Two-photon absorbing water-soluble fluorescent probe as a near-neutral pH indicator

6-24-2014

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ABSTRACT

Disclosed are fluorescent compounds useful as intracellular pH probes. In particular, the invention teaches a two-photon absorbing, water soluble, fluorescent compound, a fluorescent derivative, which is effective as a near-neutral pH indicator and particularly as an intracellular probe. A method for chemical synthesis of the claimed compounds is provided.

6 Claims, 6 Drawing Sheets
References Cited

OTHER PUBLICATIONS

* cited by examiner
R_1, R_2, R_3, R_4 = \text{OH, antibodies/antigens, oligopeptides, DNA/RNA fragments, polyamino groups, amine-reactive groups, thio-reactive groups, etc.}

m=0-20, n=0-2.

R_1, R_2, R_3, R_4 may or may not be the same.

1a R_1=phenyl, R_2=C_{16}H_{31}
1b R_1=phenyl, R_2=C_2H_4OC_2H_4OCH_3
1c R_1=ethyl, R_2=C_2H_5COOH

FIG. 1
FIG. 2 A
FIG. 4
**SUMMARY OF THE INVENTION**

The present invention relates to the field of cellular and biological chemistry and, more particularly, to compounds useful as fluorescent pH probes in biological systems.

**BACKGROUND OF THE INVENTION**

The dynamics of intracellular pH are believed to be crucial for understanding the regulation mechanism of many physiological functions. Of the methods available to determine pH, optical methods have several advantages. These include a rapid response time and a high signal-to-noise ratio. Additionally, they are non-invasive, and they generally have excellent pH sensitivity. Since the first use of a trapped intracellular pH probe, 6-carboxyfluorescein, was described by Thomas et al., a large number of intracellular pH indicators has been reported. However, these one-photon excited fluorescent pH indicators have serious limitations, e.g. the interference of autofluorescence and scattering from biological fluids and tissue, photodamage of the samples and photobleaching of the indicators, difficulty in imaging intercellular or intracellular pH differences, and others. Recently, using two-photon fluorescence (2 PF) to measure the pH has gained attention. Advantages of using the two-photon approach include less scattering and deeper penetration in biological samples by using NIR excitation light, less photodamage and photobleaching, as well as the unique properties of obtaining 3D resolution.

In addition to normal fluorescence methods, a 2 PF indicator has also been employed to detect the pH at molecular level by using fluorescence correlation spectroscopy. In order to quantitatively measure pH, the pKₐ of the indicator needs to match with the pH of the experimental system. Since the pH in the cell cytosol is typically between 6.8 and 7.4, there is tremendous interest in the development of an efficient two-photon absorbing (2 PA), near-neutral, fluorescent pH indicator. However, near-neutral 2 PA pH indicators are rare and the 2 PA cross-sections of most commercial pH indicators in the NIR region are low. Only recently, one example of a pH indicator designed with emphasis on improving the two-photon absorbivity was reported by Charier et al. A relatively high value of 60 GM (1 GM=10⁻⁵⁰ cm² s photon⁻¹) was described, but its pKₐ of 5.7 is too low for near-neutral biological applications.

**FIELD OF THE INVENTION**

The present invention relates to the field of cellular and biological chemistry and, more particularly, to compounds useful as fluorescent pH probes in biological systems.

**RELATED APPLICATION**

This application claims priority to and is a division of U.S. patent application Ser. No. 12/168,326 filed on Jul. 7, 2008, which claims priority to U.S. Provisional Application Ser. No. 60/948,287 filed on Jul. 6, 2007, the contents of which are incorporated herein by reference in their entirety.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Some of the features, advantages, and benefits of the present invention having been stated, others will become apparent as the description proceeds when taken in conjunction with the accompanying drawings, presented for exemplary purposes and not with intent to limit the invention thereto, and in which:

FIG. 1 shows the general structure of fluorescein derivatives and a chemical synthesis of fluorescent probe 1 according to an embodiment of the present invention;

FIG. 2 graphically illustrates the absorption, emission and two-photon excitatin (2 PE) spectra of (A) protonated form 1 (B) neutral form 1c in buffer at 0.1 mM, with arrows indicating the change of the absorption intensities with pH increase—the inset shows the nonlinear fitting of the pH dependence of molar extinction coefficients at 341 nm;

FIG. 3 depicts the pH dependence of the fluorescence intensity of 1c in buffer (10-5 M) excited at 355 nm—the inset is the ratiometric calibration curves of I₃⁹/I₄⁹ (intensity at 391 nm vs. intensity at isomissive point 493 nm); and

FIG. 5 shows a 2 PF image of NT2 cells incubated with 1c.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT**

The present invention will now be described more fully hereinafter with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. Any publications, patent applications, patents, or other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including any definitions, will control. In addition, the materials, methods and examples given are illustrative in nature only and not intended to be limiting. Accordingly, this invention may, however, be embodied in many different forms and should not be construed as limited to the illustrated embodiments set forth herein. Rather, these illustrated embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. Other fea-
The synthesis of the disclosed compounds is shown in the chemical reactions depicted in FIG. 1. Converting the iodo group in 2 into cyano in 3 gave the precursor that can be easily hydrolyzed to carboxylic acid 4. The carboxylic acid was then transformed to acid chloride in situ and reacted with amine to form the benzothiazole derivative. Two propionitrile groups were then introduced to 5 by a Michael reaction. Subsequent reduction of the nitro to amino group, diethylation of the amine with triethylphosphate, and hydrolysis of the nitrile groups afforded the final product le in good overall yield. All compounds were fully characterized by NMR and elemental analysis (or HRMS in case of compounds with carboxylic acid moieties).

As expected, compound 1c exhibits adequate water solubility, i.e., >10^{-3} M at pH 1-7 and >10^{-6} M at pH 7-12. At pH 4, the absorption and emission maxima of protonated 1c are 341 nm and 391 nm, respectively (FIG. 2, left panel) and a fluorescence quantum yield of 0.21 was recorded. The 2 PA cross-section of the dye is very low in the excitation wavelength range of 570 nm to 750 nm, which may be attributed to very strong electron withdrawing nature of the protonated nitrogen of the diethylamino group (see the pK_a measurement). In this nearly centrosymmetric A-π-A type structure, the absorption wavelength is expected to shift to shorter wavelength and the single photon excitation allowed S_0-S_1 transition is not allowed by two-photon excitation. In contrast, at pH 10 buffer, where a neutral form of 1c predominates, the absorption maxima is red shifted 41 nm to 382 nm, and fluorescence quantum yield increased to 0.56 (FIG. 2, right panel). In addition, a dramatic increase of 2 PA cross-section to 99 GM at the excitation wavelength of 770 nm occurred. Since the neutral form of dye 1c is a D-π-A type structure, it is well-known that the single-photon excitation of the allowed S_0-S_1 also occurs via two-photon excitation, which in this case is the absorption band at 382 nm.

The pH dependence of the absorption spectra when titrated in aqueous buffer is shown in FIG. 4. With the increase of pH, the absorption peak at 341 nm, attributed to the protonated form of 1c, becomes weak and the absorption of the neutral form at 382 nm appears with a well-defined isosbestic point. It is known that the basicity of the nitrogen of diethylamino-benzene is much stronger than the nitrogen in benzothiazole. Hence, the structure protonated form of 1c is assigned as protonated at diethylamino site. The inset in FIG. 4 shows the results of the nonlinear regression of the ε at 341 nm according to an existing method which gave a pK_a value of 6.95±0.01. The pK_a value may also be calculated from pH dependence of the integrated fluorescence of 1c excited at 340 nm and 382 nm, taking advantage of well separated fluorescence bands of the two forms. By using similar nonlinear regression, nearly the same pK_a value was obtained (6.96±0.04).

In practical applications, fluorescent indicators with ratiometric properties are highly desirable since the ratio of the fluorescence intensity at peak wavelength vs insensitive isoemissive wavelength is constant, regardless of the change of fluorophore concentration by photobleaching or change of the external environment, such as ion concentration. When 1c was excited at the wavelength of the isosbestic absorption point (355 nm), the fluorescence from both protonated and neutral forms was observed, as shown in FIG. 4. The distinguished isoemission point at 493 nm makes 1c an excellent ratiometric pH indicator. Under two-photon excitation, the fluorescence from the protonated form disappeared due to its low 2 PA cross-section, rendering it unsuitable as a two-photon ratiometric pH indicator. However, the high 2 PA cross-section and high fluorescence quantum yield of the neutral form makes it promising for a number of important applications in fluorescence correlation spectroscopy and intra and extracelluar pH sensing.

For intracellular applications, one major concern for the probes is the cell permeativity of the indicator. Therefore, 1c was incubated with NT2 (NEIRA-2 cl.D1 [NT2/D1]) cells. The two-photon fluorescence image of the stained cells is shown in FIG. 5. The dye has good permeativity and disperses in the cytosol well. The cytotoxic effect of 1c on proliferating cells is another parameter of primary interest, particularly for any live-cell fluorescence imaging applications. An Alamar Blue (AB) reduction analysis was used to assess the cytotoxicity of 1c on proliferating NT2 cells. The cells were treated with different concentrations of compound 1c (0.1 µM-100 µM) dissolved in buffer, and were also treated with 10% AB solution. The observed fluorescence intensity of AB reduction by cells treated with various doses of 1c was similar to that observed for cells untreated without any fluoresce probe (control) after 48 h, indicating low toxicity of 1c over a relatively wide concentration range.

In summary, fluorene derivatives 1a, 1b and 1c were synthesized and tested. Derivative 1c functions as a near-neutral pH indicator with pK_a of 6.96, confirmed by both absorption and fluorescence methods. The distinct isoemissive point in the fluorescence spectra at different pH levels, good dispersion in the cell cytosol, and low cytotoxicity indicates that 1c satisfies all the criteria for an excellent ratiometric fluorescent pH indicator. Furthermore, its high 2 PA cross-section also shows it has great potential for 3D pH fluorescence mapping in live and fixed cells, as demonstrated in FIG. 5.

The invention, therefore, includes a fluorescent compound preferably according to formula 1c, as shown below, wherein R^1=ethyl and R^2=C_2H_4COOH.
The invention additionally includes a biologically compatible composition containing preferred compound 1c, but the composition may contain compounds 1a, 1b, or combinations of all three compounds. A biologically compatible composition is one which is compatible with the fluorescent compounds herein disclosed, for example, by not quenching the fluorescence, and which can safely be administered to living cells without causing undue toxicity to the cells. An example of such a biologically compatible composition would be a buffering solution.

Another aspect of the invention is a method of sensing pH inside a cell, the method comprising introducing the fluorescent compound into the cell and irradiating the cell with a near-infrared wavelength. More broadly, a method of sensing pH inside a cell may comprise introducing the fluorescent compound into the cell and irradiating the cell with a wavelength effective for exciting the compound to fluoresce approximately at a predetermined pH. On a larger scale, the method of sensing pH may be applied to a biological sample, the method comprising contacting the biological sample with the fluorescent compound and irradiating the biological sample with a near-infrared wavelength. Likewise, in the method comprising contacting the biological sample with the fluorescent compound, irradiating the biological sample may be accomplished with a wavelength effective for exciting the compound to fluoresce approximately at a predetermined pH.

The present invention also includes a method of synthesizing a two-photon absorbing, near-neutral, pH indicator fluorescent when excited by light of a near-infrared wavelength and having a formula selected from compounds 1a, 1b and 1c. The method of synthesis comprises a reaction sequence according to Scheme 1, as set forth in FIG. 1.

Accordingly, in the drawings and specification there have been disclosed typical preferred embodiments of the invention and although specific terms may have been employed, the terms are used in a descriptive sense only and not for purposes of limitation. The invention has been described in considerable detail with specific reference to these illustrated embodiments. It will be apparent, however, that various modifications and changes can be made within the spirit and scope of the invention as described in the foregoing specification and as defined in the appended claims.

REFERENCES CITED


That which is claimed:

1. A method of sensing pH inside a cell, the method comprising introducing a fluorescent compound having the following structure

![Chemical Structure](image)

into the cell, irradiating the cell with a near-infrared wavelength, detecting fluorescence from the compound, and associating the fluorescence with a pH, wherein R<sup>1</sup>=ethyl and R<sup>2</sup>=C<sub>2</sub>H<sub>5</sub>COOH.

2. The method of claim 1, wherein the near-infrared wavelength is effective for exciting the compound to fluoresce.

3. The method of claim 1, wherein associating the fluorescence with a pH comprises comparing the detected fluorescence with a set of pre-determined pH values corresponding to fluorescence from the compound measured at a plurality of pHs.
4. A method of sensing pH in a biological sample, the method comprising contacting the biological sample with a fluorescent compound having the following structure:

![Chemical Structure]

irradiating the biological sample with near-infrared radiation, detecting fluorescence from the compound, and associating the fluorescence with a pH, wherein $R_1 = \text{ethyl}$ and $R_2 = \text{C}_2\text{H}_4\text{COOH}$.

5. The method of claim 4, wherein the near-infrared radiation is effective for exciting the compound to fluoresce.

6. The method of claim 4, wherein associating the fluorescence with a pH comprises comparing the detected fluorescence with a set of pre-determined pH values corresponding to fluorescence from the compound measured at a plurality of pHs.

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