

Molecular fluorescent probes for monitoring pH changes in living cells

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The availability of synthetic fluorescent probes and the development of powerful new approaches to microscopy have made fluorescence microscopy an essential tool for biomedical science and biology. Intracellular pH plays vital roles in physiological and pathological processes (e.g., receptor-mediated signal transduction, cell growth and apoptosis, ion transport, and homeostasis). Due to the presence of an H^+ acceptor linked to the fluorophore, some small molecular fluorescent probes display pH-sensitive absorption and fluorescence emission. Such behavior has been employed to engineer pH indicators for studies of pH regulation *in vivo*. The review summarizes advances in the creation of novel molecular fluorescent pH probes and their applications in biomedicine and cell biology, especially focusing on the design and the synthesis of small molecular probes for monitoring pH changes in living cells.

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1. Introduction

Since fluorescence emission from an indwelling patch can be detected without direct contact, the measurement can be made non-invasively [1]. Non-invasive measurement offers advantages, including high sensitivity, good selectivity, short response time, real-time monitoring, and *in situ* observation [2]. Synthetic organic chemistry furthers many fields of science, because organic synthesis technology can achieve the clean formation of new desired compounds [3], so a promising synthetic strategy can deal with a troublesome biomedical problem successfully. The availability of various synthetic fluorescent probes, and the development of new powerful approaches to microscopy [e.g., confocal laser scanning microscopy (CLSM)] has given fluorescence microscopy an essential, critical role in biomedical science and biology [4]. Proceeding

from this point, the symbiotic relationship between synthetic chemistry and biological imaging continues to spur synergistic developments in probe design and instrumentation [5].

Fluorescent pH probes usually suffer from optical changes in terms of emission-spectra variation and fluorescence intensity [4], but they have proved effective tools for investigating the role of intracellular pH in diverse physiological and pathological processes, including receptor-mediated signal transduction, enzymatic activity [6], cell growth and apoptosis [7], ion transport and homeostasis [8], calcium regulation, endocytosis, chemotaxis, and cell adhesion [9]. They also afford much greater spatial sampling capability and non-invasive measurement, compared with microelectrode techniques [4,10].

Under normal physiological conditions, extracellular hydrogen-ion concentration is maintained within very narrow limits. The normal value is about 40 nmol/L at pH 7.4 and varies by about 5 nmol/L in the pH range 7.35–7.45. Deviation by 0.10–0.20 pH units in either direction can cause cardiopulmonary and neurologic problems (e.g., Alzheimer's disease) [11, 12], and more extreme variations can be fatal [12]. H^+ is therefore one of the most important targets among the species of interest in the biomedical field.

This review summarizes progress in the invention of new molecular fluorescent pH probes and their applications in the biomedical field and cell biology, particularly design and synthesis of small molecular probes for monitoring pH changes in living cells. We review the chemical tools

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and strategies available. We also summarize current and future challenges in this field from the perspective of probe development and application.

2. Designing H⁺-responsive molecular fluorescent probes

In order to quantify pH, it is important to match the pK_a of probes to the pH of the research system [4]. In this regard, these biological pH probes can be divided into two types for:

- (1) cytosol, which works at pH values of 6.80–7.40; and,
- (2) acidic organelles (e.g., lysosomes and endosomes), which function in the pH range 4.5–6.0.

We emphasize that, under the conditions of detection, whether near-neutral or acidic, the desirable probes should respond remarkably to a minor change in pH, give dependable results, and meanwhile particularly avoid interference from native cellular species. Effective fluorescent probes for monitoring pH changes must therefore meet several strict requirements, including good selectivity, high sensitivity, good photostability, and the ability to work within the appropriate pH range. Most importantly, a probe should be selective for H⁺ over other biological species. Probes must be matched with acid-dissociation constants (pK_a) appropriate to the system under study. Moreover, high fluorescence quantum yield can lower the amount of dye required for cellular applications, and that minimizes the effect of altering the distribution of intracellular species. Fluorophores with long-wave excitation and emission are desirable in order to minimize photo damage and to avoid the influence of cell autofluorescence. Finally, probes must also be compatible with biological systems (e.g., water solubility and low bio-toxicity).

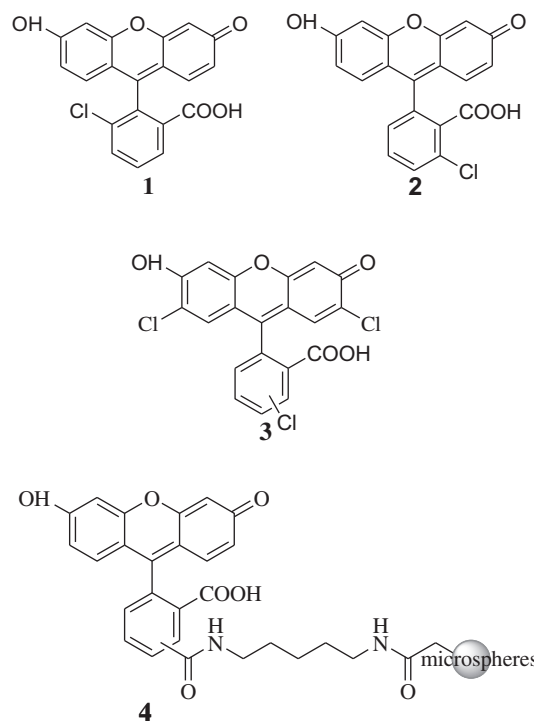
In the design of such probes, attention should be paid to both recognition and fluorophore moieties [13]. The fluorophore moiety acts as a signal transducer, which converts the recognition information into a change in optical signal. Operating mechanisms [e.g., charge transfer (CT), photo-induced electron transfer (PET), monomer-excimer, and electronic energy transfer (EET)] are often employed [14].

As regards the recognition moiety, it undertakes responsibility for selectivity and efficiency of binding the analyte. It is worth pointing out the problem that the fluorophore moiety can be linked to the recognition moiety via a spacer or not. Even in the latter case, some atoms of the fluorophore may participate in the complexation, so the change in optical signal often results from the whole molecular structure, including both fluorophore and recognition moieties.

3. Chlorinated fluoresceins

Fluorescein, the most common fluorescent reagent, has high extinction coefficients, excellent fluorescence quantum yield, good water solubility and non-toxicity. Its fluorescence excitation and emission spectra lie in the visible region [15]. The use of fluorescein derivatives allows measurement of intracellular pH in single cells [16]. However, the relatively broad fluorescence-emission spectra limit their utility in multicolor applications [4]. Moreover, when fluorescein functions in the pH range 5.0–8.5, the fluorescent intensity reaches its maximum only at pH >8.5, so accurate pH determinations cannot be achieved in acidic cells, because the pH range of the acidic cells is 4–6.

As selective substitution of chlorine for aromatic hydrogen can change the photophysical properties of the compound [17], Ge et al. [18] synthesized chlorinated fluorescein probes **1–3** to resolve the above problems. These compounds exhibit strong dependence on pH in the range 3.5–7.0, with lower pK_a values than fluorescein of **1** and **2** with pK_a 6.34 and **3** with pK_a 4.64. The fluorescent intensity can reach the maximum in the physiological pH range 6.8–7.4. Unfortunately, the probes have not yet been applied to measure acidic cells. Furthermore, these compounds need to be modified as fluorescein diacetate in order to increase cell permeability.

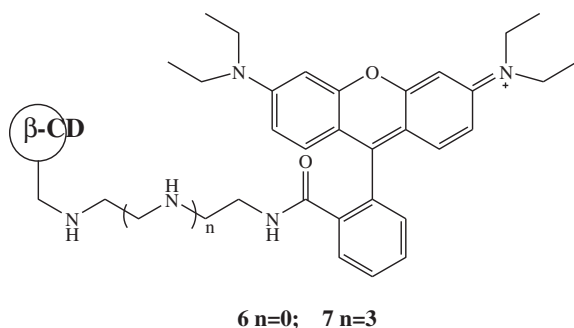
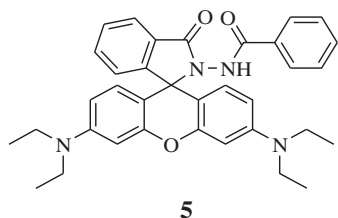


Fluorescein can rapidly leak out from cells, and that makes it difficult to quantify changes in intracellular pH. To eliminate leakage of the dye from cells, Bradley et al. [19] bound fluorescein covalently to polystyrene microspheres. The fluorescent microsphere probe **4** is used for real-time pH sensing in living HEK-293T, B16F10 and L929 cells.

4. Rhodamine dyes

Rhodamine (Rh) dyes are widely used as molecular probes in cell biology [20]. The dyes possess excellent photophysical properties (e.g., high extinction coefficients, good photostability, high fluorescence quantum yield, and elongated wavelengths of their absorption and emission to the visible region). The dyes also have good cell-membrane permeability. Based on Rh B, Tang et al. [21] designed and synthesized acidic fluorescent probe **5**. Alteration in molecular structure between spirocyclic (non-fluorescent) and ring-open (fluorescent) conformations of Rh dyes is employed as the detection mechanism. The fluorescence intensity of the probe varies more than 100-fold within the pH range 4.2–6.0. Its pK_a is 4.85. The designed probe is applied to monitor H^+ within living HepG2 cells.

Rh B has been modified with β -cyclodextrins (β -CD) by Hasegawa et al. [22]. β -CD plays the role of non-toxic cavity for guest molecules, while Rh B works as fluorescent moiety. Probe **6** monitors lysosome pH in HeLa cells. The MTT assay shows lower cytotoxicity of **6** and **7** than that of Rh B.



5. Near-infrared cyanine dyes

Near-infrared (NIR) fluorescent cyanine dyes are becoming more and more important in modern imaging techniques of biomedicine [23]. These dyes possess high extinction coefficients (>200000 L/mol/cm), wide response spectrum, good fluorescence function and high photostability. The fluorescent emission wavelength, which lies in the NIR region (>600 nm), can minimize photo damage and avoid the influence of cell autofluorescence.

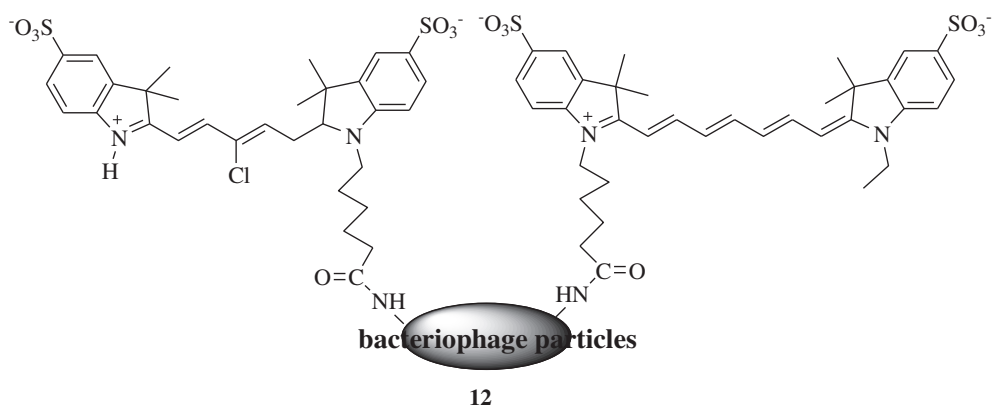
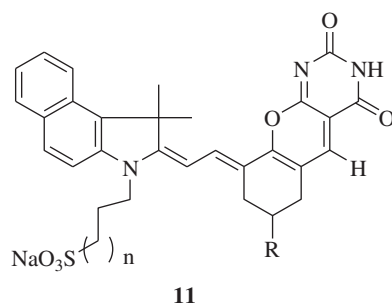
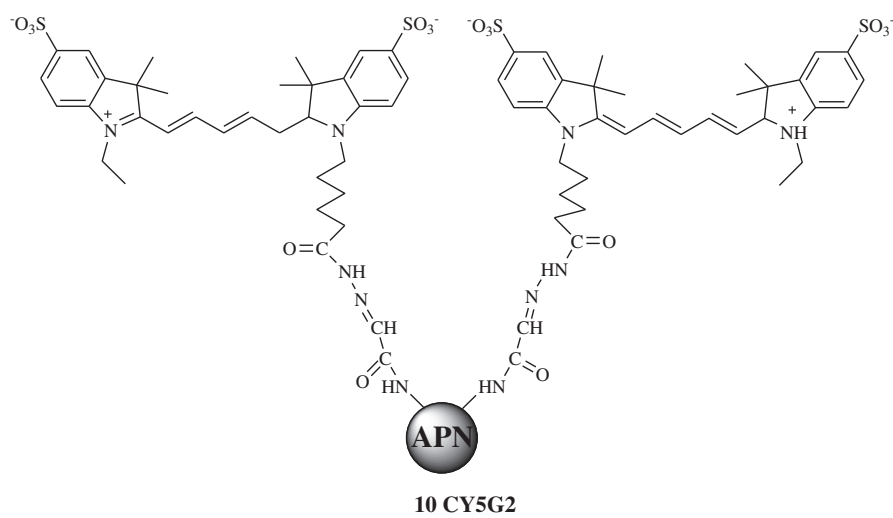
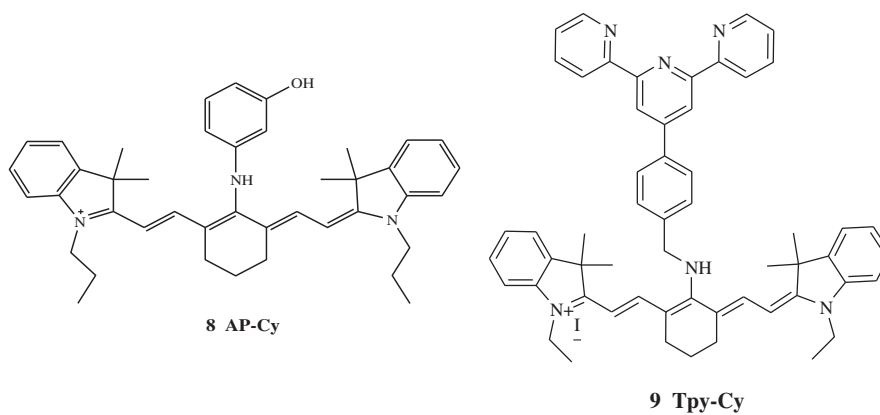
Tang et al. [24] chose cyanine as fluorophore and employed PET as the mechanism to synthesize two pH probes of AP-Cy (**8**) and Tpy-Cy (**9**). AP-Cy is a dual NIR pH fluorescent probe. Its fluorescence modulator is 3-aminophenol and the pK_a value is 5.14 under acidic conditions and 11.31 under basic conditions. AP-Cy is applied for pH imaging in HepG2 cells. Tpy-Cy takes terpyridine (Tpy) as H^+ -receptor, with a pK_a of ~ 7.1 . The probe was successfully used for the real-time imaging of cellular pH and the detection of pH *in situ* in living HepG2 and HL-7702 cells.

Galande et al. [25] reported an aminopeptidase N (APN)-targeted fluorescence pH probe **10** (CY5G2). Cy5 dyes were chosen as fluorophore. Due to self-quenching, the probe exhibited weak fluorescence at a physiological pH of 7.4, while enhanced fluorescence at pH of 4.5. Because APN is over-expressed in tumor vasculature, CY5G2 demonstrates potential applications in monitoring pH changes in cancerous cells.

Lee et al. [26] demonstrated an unusual barbiturate-mediated synthesis of NIR fluorescent pH-sensitive probe **11** (pK_a of ~ 3.5). The structural feature of this fluorescent pH probe suggests a novel substitution/elimination pathway, which is different from a well-established S_N1 mechanistic pathway using the meso-chlorine atom of heptamethine dyes.

Hilderbrand et al. [27] labeled bacteriophage particles with HCyC-646 and Cy7 dyes to get an NIR fluorescence ratiometric pH probe **12**. The emission of Cy7 remains stable in the range pH 5–9. However, the emission signals of HCyC-646 with pK_a of 6.2, vary with pH changes. The probe applies to fluorescence-based pH imaging, for intracellular measurements and through optically-diffuse biological tissue.

Povrozin et al. [28] investigated a commercial pH-sensitive dye Square-650-pH (K8-1407). The probe exhibits its absorption and emission in the NIR region. Compared with the protonation form, the deprotonation form displays a blue shift of the excitation spectra. Labeled on antibody, the protein-conjugated conformation acts differently from the dye. The free probe has a pK_a of 7.11, while the pK_a of the antibody-conjugated label is 6.28. The novel method of conjugating probe to microorganisms indicates great potential to study phagocytic events and monitor pH changes in cells.



at 600 nm) and pH 7–8 (giving red fluorescence at 525 nm). The probe comprised two fluorescein molecules as energy donors and one BODIPY molecule as energy acceptor. The energy transfer from the donors to the BODIPY core was employed to make the whole energy cassette sensitive to pH changes under physiological processes. The dye has been used for imaging protein-dye conjugates in living COS-7 cells.

Urano et al. [33] developed pH-activable fluorescence BODIPY probes for selectively imaging *ex vivo* and *in vivo* by using labeled macromolecule conjugates. The acidic pH in lysosomes was maintained by the energy-consuming proton pump only in viable cancer cells, so the dyes were almost non-fluorescent at pH 7.4, and became highly fluorescent at pH <6, which minimized photo damage and avoided the influence of cell auto-fluorescence. The dyes could therefore covalently bind the targeting antibody and tune to different pK_a values.

Urano et al. [33] selected anilines as the recognition moiety toward H^+ and 2,6-dicarboxyethyl-1,3,5,7-tetramethyl boron-dipyrromethene as fluorophore. They again employed PET. Herein, two carboxylic groups could be used for coupling proteins. The pK_a values of **19**, **20**, **21** and **22** were 3.8, 4.3, 5.2 and 6.0, respectively. The dyes are applied to image HER2-positive cells and HER2-positive lung metastases for real-time monitoring of pH changes *ex vivo* and *in vivo*.

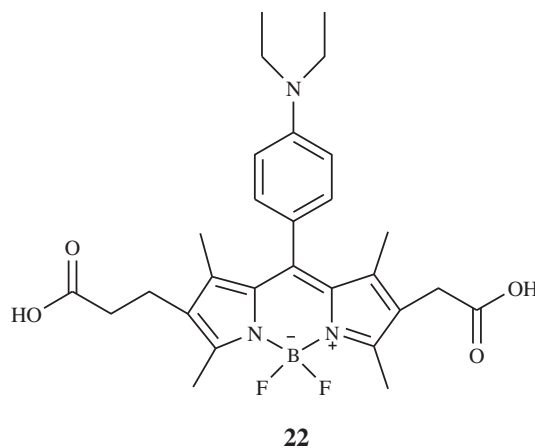
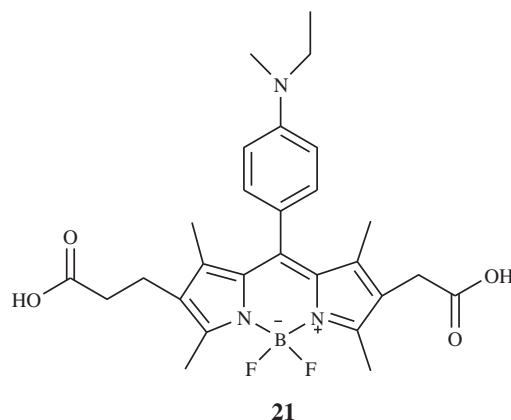
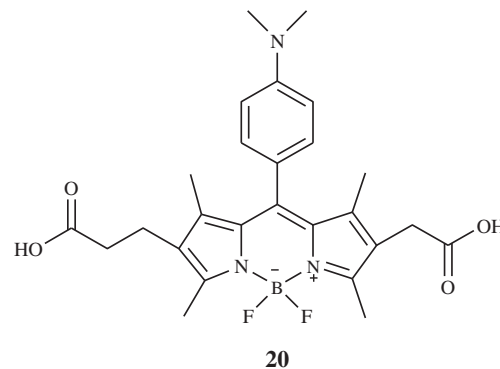
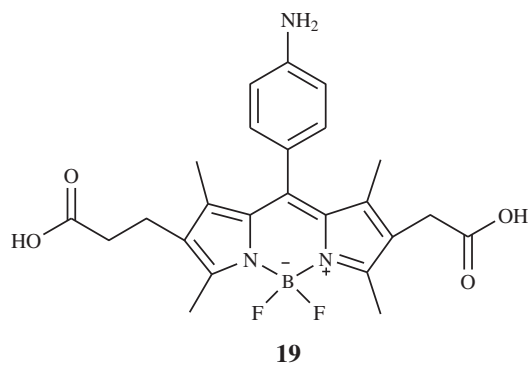
7. Other fluorescent pH probes

Fluorene and its derivatives are famous for exhibiting high fluorescence quantum yields and excellent photostability [34].

Yao et al. [35] reported a near-neutral pH indicator (pK_a 6.96) based on donor- π -acceptor fluorene derivative **23**. The probe is used as a sensitive ratiometric pH indicator with a distinctive isoemissive point. The dye also shows nice properties as excellent two-photon fluorescent (2PF) materials. 2PF images NT2 cells.

Bergen et al. [36] employed acridizinium fluorophore as a water-soluble substitute for anthracene. Acridizinium derivatives **24** and **25** with pK_a of 3 and 3.2, respectively, are intended to be used complementary to anthracene in fluorescent pH probes. PET probes and switchable donor-acceptor systems can be constructed, acting as pH-sensitive probes at low pH values by an appropriate substitution.

Few fluorescent pH probes based on non-covalent forces, namely intramolecular hydrogen bonding, have been reported. Cui et al. [37] synthesized 4-piperidine-naphthalimide derivatives **26** based on 4-aminonaphthalimides, which contain potential acceptors for hydrogen bonding. Their pK_a values were estimated to be about 6.4–7.5. These pH probes have great potential in biological and supramolecular systems.



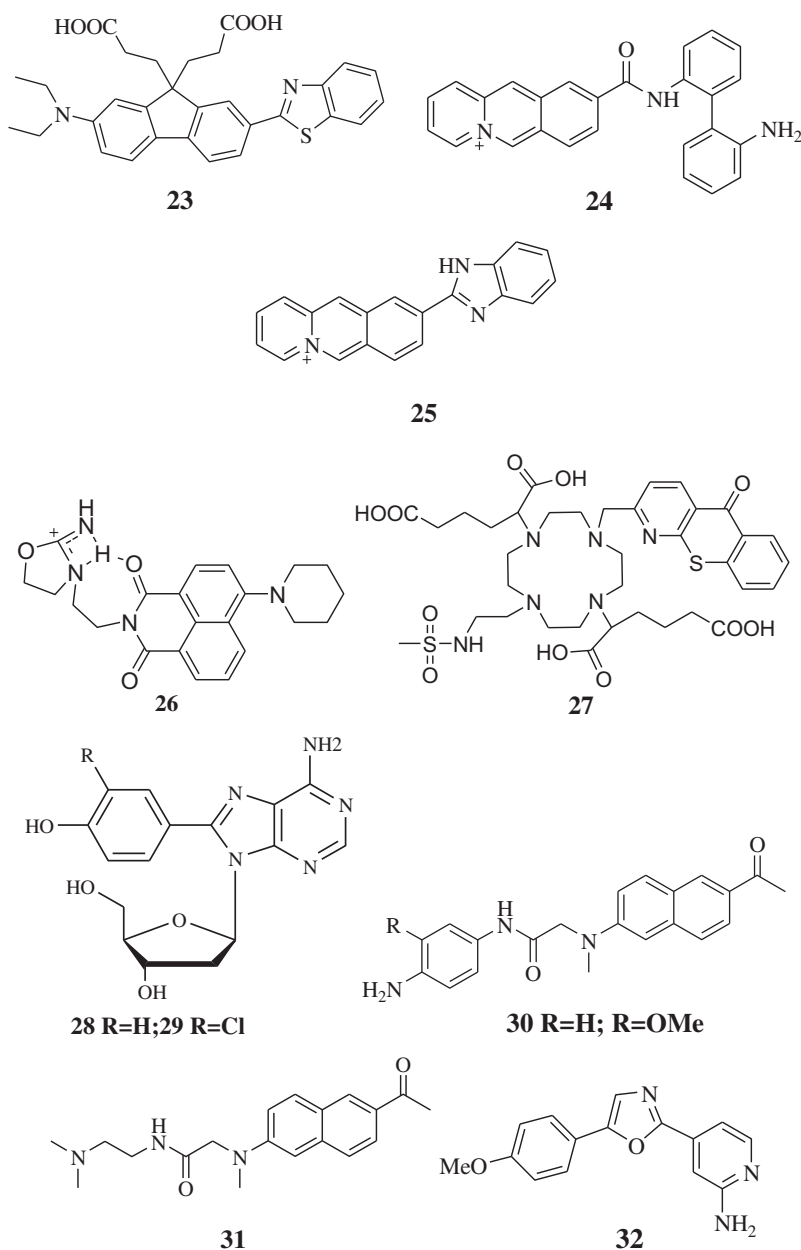
Pal and Parker [38] described a cell-permeable macrocyclic Eu(III) complex ratiometric pH probe **27** that

incorporates an N-methylsulfonamide moiety. Eu emission spectra depend on variations in pH, and the intensity ratio of the two wavelengths (680:587 nm) fits the observed data for cells. The pK_a value is 6.15. Confocal fluorescent microscopy images of NIH 3T3 cells have been achieved.

Sun et al. [39] modified nucleobase stems from research on DNA adduction by phenolic carcinogens, and synthesized biomarkers for phenol carcinogen exposure to act as pH-sensing fluorescent probes. A pK_a of 7.3 was determined for **29**. The proposed mechanism was that the purine base acts as the donor while the phenolate is the electron acceptor. The probes may be used to establish the basis of phenol-mediated carcinogenesis.

Kim et al. [40] designed and synthesized a two-photon (TP) pH probe **30** and a TP lysotracker **31** derived from 2-acetyl-6-(dimethylamino)naphthalene. The proton-binding site, aniline, o-methoxy aniline, and tertiary amine were introduced through an amide linkage to the fluorophore. Probe **30** showed a pK_a value of ~ 4.5 . TP microscopy images have been obtained for individual macrophages and rat hippocampal tissue.

Charier et al. [41] chose 2-(4-pyridyl)-5-aryloxazole as backbone and designed a donor-acceptor fluorescent ratiometric pH probe **32** with pK_a of 5.7. The fluorescent indicator can measure pH in the range 3–8 in aqueous solutions by fluorescence emission after one- or two-photon excitation. The dye **32** would find interesting applications as intracellular pH probes.



8. Conclusions

Molecular fluorescent pH probes can potentially be used as *in vitro* and *in vivo* tools for evaluating intracellular receptor kinetics, cancer detection, cell viability, and real-time monitoring of cell apoptosis and therapy. Molecular fluorescent pH probes, particularly those

with good water solubility, have become more and more important in monitoring biochemical processes *in vivo*.

Table 1 summarizes the photophysical properties of typical pH fluorescent probes and Table 2 highlights their applications. Though the availability of fluorescence is increasing, with even some commercial appli-

Table 1. Photophysical properties of pH fluorescent probes

Probes ^a	pK _a	$\lambda_{\max,abs}$ (nm)	$\lambda_{\max,em}$ (nm)	Quantum yields, ϕ	Ref.
Fluorescein 1	6.34	499	518	0.93	[18]
Fluorescein 3	4.64	512	528	0.87	[18]
Rhodamine Dyes 5	4.85	562	582	NA ^a	[21]
AP-Cy	5.14 (acidic conditions) 11.31 (basic conditions)	dual absorption 431 and 750	800	NA	[24a]
Tpy-Cy	7.10	650	750	0.008 (pH 10)	[24b]
11	3.5	605 (MeOH, pH <4)	700	0.079 (MeOH)	[26]
BODIPY 13	8.75	505 (MeOH)	517 (MeOH)	0.28	[30]
BODIPY 14	9.34	508 (MeOH)	521 (MeOH)	0.19	[30]
BODIPY 16	7.75	502 (Cyclohexane)	512 (Cyclohexane)	0.099	[31]
BODIPY 17	7.38	503 (Cyclohexane)	515 (Cyclohexane)	0.043	[31]
18		576	600	NA	[32]
23	6.96	341	391	0.21	[35]
28	8.70	280	391	0.56	[39]
29	7.29	290	383	0.22	[39]
31	NA	364	496	0.72 (pH 3.2)	[40]

^aNot available.

Table 2. Highlighted applications and performances of pH fluorescent probes

Probes	Applications	Advantages/Disadvantages	Ref.
Chlorinated fluoresceins	Real-time pH sensing in living HEK-293T, B16F10 and L929 cells in pH range of 3.5–7.0	High extinction coefficients, excellent fluorescence quantum yield, good water solubility, non-toxicity, fluorescence excitation and emission spectra in visible region/Too broad fluorescence emission spectrum, rapid leakage of the dye from cells and little Stokes shift	[15–19]
Rhodamine dyes 5	Monitoring H ⁺ within living HepG2 cells <i>in vivo</i>	High extinction coefficients, good photostability, high fluorescence quantum yield/Weak interactions between probe and targeted biomolecule	[20,21]
Rhodamine dyes 6 and 7	Monitoring lysosome pH in Hela cells <i>in vivo</i>	Cell membrane permeability/Short fluorescence lifetime	[22]
NIR cyanine 8	pH imaging in HepG2 cells		[24]
NIR cyanine 9	Detecting pH <i>in situ</i> in living HepG2 and HL-7702 cells	High extinction coefficients (>200000 L mol ⁻¹ cm ⁻¹), wide response spectrum, good fluorescence function and high photostability/Inferior photostability and thermal stability	[24]
NIR cyanine 12	Fluorescence-based pH imaging, both intracellular and through photodiffusion in biological tissue within pH ranges of 5.0–9.0		[27]
Square-650-pH	Studying phagocytic events and monitoring pH changes in cells	Commercial, absorption and emission in the NIR region/NA	[28]
BODIPY 13–17	Detecting pH in cells		[4,29–31]
BODIPY 18	Working within pH 4.0–6.5 and pH 7.0–8.0, imaging protein-dye conjugates in living COS-7 cells	Uniquely small Stokes shift, high, environment-independent fluorescence quantum yields, and sharp excitation and emission peaks contributing to overall brightness/NA	[32]
BODIPY 19–22	Real-time monitoring pH changes in HER2-positive cells <i>ex vivo</i> and <i>in vivo</i>		[33]

cations, there are still numerous challenges and opportunities remaining for development of new fluorophores and practical applications in biological systems. Probes that monitor minor pH fluctuations with a pK_a value near physiological pH are rarely available, especially those that can be used to quantify near-neutral pH in aqueous solutions and *in vivo*.

Molecular fluorescent pH probes that can directly target specific subcellular locations are desirable. New candidates for ratiometric fluorescence imaging would allow quantitation of H^+ changes in cells. Both reagents that can assist in achieving multicolor imaging applications and probes that exhibit NIR excitation and emission profiles would maximize signal-to-noise ratios.

Acknowledgements

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