Strategies for Developing pH Sensitive Fluorescent Probes

Mikhail Y. Berezin1*, Kevin Guo1, Hyeran Lee1, Walter Akers1, Adah Almutairi2, Jean M.J. Fréchet2, Samuel Achilefu1,3

1Department of Radiology and 3Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri, USA 2College of Chemistry, University of California, Berkeley, California, USA

ABSTRACT

Many physiological processes function efficiently within a well-controlled pH range. Higher acidity level has been implicated with a number of systemic pathologies. The potential of pH sensitive fluorescent probes for reporting on biological environments has been widely utilized in a variety of cell studies and has been recently recognized as a powerful technique for in vivo imaging of diseases associated with elevated acidity level. We present several strategies for the development of pH sensitive probes suitable for in vivo imaging. The strategies include incorporation of pH sensitive functionalities in known fluorophores, synthesis of novel pH sensitive skeletons, and design of pH sensitive nanoparticles using acid-degradable polymers.

1. INTRODUCTION

The potential of pH sensitive fluorescent probes for reporting on biological environments has been widely utilized in a variety of cell studies [1] and has been recently recognized as a powerful approach for in vivo imaging of diseases associated with elevated acidity [2, 3]. Higher acidity level (acidemia and acidosis) has been implicated with a number of systemic pathologies [4], such as renal acidosis, metabolic disorders, intoxications, diabetes and emphysema, as well as localized hyperacidity such as infections, inflammation and cancer. In particular, solid tumors with pH ranging from 5.8 to 7.7 [5, 6] are on average 0.5 units lower than the pH of normal tissue due to a higher level of anaerobic metabolism through glycolysis. After being excreted from tumor cells, the acidic products accumulate in the extracellular, extravascular space (EES) due to the poor lymphatic drainage [7]. This dramatic difference in pH between normal and cancerous tissue gives powerful potential to an optical imaging modality with a suitable pH indicator. To be used in vivo, the indicator must be optically active within a relatively narrow NIR region and have a pKa in the physiological range which we loosely defined between 5.5 and 8. Interest in these probes has recently spurred the synthesis of novel NIR pH indicators that have had success in vitro and could be suitable for in vivo imaging [8-14]. From the variety of different approaches, we present here strategies to develop pH sensitive probes based on incorporation of a pH sensitive functionality into existing fluorophores, and pH sensitive nanoparticles with an emphasis on fluorescence lifetime.

2. MATERIALS AND METHODS

2.1 Synthesis

General procedure for conjugating imidazolecarbaldehydes to benzo[e]indolium. To imidazole carbaldehyde (1.0-1.1 eq.) in methanol, 3-(2-carboxyethyl)-1,1,2-trimethyl-1H-benzo[e]indolium 1 bromide [15] (1.0 eq.) was added, followed by the addition of sodium
acetate (1.5 eq.) The reaction was stirred for 20 h at room temperature in the dark. The products were purified by flash chromatography.

**General procedure for synthesis of fluorescent barbiturates.** A mixture of 6 (0.12 mmol) and barbituric acid derivative 7 was stirred at room temperature in the presence of TEA in acetonitrile. The reaction progress was monitored by UV-Vis spectroscopy. The product was precipitated from the reaction mixture upon addition of diethyl ether followed by filtration. The precipitate was further purified on the Biotage SP4 purification system eluting water/ACN. Fractions with an absorption maxima at 690 nm were collected and solvent was removed by lyophilization to give blue solid 8 and 9.

**Procedure for pH sensitive nanoparticles.** The dendrimer core pentaerythritol was conjugated to Boc-L-(4-acetyl)-phenylalanine in the presence of DMAP in DMF. The reaction mixture was cooled to 0 °C and then EDCI was added, stirred 20 h, diluted with diethyl ether, and washed with NaHSO4(aq), NaOH(aq) and water. The organic layer was dried with Na2SO4, filtered, and the solvent was removed. The product was purified by precipitation from EtOAc/hexanes. After deprotection with TFA/CH2Cl2 and pegylation with PEO-PNP (DMAP, triethylamine, pyridine and toluene (1.6 mL) piperidine was added (after 3 days) to quench excess PEO-PNP, and the reaction was stirred for an additional 16 h. The solvents were removed, the product was dialyzed against water with MW 3500 cut-off and lyophilized to yield a dendrimer 10 as white powder. Cypate-hydrazide 11 was prepared from cypate Boc-Carbazate in dry DMF and the presence EDC and HOBt and deprotected with TFA/CH2Cl2. After aqueous work-up the desired product was purified by column chromatography. NIR nanoparticles 12 were prepared by mixing 10 in a 3:1 mixture of pyridine:acetic acid in methanol. The product was purified on a PD-10 column using water as the eluent. The collected dark green fraction was lyophilized to yield a green powder.

**2.2 Optical measurements**

UV/Vis spectra of samples predissolved in methanol and diluted with water were recorded using Beckman-Coulter DU-640 spectrophotometer. Fluorescence spectra were recorded using Fluorolog III (Horiba Jobin Yvon Inc., Edision NJ). For 3D excitation-emission spectra, the emission signal was corrected by lamp intensity. Fluorescence lifetime was measured using TCSPC technique with excitation source NanoLed (Horiba) and R928P detector (Hamamatsu). The instrument response function (IRF) was obtained using Rayleigh scatter of Ludox - 40 (Aldrich) (0.03% in MQ water) in a quartz cuvette. DAS6 v6.1 decay analysis software (Horiba) was used for lifetime calculations. The fit was judged by $\chi^2$ values and Durbin-Watson parameters and visual observations of fitted line, residuals and autocorrelation function. The lifetime was recorded on 50 ns scale.

**2.3 Titration experiments and data analysis**

Compounds 2-5, 8, 9 were dissolved in methanol (0.2 mL) and added into a beaker with water (100 mL) under stirring. Flow of argon was constantly delivered to the top of the solution to reduce dissolved CO₂. The solution was basified with diluted aqueous NaOH and the desired pH was attained by titrating with aqueous HCl, or backwards at relatively low ionic strengths (I = 0.02-0.05 M). The pH of the solution was continuously measured using Accumet pH meter AB15 (Fisher Sci.) Fluorescence lifetime titrations were recorded in water (nanoparticles) or in
acidic (TFA) and basic (TEA) solutions of DMSO (barbituric derivatives). pKa values were calculated using principal component analysis software (DATAN 3.1, MultiD Analyses AB, Sweden) or sigmoidal dose-response curve fit implemented in software Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

3. RESULTS AND DISCUSSION

3.1 Imidazoles

The long standing interest of our lab lies in synthesis of novel near-infrared probes and their applications in biological imaging. Recently, we initiated a screening of organic functionalities which could be suitable as pH sensors and synthetically appropriate for incorporation into a polymethine skeleton. Our first choice was the family of imidazoles, widely utilized natural pH sensors with pKa highly dependent on the substituents and the environment. The most common imidazole derivatives, including aminoacid histidine with a pKa ~6.8 and histamine with a pKa ~5.7, naturally fall into the range of physiological relevance. Many pH sensitive fluorescent proteins such as mCherry [16], EBFP2 [16], and EosFP [17] also incorporate imidazoles in their fluorophores. Inspired by the optical properties of imidazole containing natural molecules, we incorporated an imidazole heterocycle via a methine linker into a fluorogenic π-conjugated benz[e]indolium core (compound 2 Scheme 1) [18].

![Scheme 1 Library of synthesized fluorogenic imidazole derivatives based on 5-imidazoles](image)

All prepared compounds from 5-imidazole series 2-5 were found to be fluorescent and pH sensitive with an exception of the dimethylated compound 3. An example of fluorescence pH sensitivity is given in Figure 1, where 3D excitation-emission spectra for compound 2 were recorded at three buffers: acidic (pH 3), neutral (pH 7) and basic (pH 10). At pH 3, the emission maximum was located at 370 nm excitation and about 600 nm emission (ex/em: 370/600). Upon increase of pH from acidic to neutral, the position as well as intensity of the emission band changed: emission experienced a blue shift to ~ 360/560 nm with the concomitant decrease in intensity. Additional basification to pH ~10 caused the emission band to shift to 450/550 nm with further decrease in band intensity. The presence of three distinct emission properties indicated the presence of at least three emitting species. The assignment of these emitters is given in Scheme 2.
Figure 1 3D excitation-emission plot of compound 2 in aqueous buffered solution. Emission signal was corrected by lamp intensity. Rayleigh scattering was digitally removed.

From studying the library of fluorescent imidazoles, several observations can be made as follows: N-methylation substantially alters the emission profiles, non-methylated imidazoles have at least three emitting species, and substitution of one of the nitrogens on the imidazole ring decreases this number to two emissive species as shown in Scheme 2. As expected, dimethylated 3 was not pH sensitive which correlated well with the lack of “active solvation centers” [19] in the molecule.

Table 1 pKa of fluorogenic imidazoles

<table>
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<th>Non N-methylated pKa</th>
<th>N-1 methylated</th>
<th>N-3 methylated pKa</th>
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3.2 pH sensitive barbiturates

The continuation of this strategy was the incorporation of the pH sensitive barbituric functionality into a polymethine structure [11]. Instead of expected Suzuki coupling in the meso-position of a chlorosubstituted cyanine dye 6, an unusual barbiturate-mediated debenzoindolation and subsequent heteroannulation occurred leading to NIR pyrimidine-fused fluorescent probes (Scheme 3) [11].

Spectrophotometric analysis of 8 at various pHs revealed that both absorption and fluorescence emission are pH-dependent. The absorption spectra of 8 exhibited an intense peak
at 690 nm in neutral pH with a hypsochromic shift to 605 nm at acidic pH with overall changes in intensity at 680 nm as shown in Fig. 2A. The fluorescence peak was located at ~700 nm with intensity minimal below pH 2 but increased >6-fold as pH increased from 3 to 6 (Fig. 2B). Fluorescent lifetime slightly rose from 1.28 ns to 1.4 ns at higher pH (Fig. 2C).

Scheme 3 Synthesis of fluorescent derivatives of barbituric acid

The pKa values of 8 determined by absorption and fluorescence titration plots (Fig. 2) were found to be comparable at ~3.5, suggesting similar acidity in the ground and excited states. A drastically low pKa (~3.5) for N-H dissociation equilibrium of 8 compared to the typical pKa of ~9.5 for uracile derivatives was attributed to the presence of delocalized positive charge across the cyanine molecular framework in which deprotonation results in the loss of net charge. This process is energetically favorable and thus lowers the pKₐ values. Deprotonation of one of the two nitrogens with the formation of uracilate and its resonant form is shown in Scheme 4.

In contrast to non-methylated 8, the absorption spectra of dimethylated 9 did not exhibit spectral sensitivities, indicating the absence of a deprotonation site similar to imidazole 3. The optical properties of 9, including the absorption and emission spectra and quantum yield, are almost identical to those of 8H (acidic), suggesting that both 9 and 8H have similar electronic distributions. Thus, it is evident from these studies that the presence of a free -NH moiety is crucial in inducing pH-sensitivity.
3.3 pH sensitive NIR nanoparticles: lifetime approach

In this strategy, we utilized specially designed pH sensitive nanoparticles and took advantage of their fluorescence lifetime sensitivity. Previously, we demonstrated the fluorescence lifetime of NIR cyanine dyes is extremely sensitive to the solvent polarity [20] and that dendrimeric nanoparticles are biodegradable [21]. Herein we implemented both concepts and formed NIR nanoparticles with fluorescence lifetime sensitive to pH. For that, the dendrimer 10 was conjugated with NIR dye cypate via an acid sensitive hydrazone bond to give a NIR fluorescent nanoparticle 12 with NIR dyes located outside the nanoparticle (Scheme 5) [21]. If the nanoparticle is injected into a blood stream and is intact, it cannot be opsonized by albumin due to the large size (MW ~ 40,000). In that case the lifetime of the nanoparticle in blood would reflect the lifetime of the dye in the aqueous environment [20, 22].

Indeed, fluorescence decay at pH 7 in water and in 4% albumin/water solution were indistinguishable (Fig. 3A) with very similar lifetime values of 0.34 and 0.36 ns respectively (Table 2). The observed short lifetime is characteristic of cypate under hydrophilic conditions such as water. The stability of the lifetime indicates negligible hydrolysis of the hydrazone bond.

Table 2 Average fluorescence lifetime of nanoparticles
At low pH, the hydrazone bond undergoes cleavage, leading to the release of cypate from the surface of the nanoparticles. Once the fluorophore is released, it is free to interact with \textit{in vivo} blood proteins such as albumin which quickly opsonizes the dye by the hydrophobic binding site. This hydrophobic environment is expected to cause an increase in fluorescence lifetime. Indeed, at pH 4 without albumin, the hydrolysis might take place, but the changes in lifetime could not be detected because the dye is still in hydrophilic media. In the presence of albumin, the released free dye is immediately bound as indicated by longer decay (Fig. 3B) with the change in lifetime from 0.36 ns to 0.54 ns (Table 2). Overall, the change in fluorescent lifetime acts as a reporter of the low pH.

4. CONCLUSIONS

Several strategies to form pH sensitive fluorescent probes have been explored. Incorporation of pH sensitive functionalities such as imidazole and barbituric acids into fluorophores leads to the pH sensitive fluorescent constructs with a range of pKa. Although the resulting pKa values for the presented limited library lie outside the physiological pH, the strategy holds promise since it allows modification of chemical structures and therefore pKa tuning. Additional optimization, as well as incorporation of other pH bearing functionalities into fluorophores including NIR dyes, is currently under investigation. Acid degradable nanoparticles also constitute a promising strategy. As we demonstrated, such a strategy can be utilized in conjunction with fluorescence lifetime.

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REFERENCES


