Fluorescent Probes

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Synthetic Macrocyclic Peptidomimetics as Tunable pH Probes for the Fluorescence Imaging of Acidic Organelles in Live Cells**

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Synthetic fluorescent probes have found widespread applications in cell biology for the intracellular measurement of several species, from the zinc(II) cation[1] and citrate anion[2] to singlet oxygen[3] and nitric oxide.[4] In recent years, several reviews have appeared that deal with synthetic fluorescent chemosensors.[5,6] Protons are one of the most important targets among the intracellular species of interest, as it is well-known that pH plays a central role in many cellular events.[7] In general terms, two broad ranges of pH values are found in cells. Consequently, two types of synthetic probes have been developed,[8] namely, probes for cytosol that work at a pH of about 6.8–7.4, and probes for the so-called acidic organelles (for example, lysosomes) that function over the pH range of about 4.5–6.0. It is possible to find a large array of commercial probes for the former pH range; however, this is not the case for acidic organelles. Many reports have appeared that connect some cellular dysfunction with abnormal pH values in acidic organelles. For instance, defective pH regulation of acidic compartments has been observed in human breast cancer cells.[9] Another illustrative example is the finding of elevated lysosomal pH in neural ceroid lipofuscinoses, a common inherited neurodegenerative disorder that affects

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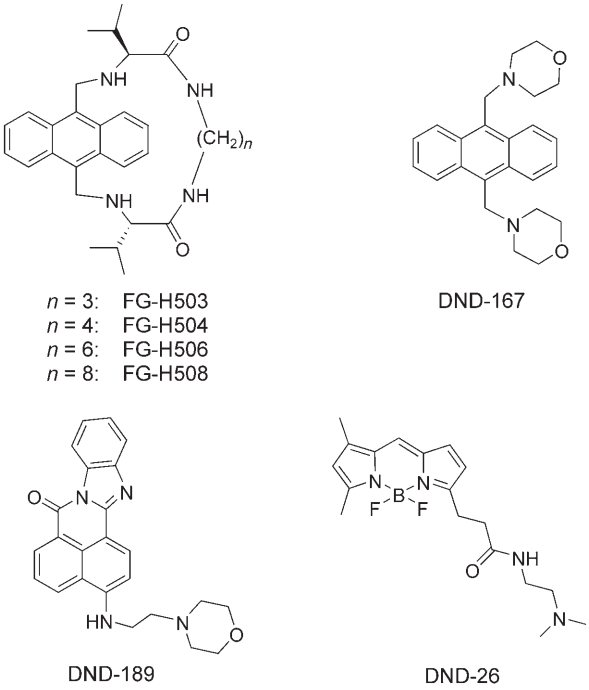
children.[10] It would, therefore, be extremely desirable to have access to a broad variety of "acidic" probes to study these and other conditions. Unfortunately, as a consequence of the current unavailability of probes for the acidic pH range, there is a research bottleneck as regards progress in some areas of cell biology or medicine. Additionally, in the light of the above discussion, a further fundamental criterion emerges for the design of new probes. This criterion is the possibility of chemical control of the pH reporter probe under extracellular conditions, that is, the potential for precise control (and prediction) of the pK_a value of the probe by adjusting the appropriate elements in the molecular architecture.

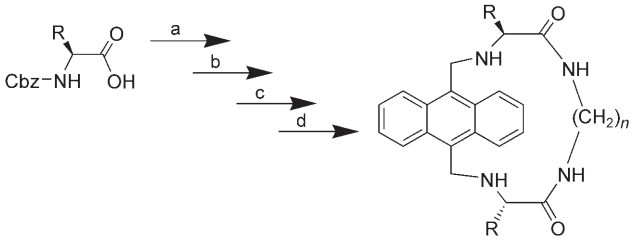
Herein, we present a new family of fluorescent macrocyclic probes (FG-H series)[11] that consist of a 9,10-anthracene subunit linked by a C_2-symmetric peptidomimetic chain (Scheme 1). The probes differ only in the size of the chain



Scheme 1. Structures of the fluorescent probes studied.

($n = 3,4,6,8$), but this structural variation produces a smooth shift in the pK_a values, which is an essential feature that enables a range of pH "windows". The synthesis of the FG-H family members is facile, and the compounds exhibit good photostability and present excellent cellular uptake without the need for derivatization to improve crossing of the cellular membrane. Moreover, the series extends the range of pH utility by about 0.5 pH units (toward a lower pH) over that of currently available probes.

Compounds FG-H503, FG-H504, FG-H506, and FG-H508 were synthesized according to a procedure developed for the preparation of peptidomimetic macrocycles in high yields.[12,13] It has been previously demonstrated that the β-turn conformation adopted by the synthetic intermediates (arising from the presence of two α-amino acids as building blocks) allows the synthesis of the peptidomimetic macrocycles in moderate to high yields without the need for high-dilution conditions. In the case of the FG-H series shown in Scheme 1, two units of L-valine were selected because of their reported ability to induce the formation of β sheets.[14] However, it should be possible to introduce virtually any α-amino acid with an appropriate protecting group into the general synthetic procedure (Scheme 2) to obtain a new



Scheme 2. a) Acid activation; b) coupling with 1,*n*-diaminoalkane; c) deprotection; d) ring-closing with 9,10-bis(bromomethyl)anthracene. Cbz = carbobenzyloxy.

family of peptidomimetics with different properties as pH probes. The 9,10-dimethylanthracene moiety was chosen as a fluorophore as it is known to be photostable and also to allow comparison with the widely used pH probe for acidic organelles, LysoSensor Blue DND-167 (Scheme 1). DND-167, which was originally studied by de Silva et al.,[15] is also an anthracene derivative that shares with the compounds in the FG-H series the presence of two amine groups decoupled electronically from the fluorophore by both methylene spacers. However, in the case of DND-167, there is the possibility that more flexible configurations could be adopted. For comparison a second commercial probe, LysoSensor Green DND-189, which has a different photochemical nature, was also selected. LysoTracker Green DND-26 (see Scheme 1)[16] was also used for fluorescence imaging experiments in vivo. This latter compound was employed to certify the acidic nature of the organelles, as it is known to accumulate in lysosomes. (Note, however, that LysoTracker Green DND-26 is not a pH "reporter", as its fluorescence emission does not vary with changing pH.[8a])

Standard fluorescence pH titrations were performed for all the compounds in Scheme 1 in buffered aqueous media (containing 0.2% DMSO from the initial stock solution) at a probe concentration of 2 μM, with measurement at pH intervals narrow enough to allow a precise determination of the apparent pK_a value. Figure 1 shows the absorption spectrum of FG-H503 (Figure 1a) along with the fluorescence emission spectra at different pH values (Figure 1b–g). The fluorescence changes in systems composed of fluorophore–CH_2–amine moieties, such as the FG-H series, can be rationalized according to the photoinduced electron-transfer (PET) scheme developed by the research groups of de Silva and Czarnik:[6] Under basic conditions the amino groups are not protonated, and hence the PET process takes place (if thermodynamically allowed by the Rehm–Weller formalism[17]) which leads to fluorescence quenching. In acidic media the electron pair in each amine is protonated, thus preventing

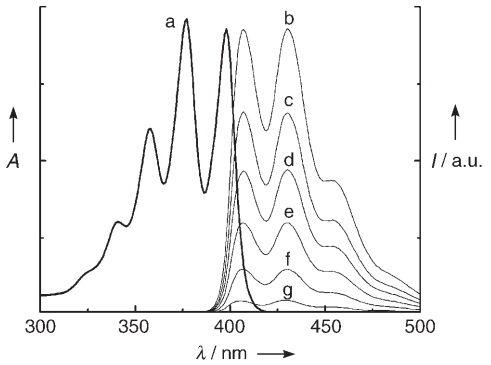


Figure 1. FG-H503 (2 μM) in aqueous solution (0.2% DMSO). a) Normalized absorption spectrum at pH 1.02, and fluorescence spectra (λ_{ex} = 377 nm) at b) pH 2.55, c) 4.70, d) 5.10, e) 5.41, f) 5.83, and g) 6.90.

Table 1: Spectral characteristics and pK_a values of the studied systems.

Entry	Compound[a]	λ_{ex} [nm]	λ_{em} [nm]	pK_a
1	FG-H503	377	430	5.06 ± 0.02
2	FG-H504	377	430	5.24 ± 0.02
3	FG-H506	377	430	5.41 ± 0.01
4	FG-H508	377	430	5.43 ± 0.01
5	DND-167	374	427	5.61 ± 0.01
6	DND-189	443	505	6.06 ± 0.06
7	FG-H503 + Cu^{II}	377	430	5.02 ± 0.02
8	DND-167 + Cu^{II}	374	427	5.57 ± 0.01
9	DND-189 + Cu^{II}	443	505	6.01 ± 0.03
10	FG-H503 + Zn^{II}	377	430	4.95 ± 0.03
11	DND-167 + Zn^{II}	374	427	5.52 ± 0.01
12	DND-189 + Zn^{II}	443	505	5.70 ± 0.03

[a] Probe concentration: 2 μM in aqueous solution (0.2% DMSO); metal concentration: 200 μM.

PET from occurring, and hence emission from the fluorophore takes place.

In the case of the FG-H series, a minimal variation in the chemical structure of the probe causes an appreciable change in the titration curve (see Figure 2 for some of the titration

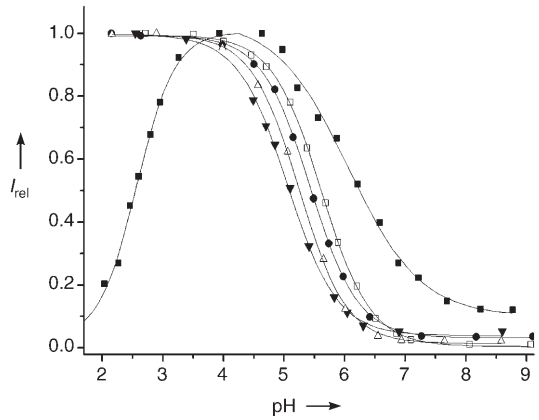


Figure 2. Fluorescence versus pH titration curves for the probes under study. Data are normalized at the maximum emission (I_{rel}). Probe concentration: 2 μM in aqueous solution (0.2% DMSO); excitation wavelengths as indicated in Table 1. FG-H503 (▲), FG-H504 (△), FG-H508 (●), DND-167 (□), DND-189 (■).

curves; complete spectra and titration results can be found in the Supporting Information).[18,19] The pK_a values are derived from the nonlinear curve-fitting of the data and are shown in Table 1 along with the excitation and emission wavelengths.

It can be seen in Figure 2 that the DND-189 probe exhibits a fluorescence intensity versus pH profile that is not the same as the other profiles, which reflects its different chemical nature with respect to the anthracene framework. Additionally, a sharp decrease in the fluorescence intensity is measured with DND-189 at acidic pH values.

To examine the useful pH range for each probe, the pH interval for which the tested compound changes from 20 to 80% of its maximum fluorescence was determined (Figure 3; the maximum is measured at pH 2 for the FG-H series and

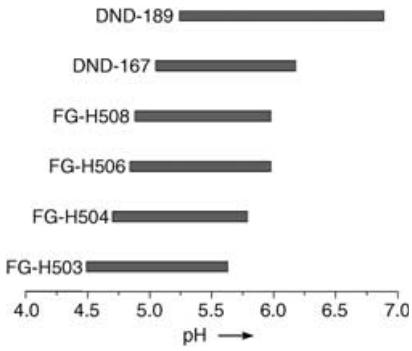


Figure 3. Schematic representation of the pH intervals where the fluorescence emission of each probe experiences a change between 20 and 80% of its maximum intensity. Probe concentration: 2 μM in aqueous solution (0.2% DMSO); excitation and emission wavelengths as indicated in Table 1.

DND-167, and at pH 4 for DND-189). Thus, it can be seen that the peptidomimetic macrocycles are complementary to the DND probes, as they extend the pH range of measurement toward the acidic side.

On account of the complexity of the intracellular environment, an additional examination of the probes was performed to determine whether other ions were potential interferents. For example, it is well-known that amines can bind many metal cations in solution.[6] In fact, many chemosensors for metal ions use the ability of amines to coordinate the vacant orbitals of such analytes. Among them, zinc(II) and copper(II) are the types of species likely to be targeted by free amines in solution. Consequently, control experiments with FG-H503, DND-167, and DND-189 in the presence of an excess of Zn^{II} and Cu^{II} ions were performed to assess such possible coordination. As can be seen in Table 1, the effect of such metals on the peptidomimetic macrocycle is negligible, particularly when considering that the concentrations used for the experiment were significantly higher than those present in the intracellular environment.

FG-H503 was investigated as a fluorescent probe in cultured cells and was compared to DND-189. The probe

DND-167 did not produce images with sufficient resolution; therefore, the additional probe LysoTracker Green DND-26[16] was also used which is known to be retained specifically in acidic organelles. Two series of experiments were performed: Raw 264.7, a mouse macrophage cell line, was loaded with FG-H503 and with either DND-189 or DND-26. As shown in Figure 4b,g, FG-H503 exhibited a vesicular distribution. The probe predominantly co-localized with both DND-189 and DND-26, which indicates that the probe is taken up by the cells and that it localizes within acidic organelles. Note that the different excitation and emission wavelengths of FG-H503, as compared to those of the commercial probes, allow simultaneous visualization of both probes (FG-H503 and either DND-189 or DND-26) from the same intracellular compartment. Interestingly, the fluorescence intensity of FG-H503 appears to match that of DND-189: areas of low and high fluorescence (light and dark blue arrows, respectively, in Figure 4b–d) of the probe match those of DND-189, which indicates that the FG-H probe can distinguish between different pH values in the cell in a manner similar to the commercial probe. Analogous results are obtained from the joint use of FG-H503 and DND-26. Composite images of the fluorescence and differential interference contrast (Figure 4e, j) confirm that FG-H503 is located within acidic compartments of this macrophage cell line.

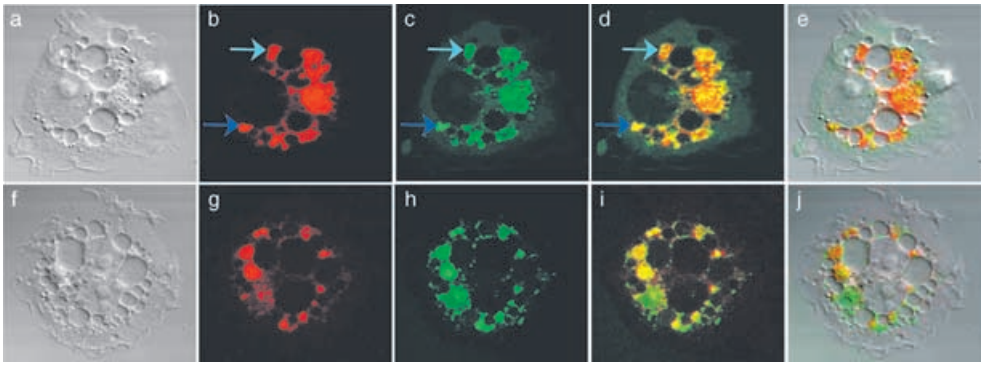


Figure 4. Distribution and co-localization of FG-H503 with lysosomal probes DND-189 (a–e) and DND-26 (f–j) in Raw 264.7 cells. Differential interference contrast (DIC; a, f) and fluorescence images of FG-H503 (b, g) and DND-189 (c) or DND-26 (h) were collected with a confocal laser scanning microscope in the DIC, DAPI, and FITC modes. d), i) Merged images of red and green channels; e), j) composite images of red, green, and DIC channels. DND-189 (a–e) and DND-26 (f–j) are from a different set of experiments.

In summary, a new family of fluorescent macrocyclic peptidomimetic compounds has been synthesized, and their abilities as pH probes have been tested both in solution and in live cells with positive results. The synthetic modular scheme is an excellent approach for the development of new tailor-made probes with targeted pH "windows" ideal for measurement of the changing acidic environment of intracellular organelles. Current efforts involve the expansion of the FG-H family with other α-amino acids, spacers, and fluorophores, and determination of the extent by which the peptidomimetic nature of the new probes favors their performance in biological media.

Experimental Section

Synthesis of the peptidomimetic macrocycles: The appropriate 1,*n*-diaminoalkane was reacted with *N*-protected (Cbz) L-valine through the formation of an *N*-hydroxysuccinimide ester.[12] Yields of these intermediate peptidomimetic compounds were about 70%. The cyclization step was performed by treating 9,10-bis-bromomethylanthracene with the diamine formed by *N*-deprotection of the intermediates with HBr/AcOH (deprotection yields over 90%). The products of the cyclization step (at reflux in acetonitrile as the solvent, and anhydrous K_2CO_3 as the base) were purified by column chromatography to afford the final products in about 30% yield (see Supporting Information for chemical characterization).

Fluorescence measurements: The probes were dissolved in dimethyl sulfoxide (DMSO) to obtain 1 mM stock solutions, and then aliquots were diluted to 2 μM with water containing a mixture of several buffers to facilitate titrations between pH 9 and 2 (40 mM of each sodium salt: acetate, phosphate, borate, and carbonate). All the measurements were performed in the presence of 100 mM NaCl to maintain a constant ionic strength. Slight variations in the pH of the solutions were achieved by adding the minimum volumes (typically 10 μL added to 10 mL) of 0.1–1.0 M NaOH or 0.1–1.0 M HCl, in such a way that dilution effects were negligible. Fluorescence spectra were recorded (Spex Fluorolog Max-2) with excitation at the wavelengths indicated in Table 1. Plots of the fluorescence intensity versus pH were fitted by using a nonlinear curve-fitting technique (Microcal Origin 6.0). All the measurements were carried out at ambient temperature and in air-equilibrated solutions. Copper(II) and zinc(II) were added as the chloride and nitrate salts, respectively (200 μM).

Confocal fluorescence imaging: Raw 264.7, a mouse macrophage cell line, was cultured on 42-mm-diameter round glass coverslips in Dulbecco's modified Eagle's medium (MEM) containing fetal bovine serum (10%), L-glutamine (2 mg mL^{-1}), and antibiotics (penicillin/streptomycin). The cells were stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) for 16 h, loaded with the dyes for 15 min in serum-free media, and then mounted on a stage at 37°C after changing to phenol-red-free L15 medium containing the above supplements. The cells were observed with a confocal laser scanning microscope (Carl Zeiss, LSM 510 meta) with 350- and 488-nm laser excitation for the 4,6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC) channels, respectively. An appropriate emission band was selected for the two fluorescent channels. A 63×, 1.4-NA objective was used to ensure high-resolution images. DIC images were collected simultaneously with transmitted light by excitation at 488 nm. Sequential rather than simultaneous acquisition was used to avoid bleed-through between the two fluorescent channels. Images were processed, and double- and composite-merged images were made with Adobe Photoshop.

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[18] Such a different protonation behavior must occur for a combination of reasons. To partially explain the phenomenon, the observation made years ago for related systems (ref. [15b]) is relevant: The protonation of the second amine in a 9,10-bis(aminomethyl)anthracene derivative is negatively influenced by the first protonated amine, as a result of the repulsion existing between its ammonium group and the second incoming proton. In this way, larger macrocycles like those presented here would protonate the second amine (and hence lead to restoration of the fluorescence) at a lower concentration of protons (higher pK_a) than the smaller macrocycles, as the former macrocycles would accommodate the second positive charge more easily than the latter. However, other contributions should not be disregarded, in particular solvation effects in protonated species. A more extensive study of a larger family of peptidomimetic molecules is in progress.

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