalic anhydride (2.96g, 0.020 mol) were crushed and melted under dry nitrogen at 140 °C. Fused anhydrous $ZnCl_2$ (7.09g, 0.052 mol) was added slowly and the temperature was slowly increased to 150–160 °C over 2 h until the material solidified. The yellow solid was pulverized and boiled in 100 ml of 1 M HCl for 1 h. The solid was collected on a frit, washed several times with hot water, and then dried in vacuo. The yellow product 4.53 g was obtained in 56% yield.

^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.09 (s, 2H), 8.02 (d, J=7.5 Hz, 1H), 7.86 7.73 (m, 2H), 7.34 (d, J=7.8 Hz, 1H), 6.91 (s, 2H), 6.66 (s, 2H); ^{13}C NMR δ: (300 MHz, DMSO-d_6) δ: 168.97, 155.82, 152.19, 150.75, 136.62, 131.22, 128.90, 126.58, 125.78, 124.65, 116.93, 111.13, 104. 38, 82.16; MS (ESI-TOF): m/z=401.2 (M+H)[+], calcd 399.9.

Preparation of 4-chlorofluorescein (2) and 7-chlorofluorescein (3)

Following the procedure described above for compound 1, 3-chloro-phthalic anhydride (3.67 g, 0.020 mol) and resorcinol (5.50 g, 0.050 mol) gave 7.1g (76% yield) the mixture of 4(7)-chlorofluorescein. The mixture was separated by column chromatography on silica with the eluent of 1:20 concentrated ammonia/ethanol. The pure regioisomers 4-chlorofluorescein and 7-chlorofluorescein as the red solid were confirmed by MS, ^{1}H NMR and ^{13}C NMR.

Compound 2 (4-chlorofluorescein): Mp 244–246 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.12(2H, s), 7.80(1H, d, J=7.2 Hz), 7.82(1H, d, J=7.6 Hz), 7.74(1H, t, J=7.6 Hz), 6.66(2H, d, J=2 Hz), 6.63(2H, d, J=8.4 Hz), 6.54(2H, dd, J=2.4, 8.8 Hz); ^{13}C NMR(400 MHz, DMSO-d_6): δ 167.51, 159.61, 151.88, 147.90, 135.93, 132.44, 128.73, 128.32, 128.28, 123.91, 112.58, 107.44, 102.25; MS(ESI-TOF): found 367.3 (M+H)[+], calcd 366.8.

Compound 3 (7-chlorofluorescein): Mp 242–243 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.12(2H, s), 7.79(1H, d, J=7.4 Hz), 7.81(1H, d, J=7.2 Hz), 7.73(1H, t, J=7.8 Hz), 6.67(2H, d, J=2.2 Hz), 6.62(2H, d, J=8.7 Hz), 6.50(2H, dd, J=2.6, 8.9 Hz); ^{13}C NMR(400 MHz, DMSO-d_6): δ 167.31, 159.89, 152.63, 147.96, 135.73, 133.56, 132.21, 128.73, 128.38, 127.91, 111.43, 105.94, 102.23; MS(ESI-TOF): found 367.3(M+H)[+], calcd 366.8.

Preparation of 2′,7′-dichloro-4(7)-chlorofluorescein (4)

This compound was prepared according to the procedure described above for compound 1, except with 3-chloro-phthalic anhydride instead of phthalic anhydride. The title compound was obtained 3.92 g (45% yield) as yellow powder. MS (ESI-TOF): m/z=367.3 (M+H)[+], calcd 366.8.

Fig. 1 The structure of chlorinated fluoresceins
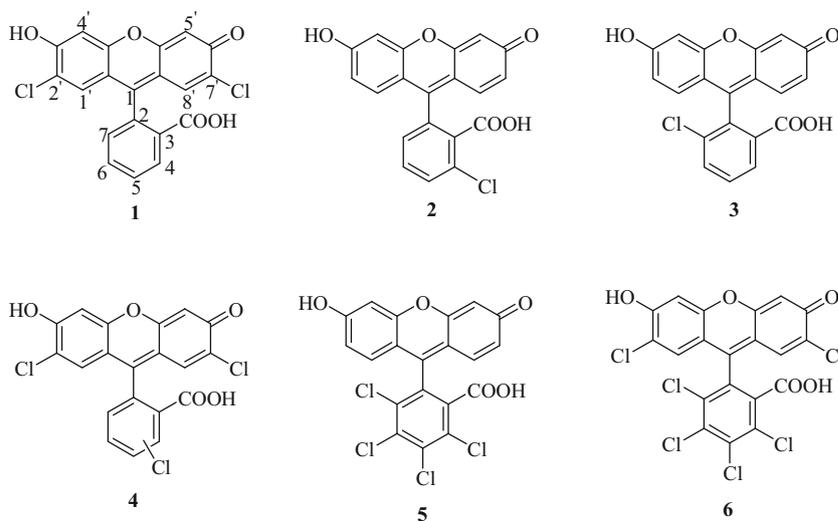
Preparation of 4,5,6,7-tetrachlorofluorescein (5)

Following the procedure described above for compound 1, 4,5,6,7-tetrachlorophthalic anhydride (5.72 g, 0.020 mol) and resorcinol (5.50 g, 0.050 mol) gave 9.24g (98% yield) of the crude product as orange solid. Then the crude product was purified by flash chromatography eluting with 1:10 concentrated ammonia/ 2-ethanol.

^{1}H NMR (300 MHz, DMSO-d$_6$) δ: 9.12 (s, 2H), 7.00 (d, J=9.0 Hz, 2H), 6.70 (s, 2H), 6.60 (d, J=8.7 Hz, 2H); ^{13}C NMR (300 MHz, DMSO-d$_6$) δ: 164.34, 153.35, 146.26, 138.14, 135.18, 130.64, 129.87, 128.32, 127.74, 114.58, 108.11, 103.07; MS (ESI-TOF): m/z=367.3 (M+H)$^+$, calcd 366.8.

Preparation of 2′,7′-dichloro-4,5,6,7-tetrachlorofluorescein (6)

Following the procedure described above for compound 1, 4,5,6,7-tetrachlorophthalic anhydride (5.72 g, 0.020 mol) and 4-chlororesorcinol (7.24 g, 0.050 mol) gave 8.00 g (85% yield) of the crude product as sad pink solid. Then the crude product was purified by flash chromatography eluting with 1:10 concentrated ammonia/ 2-ethanol.

^{1}H NMR (300 MHz, DMSO-d$_6$) δ: 10.10 (s, 2H), 7.37~7.12 (m, 2H), 6.89 (s, 1H), 6.24 (s, 1H); ^{13}C NMR (300 MHz, DMSO-d$_6$) δ: 173.31, 165.46, 156.80, 133.38, 130.94, 129.93, 128.04, 127.64, 108.72, 103.89, 62.69; MS (ESI-TOF): m/z=539.5 (M+H)$^+$, calcd 539.0.

Photophysical properties

UV-Visible spectroscopy

Absorption spectra were recorded on a UV-2450 (Shimadzu). ε was denoted molar extinction coefficient. It was calculated by following Eq. 1.

$$\varepsilon = \frac{A}{bc} \tag{1}$$

Fluorescence spectroscopy

Fluorescence spectra were recorded on JASCO FP-750 spectrofluorimeter. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by rhodamine correction curve. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath. All emission spectra of these compounds were detected by the same instrument and the same concentration.

1. Quantum yield. ϕ denotes the quantum yield for fluorescence. It was obtained by comparison of the integrated area of the corrected emission spectrum of the sample with that of a solution of fluorescein in 0.1 N NaOH, which had a quantum efficiency of 0.9 [14]. ϕ can be calculated from multiple measurements (N=3) with the following Eq. 2 (The Abs was obtained from the absorption spectra and $\int F$ was calculated by summation of fluorescence intensity).

$$\phi_{\text{sample}} = \frac{\text{Abs}_{\text{standard}} \phi_{\text{standard}} \int F_{\text{sample}}}{\text{Abs}_{\text{sample}} \int F_{\text{standard}}} \tag{2}$$

2. Stokes shift. The parameter was calculated with the following Eq. 3.

$$\text{Stokes shift} = 10^7 \left(\frac{1}{\lambda_{\text{ex}}} - \frac{1}{\lambda_{\text{em}}} \right) \tag{3}$$

pH-Dependent fluorescence studies

The fluorescence emission of chlorinated fluoresceins at maximum excitation wavelength were measured for pH variations ranging from 2 to 10.

pK_a values of the chlorinated fluoresceins were obtained by linear regression analysis of the fluorescence data to fit the Eq. 4 [15, 16].

$$\text{pH} = \text{p}K_a + c \left[\log \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right] + \log \frac{I_a}{I_b} \tag{4}$$

Where c is the slope (positive for the basic forms of the compound and negative for the acidic forms), the pK_a of the compound is the intercept, and R is the ratio of emission intensity at two wavelengths at a given pH, R_{max} and R_{min} represent limiting values of the ratio at extremes of acid or alkaline pH, respectively. I_a/I_b is the ratio of emission intensity in acid to the intensity in base at the wavelength chosen for the denominator of R.

Discussion

Syntheses of chlorinated fluoresceins

In the preparation of chlorinated fluoresceins, there were two potential approaches to obtain the title compounds. One approach was the direct chlorination of fluorescein; The other was chlorination of the building blocks of fluorescein and then preparation of chlorinated fluoresceins. Because the structure of fluorescein had several active positions, fluorescein could be readily chlorinated by electrophilic chlorination reagents. Moreover, the isolation of pure products from different chlorinated fluoresceins proved to be a difficult problem. Therefore, we selected the regiospecific synthesis of chlorinated materials as a means

Table 1 Photophysical properties of chlorinated fluoresceins

Substrate	f	1	2(3)	4	5	6
λ_{ab} (nm)	492	503	499	512	511	522
A	0.0392	0.0515	0.0242	0.0469	0.0458	0.0329
ε (mol/cm·1×10^4)	7.84	10.30	4.84	9.38	9.16	6.58
λ_{ex} (nm)	492	503	499	512	511	522
λ_{em} (nm)	514	522	518	528	527	537
Stokes shift (cm^{-1})	870.0	723.6	734.9	591.7	594.1	535.1
F	586.856	778.040	399.813	726.410	699.557	512.459
ϕ_F	0.90	0.86	0.93	0.87	0.84	0.83
pK_a	6.43	4.72	6.34	4.64	5.97	4.28

f Fluorescein; 1 2′,7′-dichlorofluorescein; 2(3) 4(7)-chlorofluorescein; 4 2′,7′-dichloro-4(7)-chlorofluorescein; 5 4,5,6,7-tetrachlorofluorescein; 6 2′,7′-dichloro-4,5,6,7-tertachlorofluorescein

to achieve the regiospecific synthesis of various chlorinated fluoresceins. In this paper, 4-chloro-resorcinol, 3-chlorophthalic anhydride and 4,5,6,7-tetrachlorophthalic anhydride were used as chlorinated materials to prepare a series of chlorinated fluoresceins, such as 2′,7′-dichlorofluorescein, 4-chlorofluorescein, 7-chlorofluorescein, 2′,7′-dichloro-4(7)-chlorofluorescein, 4,5,6,7-tetrachlorofluorescein and 2′,7′-dichloro-4,5,6,7-tetrachlorofluorescein.

Despite the ease of preparation of the mixture 4(7)-chlorofluorescein, it was difficult to obtain pure regioisomer 2 and 3. We found that the mixture of 4- and 7-chlorofluorescein could be separated by column chromatography on silica with the eluent of 1:20 concentrated ammonia/ethanol [17]. The pure regioisomers were confirmed by MS, ¹H NMR and ¹³C NMR.

Fluorescent properties

The fluorescent properties of chlorinated fluoresceins were investigated by comparison with fluorescein. The wavelength of absorption maxima (λ_{ab}), the absorption intensity, molar extinction coefficient (ε), the wavelength of fluorescence excitation maxima (λ_{ex}), the wavelength of fluorescence emission maxima (λ_{em}), Stokes shift, fluorescence quantum yield (ϕ) and pK_a were summarized in Table 1 for the novel chlorinated fluoresceins.

In general, chlorinated fluoresceins were found to shift the absorption spectra towards long wavelength, and shift of the spectra toward long wavelength increased with increase of chlorine. Such as 2′,7′-dichloro-4,5,6,7-tetrachlorofluorescein with six chlorine substitutions had absorption maxima at 522nm, and its shift was larger than that of other chlorinated fluoresceins. Similarly, chlorine substitution on fluorescein had the same effect on the fluorescence excitation spectra and fluorescence emission spectra. Comparison with fluorescein, λ_{ex} and λ_{em} produced a red shift with the electron-withdrawing ability of chlorine substituted group. However, the stokes shift of chlorinated fluoresceins decreased with increase of chlorine. In addition, chlorinated fluoresceins had high fluorescence quantum yield. Especially, 4(7)-chlorofluorescein had higer fluorescence quantum yield 0.93 than fluorescein 0.90.
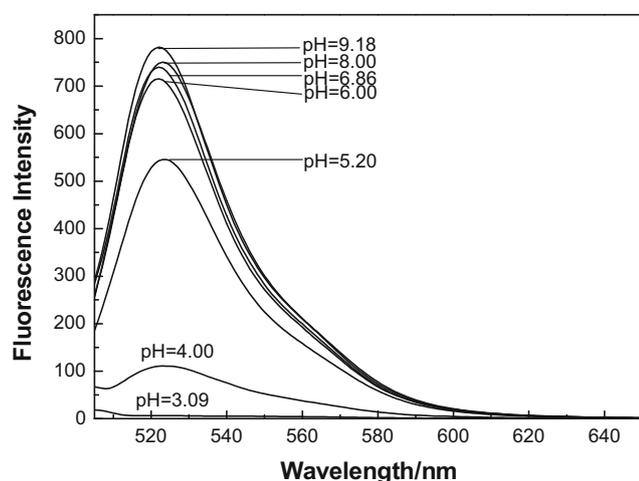
Fig. 2 Emission spectra of 2′,7′-dichlorofluorescein in buffered solution of various pH

f-----fluorescein, 1-----2′,7′-dichlorofluorescein, 2(3)-----4(7)-chlorofluorescein, 4-----2′,7′-dichloro-4(7)-chlorofluorescein, 5-----4,5,6,7-tetrafluorescein, 6-----2′,7′-dichloro-4,5,6,7-tetrafluorescein
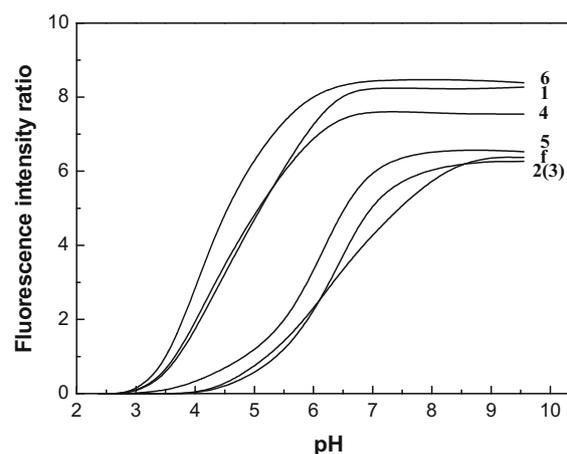
Fig. 3 Effect of pH on fluorescence intensity ratio of fluorescein derivatives with chlorine substituted
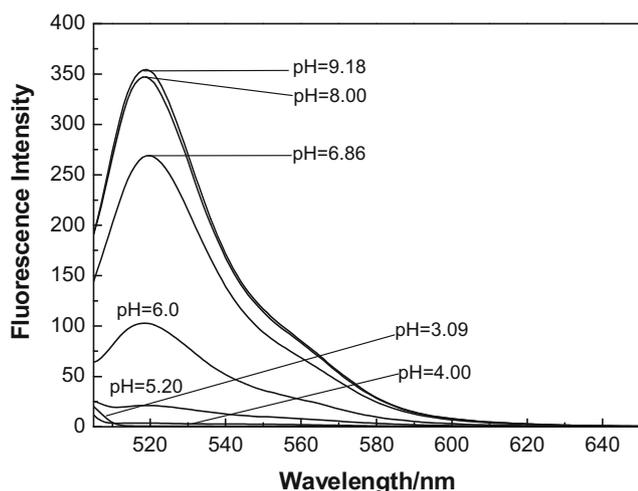
Fig. 4 Emission spectra of 4(7)-chlorofluorescein in buffered solution of various pH

Fig. 6 Emission spectra of 4,5,6,7-tetrafluorescein in buffered solution of various pH

Thus it can be seen that chlorination at 4 or 7 position will increase the fluorescence quantum yield. The reason may be that 4(7)-chlorofluorescein, which is substituted by chlorine at 4 or 7 position, has a lower population of triple relative to singlet. But chlorine substitution at the other positions on fluorescein will little or no effect on the fluorescence quantum yield.

pH-Dependent fluorescence studies

The relationship between the fluorescence intensity and pH were studied in this paper. As shown in Fig. 2, 2′,7′-dichlorofluorescein showed clear pH-dependent in the fluorescence intensity. From Fig. 3, under the condition of pH<3.5, the fluorescence intensity approached zero and 2′,7′-dichlorofluorescein had no fluorescence. With the increase in pH value, fluorescence intensity of 2′,7′-dichlorofluorescein increased. When pH was 6.5, the

fluorescence intensity reached the maximum. And when pH was also raised, the fluorescence intensity did not change. Therefore, 2′,7′-dichlorofluorescein was strongly pH dependent between 3.5 and 6.5. As can be seen from Figs. 4, 5, 6 and 7, other chlorinated fluoresceins had the similar pH-dependent relationship as 2′,7′-dichlorofluorescein. As shown in Fig. 4, 4(7)-chlorofluorescein was pH-sensitive between 4.5 and 8.0; The pH-sensitive range of 2′,7′-dichloro-4(7)-chlorofluorescein was from 3.4 to 6.3; 4,5,6,7-tetrachlorofluorescein exhibited pH-sensitive range between 4.0 and 7.0, and 2′,7′-dichloro-4,5,6,7-tertachloro-fluorescein was pH-sensitive between the pH range of 3.0 to 6.0. The reason was that the fluorescence intensity changes with difference of chlorinated fluoresceins proto-tropic forms in the various pH conditions. For example, 2′,7′-dichlorofluorescein could exit in four prototropic forms in different pH, such as cationic, neutral, anionic

Fig. 5 Emission spectra of 2′,7′-dichloro-4(7)-chlorofluorescein in buffered solution of various pH
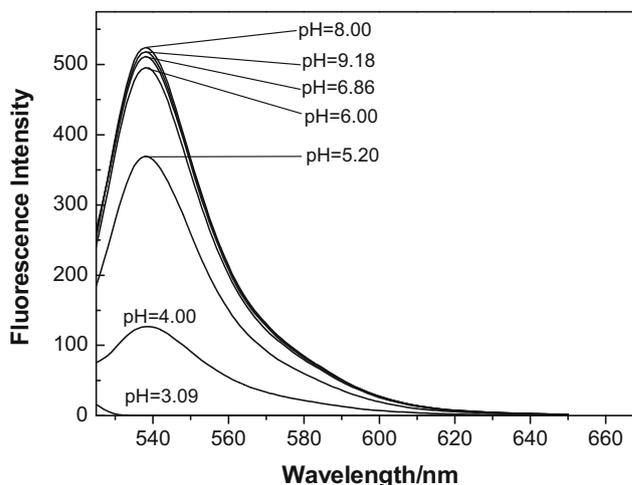
Fig. 7 Emission spectra of 2′,7′-dichloro-4,5,6,7-tetrachlorofluorescein in buffered solution of various pH

and dianionic forms (Fig. 8). But only dianionic form was the most intense fluorescence species. Therefore, the fluorescence of chlorinated fluoresceins showed strongly pH-dependent. By taking advantage of the pH dependence, chlorinated fluoresceins could be used to follow cellular pH and detect cell. Comparison with fluorescein, the results showed that the pH-sensitive ranges of chlorinated fluoresceins shifted to the acid condition with the number increase of chlorine And the relatively low pH-sensitive range was advantage for pH measurement in acidic environments, as could be used in endocytic organelles. In addition, the fluorescence intensity of chlorinated fluoresceins could reach maximum in the physiological environment of the range pH 6.8–7.4. However, fluorescein could not have the maximal fluorescence intensity in physiological environment, and its fluorescence intensity was maximal only when pH was over 8.5.

In order to accurately determine pH value, the pK_a of chlorinated fluoresceins was important parameter to determine the detected pH range. The calculated method was described as Eq. 3. As well known, the protolytic equilibrium constants of fluorescein were reported to be $pK_1 = 2.08$, $pK_2 = 4.31$ and $pK_3 = 6.43$ [18]. Because the dianion form had the most intensive fluorescence, we would only discuss the relative pK_3, which was the pK_a of the phenol. The factors that affected the pK_a of the phenol would have the largest effect on the fluorescent properties of fluorescein. It was well documented that chlorinated phenols have lower pK_a values than their parent compound because of the strong electron-withdrawing ability of the chlorine [19]. As results in Table 1, chlorinated fluoresceins showed the expected decrease of pK_a, though to a markedly lesser degree than fluorescein. As a whole, the pK_a of chlorinated fluoresceins were lower 0.09–2.17 pK_a units than that of fluorescein. Chlorination at the 2′ and 7′ positions decreased the pK_a by about 1.71 units (compound 1 comprised with fluorescein). Chlorina-

tion at the 4 or 7 position lowered the pK_a by about 0.09 units (2(3) comprised with f). Chlorination at the 4, 5, 6 and 7 positions lowered the pK_a by about 0.46 units (5 comprised with f). Interestingly, the decrease in the pK_a by further chlorination at specific positions appeared to have an additive effect. For example, using fluorescein as the parent compound, 2′,7′-dichloro-4(7)-chlorofluorescein (4) had a pK_a of 4.64, which approximated the combined effect of chlorination at the 2′ and 7′ positions in fluorescein (1.71 units lower than f) and at the 4 or 7 position (0.09 units lower than f). The estimated pK_a for compound 4 would be $6.43 - 1.71 - 0.09 = 4.63$. Similarly, 2′,7′-dichloro-4,5,6,7-tertachlorofluorescein (6) had a pK_a of 4.26, which was about equal to the combined effect of 2′,7′-dichlorination and 4,5,6,7-tertachlorination. The estimated pK_a for compound 6 would be $6.43 - 1.71 - 0.46 = 4.26$. The pK_a values of these chlorinated fluoresceins ranged from about 4.2 to 6.4, making the probe useful for the measurement in low pH environment, as encountered in endosomes, lysosomes and phagocytic vacuoles. Especially, 2′,7′-dichloro-4,5,6,7-tertachlorofluorescein exhibited pK_a value of approximately 4.26, which made it most useful for pH measurement in the range from about pH 3.0 to 5.0 in the acidic cell.

Conclusion

A series of chlorinated fluoresceins have been described. The ease of synthesis and purification made these reagents more attractive than the traditionally used fluorescent reagents. They were found to have absorption and emission maxima at long wavelengths and high fluorescence quantum yields. These fluorescent properties are highly favorable for them to be used as molecular probes. In addition, the pH dependence of chlorinated fluoresceins were measured in detail. These probes were strongly pH dependent, and their pK_a values

Fig. 8 pH dependence of 2′,7′-dichlorofluorescein equilibria
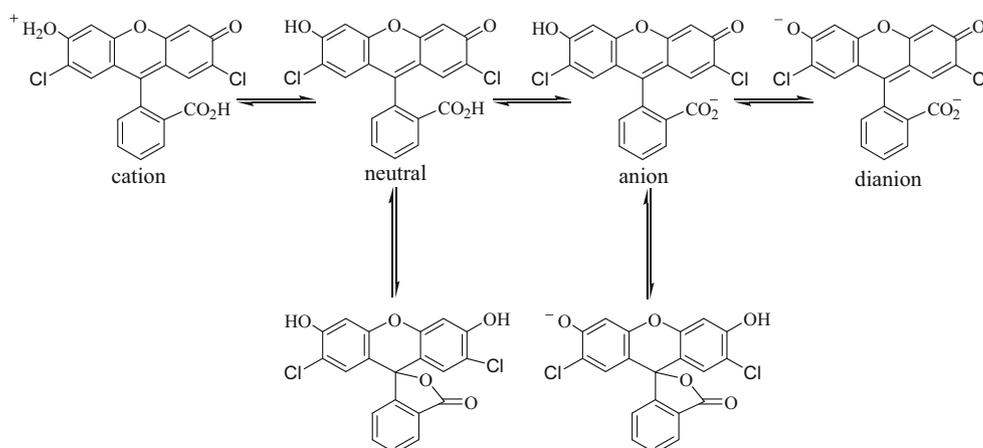
ranged from about 4.2 to 6.0, making them ideal choice for low pH environment assays. We expect these chlorinated fluoresceins will have latent application in many analytical and diagnostic techniques.

References

1. Grinstein S, Goetz JD (1985) Control of free cytoplasmic calcium by intracellular pH in rat lymphocytes. Biochim Biophys Acta 819 (2):267–270
2. Vergne I, Constant P, Lan, elle G (1998) Phagosomal pH determination by dual fluorescence flow cytometry. Anal Biochem 255:127–132
3. Diwu Z, Twu JJ, Yi G, Lavis LD, Chen Y, Cassutt KJ (2003) Fluorescent pH indicators for intracellular assays. US 20030068668A1
4. Jin SY, Xu ZC, Chen JP, Liang XM, Wu YN, Qian XH (2004) Determination of organophosphate and carbamate pesticides based on enzyme inhibition using a pH-sensitive fluorescence probe. Anal Chim Acta 523(1):117–123
5. Galande AK, Weissleder R, Tung CH (2006) Fluorescence probe with a pH-sensitive trigger. Bioconjugate Chem 17(2):255–257
6. Haugland RP (2002) Handbook of fluorescent probes and research products, 9th ed. Molecular Probes, Eugene, pp 830–833
7. Song L, Hennink EJ, Young T, Tanke HJ (1995) Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy. Biophys J 68(6):2588–2600
8. Song L, Varma CAG, Verhoeven JW, Tanke HJ (1996) Influence of the triplet excited state on the photobleaching kinetics of fluorescein in microscopy. Biophys J 70(6):2959–2968
9. Guilbault GG (ed) (1990) In: Practical fluorescence, chapter1. Marcel Dekker, NewYork
10. Graber ML, Dilillo DC, Friedman BL, Enrique PM (1986) Characteristics of fluoroprobes for measuring intracellular pH. Anal Bio 156:202–212
11. Millar D, Uttamal M, Henderson R, Keeper A (1998) Electrochemical immobilization of a pH sensitive fluorescein derivative: synthesis and characterization of a fluorescein-derivatised polythiophene. Chem Commun 447–478
12. Whitaker JE, Haugland RP, Prendergast FG (1991) Spectral and photophysical studies of benzo[c]xanthene dyes: dual emission pH sensors. Anal Bio 194:3330–340
13. Lyttle MH, Carter TG, Cook RM (2001) Improved synthetic procedures for 4,7,2′,7′-tetrachloro- and 4′,5′-dichloro-2′,7′-dimethoxy-5 (and 6)-carboxy- fluoresceins. Org Pro Res Dev 5:45–49
14. Demas JN, Crosby GA (1971) Measurement of photoluminescence quantum yields. J Phy Chem 75:991–1024
15. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450
16. Babcock DF (1983) Examination of the intracellular ionic environment and of ionophore action by null point measurements employing the fluorescein chromophore. J Biol Chem 258: 6380–6389
17. Ge FY, Yan XL, Yan FY, Pan HY, Chen LG (2005) New fluorescent labels: 4-and 7-chlorofluorescein. J Fluorescence 15 (6):829–833
18. Guilbault GG (ed) (1990) In: Practical fluorescence, chapter 6. Marcel Dekker, NewYork
19. Fujio M, Mciver RT, Taft RW (1981) Effects on the acidities of phenols from specific substituent–solvent interactions. Inherent substituent parameters from gas-phase acidities.. J Am Chem Soc 103:4017–4029