APPLICATION NOTE

INVESTIGATING THE ABSORPTION AND FLUORESCENCE PH DEPENDENCE OF FLUORESCEIN, WITH A FLOW CELL

INTRODUCTION

Fluorescein is a widely used organic dye based on a xanthene moiety, like eosin, erythrosine, and rose bengal dyes [1]. Due to its high molar absorption coefficient, fluorescent quantum yield, and photostability, fluorescein is a very useful and sensitive fluorescent dye with broad biochemical applications [2].

In aqueous solution, fluorescein can exist in cationic, neutral, mono-anionic, and di-anionic species, making its absorption and fluorescence properties essentially dependent on the pH value of the aqueous solution [3]. Under basic conditions (pH > 8.0), fluorescein exists in a di-anionic form and displays an absorption maximum of around 490 nm (76900 M-1 cm-1), with a very strong fluorescent emission, and a fluorescence quantum yield (ϕF) close to 1 [4,5]. For lower pH values (pH < 8.0), fluorescein can exist in multiple ionization states (see **Figure 1**), which can be predicted by the different structures and peaks of the absorption spectra [3,4].

Keywords:

FLEX Spectrometer;

Fluorescein Dye;

pH Dependence;

Absorbance Flow Cell;

Fluorescence Flow Cell;

Under basic conditions, the di-anionic form of fluorescein emits a strong green light (ϕF is around 0.95 in NaOH 0.1 M) with the maximum around 515 nm. The acidification of the solution gradually leads to the fluorescence emission extinction (when the excitation occurs at 490 nm, which is the absorption maximum) [5]. This extinction results from the transition of the di-anionic into the mono-anionic form, which has a lower absorbance due to a blue-shift [5].

In this application note, we study the absorption and fluorescence pH dependence of a fluorescein aqueous solution, using an absorption and fluorescence flow cell, respectively.

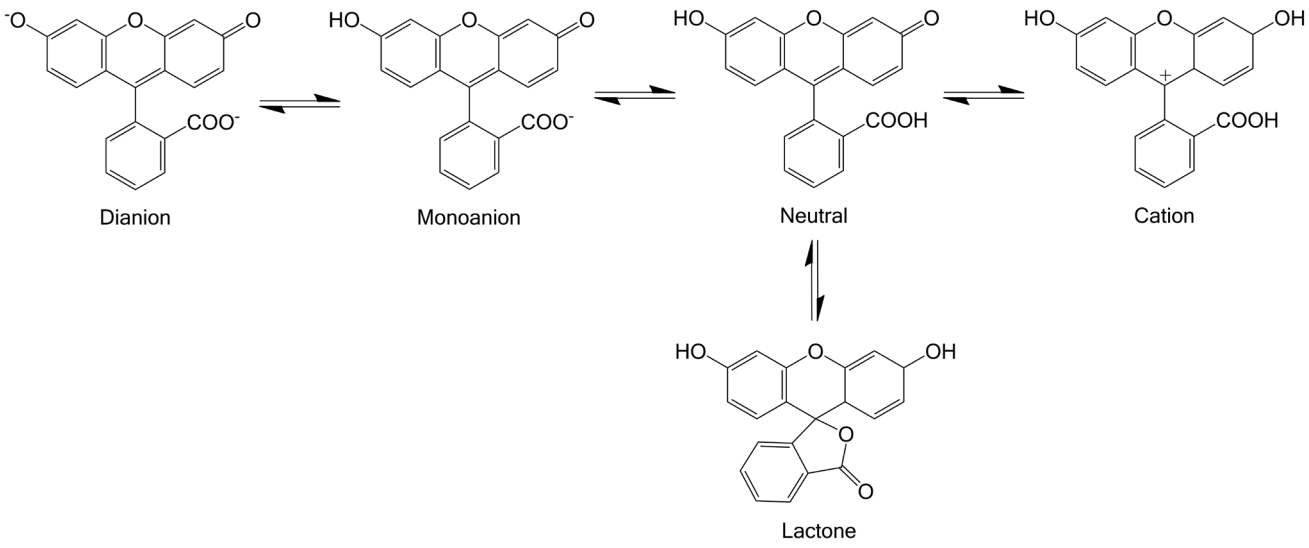

Figure 1 – Different ionic species of fluorescein [3,4].

MATERIALS & METHODS

Reagents

- Fluorescein (C20H12O5);
- Distilled Water;
- NaOH 1 M;
- HCl 1 M;

Instruments and Accessories:

- pH Meter;
- Stirring Plate;
- W20 Light Source;
- LED Light Source (with a 420 nm LED slide);
- Absorbance Flow Cell with 10 mm of pathlength;
- Optical Fibers with 600 µm of diameter;
- Fluorescence Flow Cell with 10 mm of pathlength;
- FLEX RES Spectrometer (Slit: 25 and 200 um; Grating: 600 grooves/500 nm; Detector: Toshiba 3648 pixel; Improved Sensitivity);

EXPERIMENTAL PROCEDURE

1. Add 1 L of distilled water into a large container with a circulation pump;

2. Connect the tubes to the absorption/fluorescence flow cell and turn the circulation pump on;

3. With the pH electrode submersed in the solution, adjust the pH of the solution to 11.0 with the NaOH solution;

4. Use the W20 light source in connection with the absorbance flow cell to perform absorbance measurements or the LED light source with the LED slide at 420 nm (selecting this wavelength will avoid an overlap of the excitation with fluorescence) in connection with the fluorescence flow cell to perform fluorescence measurements;

Figure 2 – W20 light source (1), LED light source (2), FLEX RES spectrometer (3), and absorbance (4) and fluorescence (5) flow cells were used to assess the pH-dependence of the absorption and fluorescence spectra of fluorescein.

5. With a continuous stirring, perform the reference and dark measurements (this step is valid for both absorbance and fluorescence measurements);

6. Add a small amount of fluorescein, and after being completely dissolved, measure the absorbance/fluorescence spectrum under basic conditions;

7. The instrument's settings selected in the LightScan software for absorbance and fluorescence measurements are given in **Table 1 and 2**, respectively;

8. Add small amounts of concentrated HCl solution (1 M) to decrease the pH values of the fluorescein solution;

9. Once the pH value is stabilized, measure the absorbance/fluorescence spectrum;

10. Perform these measurements until a pH around 3 is obtained;

Table 1 – Instrument settings used in connection with the absorbance flow cell.

Parameter	Used Settings
Integration time (ms)	5
Average	150
Smoothing	3

Table 2 – Instrument settings used in connection with the fluorescence flow cell.

Parameter	Used Settings
Integration time (ms)	800
Average	10
Smoothing	3

RESULTS

Absorbance measurements

The absorbance spectra of the fluorescein aqueous solution, obtained at different pH values, are represented in **Figure 3**.

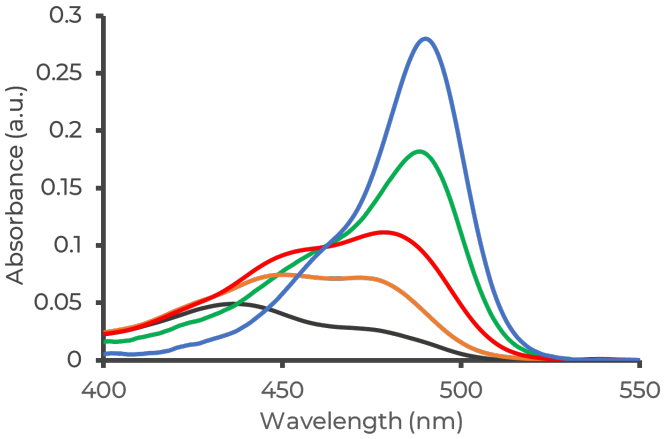

Figure 3 – Absorption spectra of the fluorescein aqueous solution, at different pH values: 3.57 (grey line), 4.58 (orange line), 5.80 (red line), 7.01 (green line), and 9.41 (blue line).

Decreasing the pH value of the fluorescein aqueous solution from 9.0 to 3.0 leads to the conversion of the di-anionic into the cationic species. As expected, this change decreases the molar absorption coefficient of fluorescein, which can be observed thought the decrease of the absorbance values and the respective wavelength, particularly at maximum values [4].

The influence of pH on the absorbance maximum of the di-anionic (around 490 nm) and anionic (at around 450 nm) forms of fluorescein can be observed in **Figure 4**.

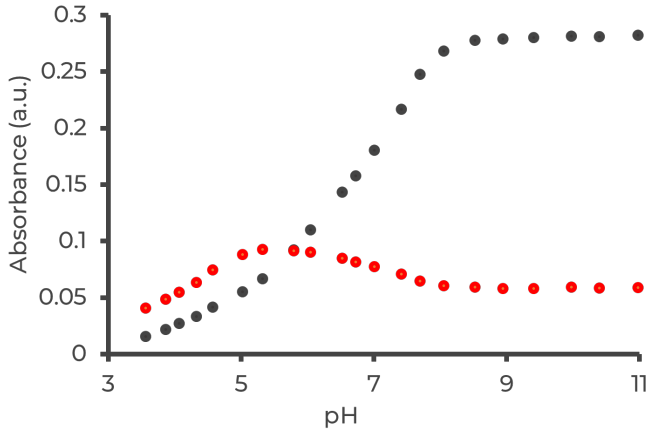

Figure 4 – Absorbance values of di-anionic (black dots, at 490 nm) and anionic (red dots, at 450 nm) forms of fluorescein as a function of pH values.

The di-anionic form of fluorescein has an absorbance maximum of around 490 nm and is strongly pH-dependent. At pH values lower than 4.0, the molar absorption coefficient of this species is almost zero. On the other hand, the absorbance maximum peak of the anionic species, is observed at lower wavelengths, around 450 nm.

Owing to an overlap of both di-anionic and neutral species absorption spectra, the pH-dependence of the absorbance is never zero (see **Figure 4**) [4].

Fluorescence measurements

The fluorescence spectra of the fluorescein aqueous solution, obtained at different pH values, are represented in **Figure 5**.

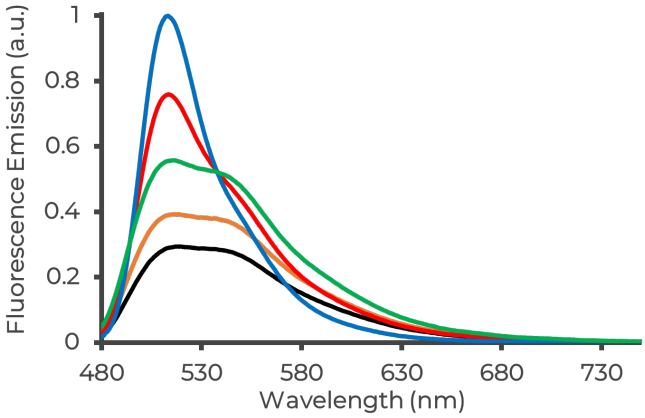

Figure 5 – Fluorescence spectra of the fluorescein aqueous solution, at different pH values: 3.46 (grey line), 4.23 (orange line), 5.16 (green line), 6.46 (red line), and 8.32 (blue line). Excitation wavelenght: 420 nm.

Due to changes in the ionic charge and chemical structure, decreasing the pH value of fluorescein aqueous solution from 8.7 to 3.5 leads to the fluorescence extinction. At basic and neutral conditions, the highly fluorescent di-anionic form stands over the other forms (mono-anionic, neutral and cationic) of fluorescein. At acidic pH, the mono-anionic form stands out and the blue-shifted absorption is followed by an decrease in fluorescence emission [6].

The influence of pH on the fluorescence maximum of the di-anionic form (around 515 nm) of fluorescein can be observed in **Figure 6**.

Changing the pH of the fluorescein aqueous solution from basic to acid leads to a decrease of the fluorescence intensity. Under irradiation at 420 nm, which corresponds to the region where both mono-anionic and neutral forms have an absorbance maximum, fluorescence is not extinct, see Figure 6. However, under irradiation at 490 nm, at lower pH values (pH < 4) fluorescein becomes non-fluorescent.

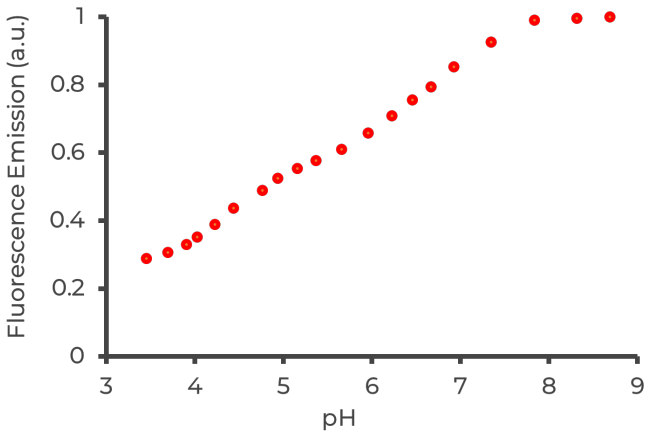

Figure 6 – Fluorescence emission at the maximum peak of the di-anionic form of fluorescein (around 515 nm), as a function of pH. Excitation wavelenght: 420 nm.

CONCLUSIONS

Absorption and fluorescence flow cells were used in this application note to investigate the pH-dependence of fluorescein absorption and fluorescence spectra. By combining a flow cell accessory with a circulation pump, it is possible to perform continuous and prompt measurements (absorption and fluorescence) of fluids in small or large containers.

This application note highlights the great accuracy, and large flexibility of Sarspec's FLEX series and flow cell accessories regarding the absorbance and fluorescence measurements of solutions in different size containers.

REFERENCES

1. D. C. Neckers and O. M. Valdes-Aguilera, Photochemistry of the Xanthene Dyes (1993). In D. H. Volman, G. S. Hammond and D. C. Neckers (Eds.), Advances in Photochemistry, vol 18 (pp. 315-394). First Edition. John Wiley & Sons, Inc.

2. K. H. Drexhage (1973) Structure and Properties of Laser Dyes. In F.P. Schäfer (Ed.), Dye Lasers (pp. 144-193). First Edition. Springer-Verlag.

3. E. A. Slyusareva and M. A. Gerasimova (2013) pH-Dependence of the Absorption and Fluorescent Properties of Fluorone Dyes in Aqueous Solutions, Russian Physics Journal, 56.

4. R. Sjöback, J. Nygren, M. Kubista (1995) Absorption and Fluorescence Properties of Fluorescein, Spectrochimica Acta Part A, 51.

5. M. M. Martin and L. Lindqvist (1995) The pH dependence of fluorescein fluorescence. J. Lumin. 10.

6. F. Le Guern; V. Mussard; A. Gaucher; M. Rottman; F. Prim (2020) Fluorescein Derivatives as Fluorescent Probes for pH Monitoring along Recent Biological Applications. Int. J. Mol. Sci. 21.