Dual-Ratiometric, Red Fluorescent Label for pH-Sensing Applications

Anatoliy L. Tatarets[1], Yevgen A. Povrozin[1], Larysa I. Markova[1], Vadim I. Sidorov[1], Igor P. Vysekantzev[2], Leonid D. Patsenker[1,3], and Ewald A. Terpetschnig[3]

[1] *State Scientific Institution "Institute for Single Crystals" NAS of Ukraine, 60 Lenin Ave., Kharkov, Ukraine 61001. E-mail: tatarets@isc.kharkov.com, http:// www.isc.kharkov.com/old*

[2] *Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, 23 Pereyaslavskaya Str., UA-61015 Kharkov, Ukraine. E-mail: cryo@online.kharkov.ua*

[3] *SETA BioMedicals, LLC, 2014 Silver Ct East, Urbana, IL 61801, USA. E-mail: ewaldte@setabiomedicals.com, http://www.setabiomedicals.com*

Introduction

Almost all existing fluorescent pH-labels that are employed in biomedical applications such as intracellular and extracellular studies are known to emit between 350 and 600 nm and there is only a small number of pH-labels that emit in the red or NIR spectral region. Typically, deprotonation of these dyes is accompanied not only by a bathochromic shift of the absorption maximum but also by a loss of fluorescence of the dye molecule. In general, the majority of commercially available pH-sensitive fluorescent dyes employed for intracellular studies are non-fluorescent in acidic media or the pKa of the dyes are outside the intracellular pH range (pH 5–8).

We have investigated and spectrally characterized a new pH-sensitive, fluorescent label, Square-650-pH, which is commercially available from SETA Biomedicals as a carboxy derivative or amine-reactive NHS-ester (http://www.setabiomedicals.com, Cat.# K8-1405 and K8-1407, respectively). This cyanine-type pH-label has spectral properties similar to those of the CypHer dyes but is fluorescent in both the protonated and deprotonated forms, displays an extremely large Stokes shift for the deprotonated form, and enables excitation and emission ratiometric measurement of pH.

Spectral properties of Square-650-pH free in aqueous media

The cyanine-type pH label Square-650-pH has a high extinction coefficient of 135,000 $M^{-1}cm^{-1}$, a long-wavelength absorption maximum of 653 nm and an emission maximum at 671 nm in its protonated form. Upon deprotonation the long-wavelength absorption band decreases and a new band with a maximum at 535 nm appears, resulting in an extremely large Stokes' shift of over 100 nm (λ_{em} 663 nm for the deprotonated form).

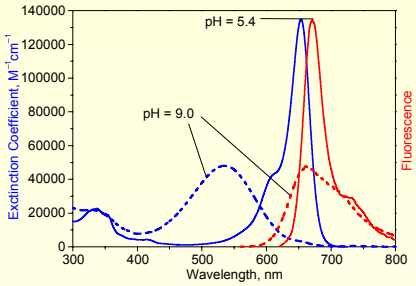

Fig. 1. Absorption and emission spectrum of Square-650-pH at pH 5.4 and 9.0

The absorption band of the deprotonated form has an extinction coefficient of 48,000 $M^{-1}cm^{-1}$. Remarkably, both forms absorb not only in the red but also in blue spectral region (around 340 nm) with extinction of ~23,000 $M^{-1}cm^{-1}$. This makes the label also suitable for use with the blue (330 nm and 370 nm) LEDs and diode lasers excitation.

Importantly both the protonated and deprotonated forms of the label are fluorescent which enables excitation and/or emission ratiometric measurements of pH.

Table 1. Spectral properties of the protonated and deprotonated forms of Square-650-pH and Square-650-pH – IgG conjugates

Compound	pH	Absorption maximum [nm]	Extinction Coefficient [M^{-1} cm^{-1}]	Emission Maximum [nm]	Quantum Yield [%]	Average Fluorescence Lifetime [ns]
Square-650-pH	2.0	653	135,000	671	16	1.17
	9.0	535	48,000	663	9	0.53
Square-650-pH – IgG, (D/P=0.8)	2.0	662	135,000	677	7	1.52
	9.0	544	48,000	665, 715	9	0.89

Spectral properties and applications of Square-650-pH conjugates

The pH-dependent properties of the Square-650-pH were also measured after conjugation to an antibody (IgG) and E.coli. Importantly, the pKa of the pH probe (pKa = 7.1) only slightly changes after conjugation to E.coli (pKa = 6.9), (Fig. 4.), and therefore the fluorescence intensity increases dramatically as the pH of its environment becomes more acidic (e.g. upon internalization in vesicles during phagocytic events (Fig. 5).

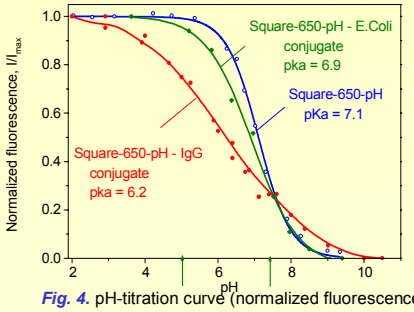

Fig. 4. pH-titration curve (normalized fluorescence intensity *vs.* pH (λ_{exc} 630 nm) of Square-650-pH, Square-650-pH – IgG and Square-650-pH – E.Coli conjugates

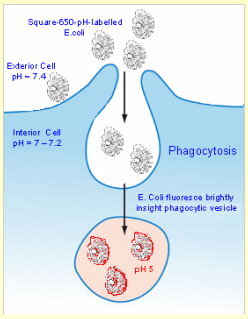

Fig. 5. Scheme for Square-650-pH – E.Coli based detection of phagocytic events

Conjugated to microorganisms (E.coli), Square-650-pH can be used as a tool to identify phagocytic events (Fig. 5). Using the no-cell background subtraction method, a specific signal is obtained from cells that ingest bacteria, thereby providing a specific index of phagocytosis.

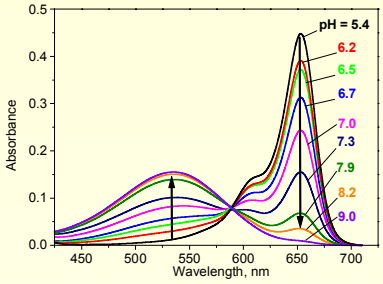

Fig. 2. Absorption spectra of Square-650-pH as a function of pH

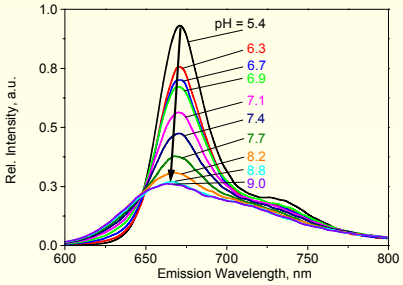

Fig. 3. Change of emission spectra of Square-650-pH *vs.* pH. λ_{exc} = 589 nm (isosbestic point)

Fluorescence lifetime measurements

We also made an attempt to measure the fluorescence lifetimes of this pH probe in various environments using the frequency-domain technique (ChronosFD from ISS).

The fluorescence lifetime of both the protonated (τ = 1.17 ns) and deprotonated (τ = 0.53 ns) forms of Square-650-pH increase upon labelling to antibodies (τ_{ave} = 1.52 ns and τ_{ave} = 0.89 ns, respectively) and the initially mono-exponential decays of the free dye become bi-exponential upon conjugation to a carrier molecule.

The conjugated label shows an 18° phase angle change between the protonated and deprotonated species at a modulation frequency of 100 MHz. This implies that the labeled form of Square-650-pH would also be suitable for fluorescence-lifetime-based pH-sensing and lifetime imaging (FLIM) applications.

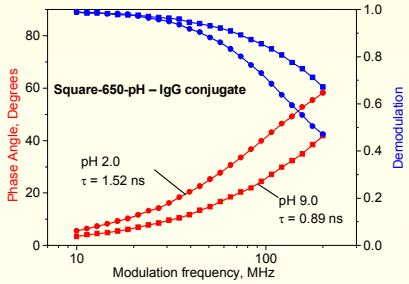

Fig. 6. Frequency response curves (phase angle and demodulation) for the Square-650-pH – IgG conjugate (D/P = 0.8) plotted for the pH 2.0 and pH 9.0

Conclusion

Due to the fact that the fluorescence intensity, spectra and fluorescence lifetime of Square-650-pH are noticeably sensitive to the pH of its microenvironment, this dye is an ideal tool for cell biology to study intra- and inter-cellular pH, phagocytic events and its regulation by drugs and/or environmental factors using both intensity and lifetime based methods.

The work was supported by the STCU grants No. 3804 and P313