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.

Keywords: Fluorescence microscopy; Fluorescent probe; Intracellular pH; Living cell; Organic synthesis; pH detection; pH indicator; pH regulation; Signal transduction; Small molecular probe

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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.